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SH3-mediated protein interactions of Mena and VASP

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Die Promovendin ist Ärztin

CONTENTS

1 INTRODUCTION

1. The vascular endothelium

The endothelium is a thin layer of polygonal, flat cells, which separate the lumen of a blood vessel from the underlying tissue. The endothelial cells have various functions: First they act as semi-permeable barrier, controlling the passage of water, proteins, and circulating blood cells. This barrier function is regulated by interendothelial cell junctions [3]. Other important functions of the intact endothelium are prevention of blood clotting, support of angiogenesis and regulation of the blood pressure by vasodilation and vasoconstriction.

There are three forms of junctions: Tight junctions (TJs), gap junctions (GJs) and adherens junctions (AJs). Tight junctions seal the plasma membranes of two neighboring cells via claudin and occluding closely. Therefore, they limit the passage of water molecules and ions via the paracellular pathway [4].

Gap junctions consist of specialized transmembrane proteins named connexins. Six of these proteins form a so called connexon, which is a hemichannel. There are about twenty different connexin isoforms, and GJs are often built up of various isoforms. One gap junction consists of two of those hemichannels. Gap junctions join cells to one functional unit regarding electrophysiological and metabolic qualities [5].

Adherens junctions connect different cells in a mechanical way. The transmembrane protein VE-cadherin is the most important protein for AJ in the endothelium. The binding of cadherins of two cells leads to the mechanical stabilization of those cells [3]. Furthermore, actin filaments are essential for the maintenance of the AJs, because destabilization of the filaments leads to a lateral diffusion of cadherins in the plasma membrane and therefore to a disruption of cadherin binding [3].

All junctions are modulated by inflammatory mediators (bradykinin, histamine for example). These mediators lead to a disruption of the junctions, which can cause swelling and edema. On the one hand the junctions are modulated

through myoendothelial contacts, which are similar to gap junctions; on the other hand the junctions are regulated through vasoactive signaling mediators such as endothelin and NO [3].

In endothelial cells, changes in the actin cytoskeleton lead to formation of defined membrane structures named lamellipodia and filopodia at the leading edge of the cell. Whereas lamellipodia show a wide and flat configuration, filopodia are thin, finger-like structures. This difference is caused by the actin filament organization: Lamellipodia contain a branched network, whereas filopodia are filled with parallel actin bundles [6]. Formation of these structures is important for movement and adherence of the migrating cells and impaired formation can lead to defects for example in wound healing, embryonic morphogenesis, and tissue repair [6].

2. Functional structure of the mammalian heart

The mammalian heart is a muscular double pump that ensures the circulation of the blood through the body. Whereas the left part of the heart pumps the blood enriched with oxygen to all parts of the body, the right part of the heart pumps the venous, low oxygenated blood to the lungs, where the oxygenation of the blood takes place.

Due to this mechanical function, the mammalian heart mainly consists of muscle tissue. Heart muscle cells, which are about 200 µm in length and 15 µm in diameter, form long chains by end-to-end joining. All cells of a chain are surrounded by the same basal membrane. The regions where the cells are in contact with each other are called intercalated discs [7]. Intercalated discs connect the heart muscle cells to work as a syncytium, meaning that the cells are supported to contract synchronically. Intercalated discs consist of three different types of adhering junctions: Gap junctions (see 1.1), desmosomes and fascia adherens. Whereas gap junctions support the electromechanical coupling of the cells, desmosomes bind intermediate filaments to join the cells together during contraction and fascia adherens serve as anchoring sites for actin filaments [8].

Concerning gap junctions in the mammalian heart, three specific connexin isoforms, could be detected: Connexin 40, 45, and 43, which is the predominant connexin isoform in the heart [9].

αII-Spectrin is a critical component of the so-called transitional junction at the intercalated disc. Transitional junctions are located at the insertion site of myofibrils into the adherens junction [10]. Due to the fact that αII-Spectrin-deficient embryos show cardiac dilation and an abnormal cardiac shape, which most likely causes the observed embryonic lethality, a critical function of αII-Spectrin in maintaining cardiac integrity can be assumed [2]. As shown by Benz et al., direct interaction of αII-Spectrin and VASP can stabilize cell-cell contacts and initiate β-actin filament assembly [11]. Therefore, a possible influence of αII-Spectrin-Mena/VASP complexes on formation and stability of cytoplasmic actin networks in the heart seems to be imaginable [2].

3. Ena/VASP proteins

Ena/VASP proteins are important regulators of the cellular actin cytoskeleton. Actin is a 43 kDa monomeric globular protein (G-actin) that is able to polymerize into filaments (F-actin). Actin enables processes like cell division, cell motility, shape change, and cell-cell adhesion and regulation of actin filament dynamics is essential for cell maintenance [1]. Ena/VASP proteins influence the actin cytoskeleton in various ways: They support elongation of the filaments by recruitment of actin to remodeling sites like lamellipodia and influence the activity of the Arp2/3 complex, which is an important regulator of the actin cytoskeleton itself. Therefore, the proteins counteract the inhibition of actin polymerization by capping proteins and they affect the branching of the actin filaments [1].

In mammalians, the Ena/VASP family consists of Mena (mammalian enabled), VASP (Vasodilator-stimulated phospho-protein), and EVL (Ena/VASP-like).

The structure of the three proteins is highly conserved. As shown in Figure 1, they contain a N-terminal Ena/VASP homology 1 (EVH1) domain, which is about 115 amino acids long and a C-terminal Ena/VASP homology 2 (EVH2) domain, which is about 150 amino acids long [1]. In the middle region of the

proteins a variable proline-rich region (PRR) is localized. The PRR of Mena consists of 64 amino acids and contains a GP_6 and a GP_9 motive. The PRR of VASP and EVL are shorter, spanning 50 amino acids in case of VASP, and 25 amino acids in case of EVL. In contrast to the PRR of Mena, EVL contains a single GP_8 motive and VASP a triple GP_5 motive (Figure 1). Furthermore, in contrast to EVL and VASP, Mena contains an additional sequence of five amino acids (LERER) between the EVH1 domain and the PRR, which is repeated 14 times [1].

Each of the domains has its own interaction partners: The EVH1 domain for example interacts with the focal adhesion proteins actA, vinculin, zyxin, and the Wiscott-Aldrich syndrome protein (WASP). This interaction is mediated by an F/LPPPP- motive [12, 13]. The PRR of Ena/VASP proteins is known to interact with the actin binding protein profilin for example. The PRR is also known to interact with src, abl and αII-Spectrin, which is mediated by the src homology 3 (SH3) domains of these proteins [14]. The EVH2 domain contains binding sites for filamentous and globular actin and mediates the tetramerization of Mena and VASP by a coiled-coil motive. The tetramerization of Mena and VASP is essential for binding to interaction partners of Mena and VASP [15, 16]. The Mena gene is alternatively spliced to generate several splice variants. The neuronal variant has a molecular weight of about 140 kDa, whereas the molecular weight of the ubiquitous variants is 80 kDa and 88 kDa respectively.

Ena/VASP proteins are well-known targets of cyclic nucleotide dependent protein kinases (protein kinases A and G) [17]. As shown in Figure 1, human VASP contains three phosphorylation sites: Serine 157 (S157), serine 239 (S239), and threonine 278 (T278) [18-20]. Whereas the cAMP-dependent protein kinase (PKA) phosphorylates the highly conserved S157 phosphorylation site, the protein kinase G (PKG) mainly phosphorylates VASP in position S239 [20]. The AMP-activated protein kinase (AMPK) phosphorylates VASP at T278 [21]. In contrast to VASP, Mena only contains PKA and PKG phosphorylation sites and in EVL only the PKA phosphorylation site is conserved.

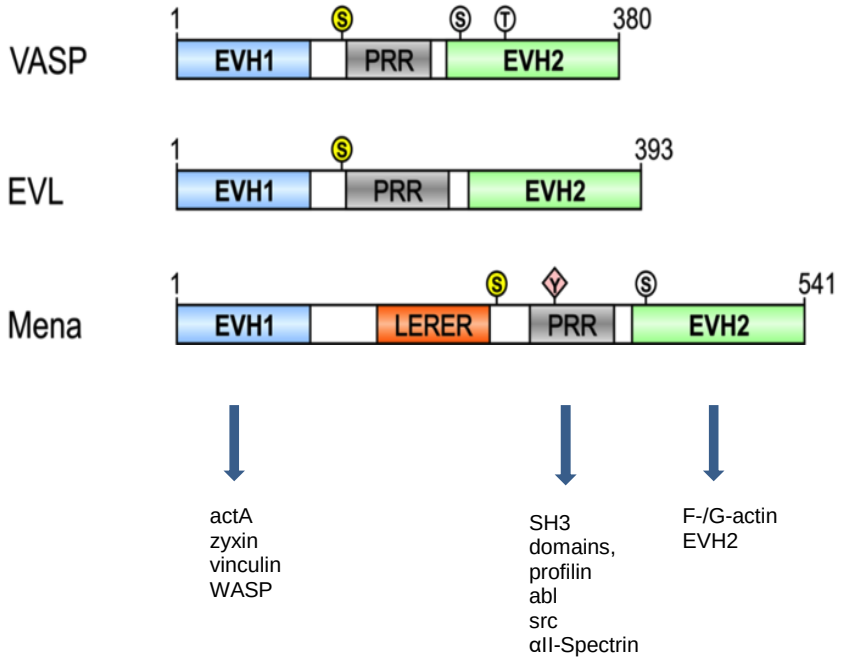
The phosphorylation of VASP at position S157 leads to a shift in the apparent molecular weight of VASP (46 kDa to 50 kDa) in SDS-PAGE in contrast to phosphorylation at the other sites where this phenomenon has not been seen [17, 20].

Important effects of phosphorylation of Ena/VASP proteins on the protein function have been described: Phosphorylation at the conserved serine phosphorylation site changes the subcellular localization of VASP and Mena and phosphorylation of VASP at S239/T278 inhibits filament elongation and bundling [22, 23]. Also it has been shown that phosphorylation of the Ena/VASP proteins can influence the interaction with other proteins. For instance, PKA phosphorylation of VASP at position S157 inhibits the interaction with SH3 domains, which are part of src, abl and αII-Spectrin [2, 14, 19, 24].

As described above, Ena/VASP proteins are involved in regulation of the cellular actin cytoskeleton. There are different mechanisms of actin filament modulation by Mena and VASP: Actin can be bundled or combined [1, 25], barbed ends of actin can be protected from capping by capZ [22] and the polymerization of the actin filaments can be supported by VASP [26].

Resulting from the regulation of the actin cytoskeleton are the physiological effects that have been described: The proteins support cell division, cell motility, cellular shape change, and cell-cell adhesion, for example [1]. Because of the importance of intact actin organization for force transduction, a possible effect of Ena/VASP proteins has been proposed in smooth muscle cells and in the heart [2, 27, 28]. Whereas the functional role of Ena/VASP proteins for smooth muscle cell contraction and relaxation remains to be investigated, an important role of Ena/VASP proteins in the mammalian heart can be assumed, because Mena/VASP deficient mice show dilated cardiomyopathy and conduction abnormalities [2]. Further analysis of the underlying structural and biochemical functions were part of this work.

A:



B:

Human EVL (V179-Q213)

VSCS**G**PPPPPPPPV**P**PPP**P**TGAT**P**PPPPP**P**L**P**AGGA

Human VASP (S157-G215)

SNAGG**P**P**A**P**P**AG**G**PPPPP**G**PPPPP**G**PPPPP**G**PPPPPGL**P**PSGV**P**AAAHGAGGG**P**PP**A**P**P**L**P**AAQG

Human MENA (G305-F378)

GIVLG**P**LA**P**PPPPP**P**L**P**P**G**P**A**QASVAL**P**PPP**G**PPPPPP**P**L**P**ST**G**PPPPPPPPPP**P**L**P**NQV**P**PPPPPP**P**A**P**P**P**L**P**ASGF

Figure 1: Domain organization, phosphorylation sites, and interacting proteins of Ena/VASP proteins.

A: EVH1: Ena/VASP homology 1, PRR: proline-rich region, EVH2: Ena/VASP homology 2, LERER: region with 14x LERER amino acid sequence; Serine-/threonine phosphorylation is indicated as circles, tyrosine phosphorylation as rhombus. The conserved PKA phosphorylation site is highlighted in yellow. Figure modified from [1].

B: Comparison of the proline-rich regions from human EVL, VASP, and Mena. Prolines are indicated in red, glycines preceding a proline-stretch in blue. PKA-mediated VASP phosphorylation at S157 (green) induces an electrophoretic mobility shift of the protein from 46 to 50 kD. The PRR of Mena is the largest of all three proteins, spanning 64 amino acids, followed by the PRR of VASP with 50 amino acids, and the PRR of EVL spanning only 25 amino acids. EVL contains a single GP_8 motive, VASP a triple GP_5 motive, and Mena a GP_6 and GP_9 motive.

4. Spectrin

Spectrins are cytoskeletal proteins, which are found in association with the plasma membrane of nearly all mature cells. The protein family was first described in erythrocytes, where Spectrins support the stabilization of the plasma membrane by modulating the actin filament organization [29]. In erythrocytes, actin filaments and Spectrin tetramers build up a meshwork, which lines the cytoplasmic face of the plasma membrane. Therefore, the Spectrin-actin network is linked to the membrane by association of Spectrin with the adaptor protein ankyrin, which itself is bound to the cytoplasmic domain of the anion exchanger [29]. Furthermore, stabilization of the Spectrin-actin complexes is mediated by protein 4.1 and adducin. Spectrins can serve as linker between membrane proteins and cytosolic proteins either directly or through adaptor proteins. This can lead to macromolecular protein complexes with various functions [30].

Spectrins are composed of two α- (280 kDa) and two β- (245-260 kDa) subunits and they form antiparallel heterodimers. The heterodimers then are used for formation of tetramers. Two α- and five β-subunits are known to date (αI, αII, βI, βII, βIII, βIV, βV) and the combination of these α- and β- subunits leads to many different functions and localizations of the tetramers [29]. Some subunits are restricted to specific cells, like the αI- and βI-subunits, which almost exclusively exist in erythrocytes. Other subunits like αII-Spectrin are expressed in many different tissues and they mainly form tetramers with βII-subunits. It has been described, that β-Spectrins associate with the Golgi mediated by ARF, a small GTPase [30].

αII-Spectrins consist of triple-helical repeats of 106 amino acids, which are aligned antiparallel side by side. The α- and β-subunits differ in the number of helix repeats: α-Spectrins consist of 20 triple-helix repeats and have a partial repeat in the N-terminus, whereas β-Spectrins comprise only 16 repeats and have a partial repeat close to the C-terminus. These partial repeats adhere to each other in case of tetramer formation in a non-covalent manner [31]. Whereas α-Spectrins at the C-terminus contain EF-hand motives, which allow a calcium-dependent conformational change, β-Spectrins harbor an actin-binding

domain at the N-terminus and a pleckstrin homology (PH) domain at the C-terminus [29] as shown in Figure 2.

As shown in Figure 2, two of the repeats of αII-Spectrins are atypical: In repeat 9 a SH3 domain is inserted and repeat 10 contains an insert that is sensitive to caspase 3 and calcium dependent calpain hydrolysis and calmodulin binding [32]. Cleavage of these sites influences Spectrin cytoskeleton stability, apoptosis and necrosis. The insert contains two potential phosphorylation sites indicating that there is a physiologically regulated function of the splice form [33, 34].

As described above, the repeats consist of three helices, named A, B and C. The SH3 domain in repeat 9 is inserted between helix B and C. SH3 domains are often found in plasma membrane proteins or proteins that are involved in signal transduction and cytoskeleton regulation [31]. For more information about the structure of SH3 domains see 1.5. Several αII-Spectrin splice variants exist, which have been identified in heart tissue [35] but have also been found in endothelial cells (personal communication Dr. Peter Benz). One isoform contains a 20 amino acid sequence insert in helix C of the SH3 domain of αII-Spectrin [35]. It has been shown that this variant α9A is localized at intercalated discs and associates with connexin 43, which is an important component of gap junctions. This implicates α9A as a potential target for stress signaling pathways that modulate intercellular communication in the heart [2, 35].

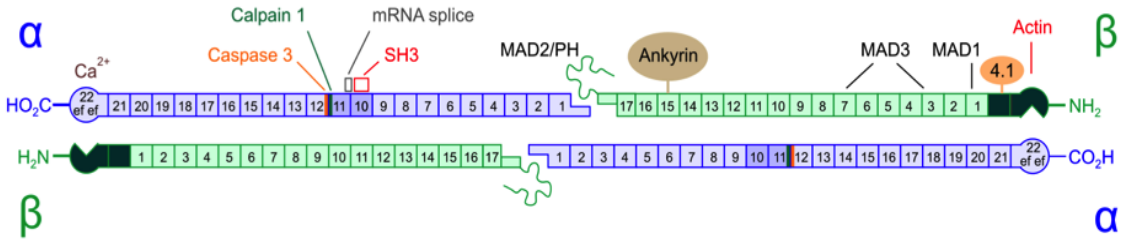


Figure 2: Domain structure of αII-βII-Spectrin tetramer.
Each subunit consists of triple helical repeats containing approximately 106 amino acids. Sites for ankyrin, band 4.1, actin binding, calpain 1 and caspase 3 cleavage, and mRNA splicing are shown. pleckstrin homology (PH) domain, the SH3 domain, located in the middle of the α- subunit, and direct membrane association domains (MAD1-3) are also indicated. Non-homologous segments are shaded. The calponin homology domains at the N-terminus of the β-Spectrins are indicated in dark green, the two EF-hand motives are shown at the C-terminus of the α-subunits. These calmodulin-like structures undergo Ca2+-induced conformational changes. One antiparallel hetero-tetramer is composed of two α- and β-subunits that first form heterodimers. Figure modified from Dr. Peter Benz.

In cardiomyocytes, αII-Spectrin is not only localized at the plasma membrane but also at Z-discs, intercalated discs, and the nucleus [2]. Because of co-localization of βII-Spectrins at intercalated discs and Z-discs with α-actinin, Spectrins may play an important role for force transduction and, by stabilizing gap junctions, for the propagation of electrical signals [36]. In vessels, complexes of αII-Spectrin and VASP have been shown to regulate cytoskeleton assembly at endothelial cell-cell junctions, which is important for endothelial barrier function [14].

5. SH3 domains

SH3 (src homology 3) domains are the most abundant protein recognition modules, hundreds are known within the human genome [37]. SH3 domains were first identified in the viral adaptor protein v-crk and the non-catalytic parts of enzymes such as the tyrosine kinases abl and src [38]. SH3 domains are mostly found in signal transduction and cytoskeletal proteins and are important mediators of transient protein–protein interactions and dynamic processes such as cytoskeletal reorganization [39, 40]. The structure of the 60-residue protein modules is described as β-barrel formed by 5 antiparallel β-strands that build up two antiparallel β-sheets. Therefore, the first strand (βA) is followed by the so-called RT-loop, connecting βA and βB strand and containing the ALYDY motive

that is one characteristic motive of SH3 domains. The following β-strands are connected by the so-called n-src (connecting βB and βC strand) and the distal loop (connecting βC and βD strand). The distal loop is followed by the highly conserved WW dipeptide, located in the βD strand and the PxxY-motive. The latter motive is situated in a 3_{10} helix that connects βD and βE [41, 42].

Early studies defined the ligand sequence PxxP, occurring for example in proline-rich regions, as minimal consensus sequence for SH3 domain binding [43]. Moreover, it has been found that amino acids neighboring this core motive are also important for binding. SH3 domains bind to ligands containing such motives in two opposite orientations: The consensus sequence of class I ligands starts with a positively charged residue (+xΦPxΦP) while class II ligands have this positively charged residue at the end of the consensus sequence (ΦPxΦPx+) (Φ: hydrophobic amino acid, x: any amino acid, P: proline, +: positively charged amino acid, usually arginine) [42].

SH3 ligands containing prolines build a left-handed polyproline II (PPII) helix and bind to three shallow pockets within a flat and hydrophobic surface of the SH3 domain. ΦP dipeptides bind to two of the shallow pockets, while the positively charged residue of the class I or II ligands named above pack into the third pocket, called specificity pocket. The positively charged residue is interacting with a negatively charged residue of SH3 [42].

Moreover it has been found that SH3 domains are capable to bind to non-PxxP containing sequences and also to bind via tertiary contacts indicating the various functions of SH3 domain mediated protein-protein interactions [37].

The tyrosine kinase abl for example binds to class I ligands with hydrophobic residues that contact the third specificity pocket [44]. Similarly, Ena/VASP protein binding to the αII-Spectrin SH3 domain is atypical; the proteins interact via hydrophobic residues within the Spectrin SH3 domain specificity pocket [14]. Another finding was that in case of the small adaptor protein CRKL that contains SH3 domains, dimerization of SH3 domains occurs [45]. Because of the comparatively low inherent specificity in many SH3 mediated interactions, there are some additional mechanisms to increase the binding specificity, like compartmentalization of binding partners in the cell, forming of multi-protein

complexes, and regulation of the interaction via posttranslational modifications [44]. Due to the complexity of the SH3 interaction, the detailed binding mechanisms for individual SH3 domains are still under investigation.

2 MATERIAL AND METHODS

1. Protein biochemistry

1.1. SDS-PAGE

SDS-PAGE is used for separation of proteins according to their electrophoretic mobility. This method was first described by Laemmli in 1970 [46]. SDS is used to linearize the proteins and to impart a negative charge to them. To separate the proteins, continuous gels containing generally 5% to 25% acrylamide (Rotiphorese Gel 30; acrylamide:bisacrylamide 37.5:1, Roth, #3029), 375 mM Tris-HCl pH 8.8, and 0.1% SDS were used. To simultaneously resolve small and large proteins, it was occasionally necessary to use a two-step gradient gel, with a higher acrylamide concentration in the lower part. To concentrate and collect the samples, gels containing 5% acrylamide:bisacrylamide 37.5:1; 125 mM Tris-HCl pH 6.8 und 0.1% SDS were used. By addition of 0.1% ammonium persulfate (APS) and tetramethylethylendiamin (TEMED) the polymerization was induced. To reduce disulfide bridges 5x SDS-loading dye (300 mM Tris-HCl pH 6.8; 10% SDS; 50% glycerol; 0.005% bromophenol blue) with 5% beta-mercaptoethanol was used. The running buffer (10x for one liter: 10 g SDS; 30.3 g Tris; 144.1 g glycine) was diluted to a 1x end concentration. As size standard PageRuler (Fermentas, SM0671) was used. Loading of bovine serum albumin (BSA) was used to quantify the amounts of protein on coomassie stained gels.

1.2. Coomassie staining

After staining of the polyacrylamide gels for at least one hour with Instant Blue (expedeon, ISB1L), the gels were destained for at least one hour with water.

1.3. Western blotting

After separation of the samples via SDS-PAGE, the proteins were transferred to a nitrocellulose membrane where they could be detected by antibodies specific to the target protein.

The blotting buffer contained 1x SDS running buffer + 20% methanol. Electroblotting was done with 2 mA/cm^2 of membrane up to 50- 60 minutes (depending on protein size). After electroblotting, the membrane was blocked in 5% non-fat dry milk in 1x PBS for at least one hour at room temperature.

Antibody concentrations:

Table 1: Antibodies used in Western blotting

Antibody	Manufacturer	Species	Concentration
Anti-VASP (IE273)	Immunoglobe	mouse	1:1000
Anti-Mena (438)	Friendly gift from Dr. Peter Benz	rabbit	1:3000
Anti-His	Novagen	mouse	1:500
Anti-GST (2.4.08)	Friendly gift from Dr. Kai Schuh	rabbit	1:500
Anti-mouse IgG	Dianova	goat	1:5000
Anti-rabbit IgG	Dianova	goat	1:15000
Anti-mouse IgG minimal	Dianova	goat	1:2500

The membranes were incubated in 5% non-fat dry milk containing the primary antibody for at least one hour at room temperature or overnight at 4°C. After washing three times with 1x PBS + 0.05% Tween 20 (PBS-T) and incubation with secondary antibodies in 5% non-fat dry milk for another hour, the membranes were again washed three times with PBS-T.

Chemiluminescent detection was used to analyze the samples. Therefore, a self-made luminol solution (ECL) was used (ratio 1:1):

Solution A: 2.5 mM luminol, 400 µM p- cumar acid in 0.1 M Tris-HCl pH 8.5;

Solution B: 5.4 mM H_2O_2, 0.1 M Tris-HCl pH 8.5

2. Peptide scan arrays

Because of the divergent proline-rich regions of Ena/VASP proteins, binding of the αII-Spectrin SH3 domain to all human Ena/VASP proteins was compared in peptide spot arrays in collaboration with Dr. Stephan Feller, University of Oxford, UK. 30-mer peptides with 27 (Mena/VASP) or 28 (EVL) amino acid (aa) overlap, spanning the entire Mena (570 aa), VASP (380 aa), or EVL (418 aa) protein sequence, were synthesized on a cellulose membrane in an array of 30 x 20 spots (Cancer Research UK Protein and Peptide Chemistry Laboratory, Oxford, UK). The membranes were incubated with GST-αII-Spectrin SH3 fusion protein (1.65 µg/ml final concentration) or an equimolar concentration of GST alone as control (1.35 µg/ml final concentration). Western blotting was used to detect bound protein with GST-specific antibodies followed by HRP-conjugated secondary antibodies.

3. DNA specific molecular methods

3.1. Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation of DNA fragments to either visualize PCR amplifications and restriction enzyme digestion or to excise and purify DNA fragments after preparative DNA digestion. For a typical agarose gel electrophoresis, 10 µl of PCR reactions and of mini preparations or 50 µl of preparative DNA digestion were mixed with 6x DNA loading buffer. The gels contained 1% agarose dissolved in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 8 µl/ 100ml ethidium bromide. Electric separation was performed for 20 to 40 minutes at 120 V in 1x TAE buffer. Following electrophoresis, DNA was visualized by UV illumination. For size comparison, 6 µl of Generuler DNA ladder (Fermentas, SM0331) were loaded.

3.2. Isolation of plasmid DNA

The mini and maxi plasmid preparations were performed according to the manufacturer's instruction of NucleoSpin® Plasmid (Macherey Nagel, 740588) or NucleoBond® PC 500 (Macherey Nagel, 740574). For mini preparation 5 to

10 ml overnight bacteria culture was used, for maxi preparation 100 ml overnight bacteria culture. Quality of the purified plasmid DNA was analyzed by agarose gel electrophoresis subsequent to a restriction enzyme digestion. DNA quantity and purity was measured by UV spectrometry.

3.3. Restriction enzyme digestion

Restriction enzyme digestion was performed using restriction enzymes from New England Biolabs (NEB, Frankfurt am Main, Germany) or Fermentas (10 to 20 units/ µl). Plasmids were digested in a 50 µl reaction containing 10 µl mini preparation DNA, one µl of restriction endonuclease, 5 µl 10 x reaction buffer, and 5 µl 10 x bovine serum albumin (BSA), if required. The mixture was incubated for 1 h at 37°C and analyzed by agarose gel electrophoresis. DNA cleavage was performed with so called double digestion meaning that two different restriction enzymes were used simultaneously in a buffer that ensured 75% to 100% for both enzymes. In case of preparative digestion, agarose gel extraction was used for purification of the DNA fragments.

3.4. Polymerase chain reactions (PCR)

Amplification of DNA fragments was performed in a final volume of 50 µl. For cloning and amplification of DNA fragments Phusion High Fidelity DNA Polymerase (Finnzymes, F-530S) with proof-reading activity was used. Primers were designed according to the manufacturer's instruction from the DNA polymerase and ordered from MWG Biotech (Ebersberg, Germany).

Standard Phusion-PCR reactions:

10 µl of 5x HF or GC buffer (providing 1.5 mM $MgCl_2$ in final reaction),
200 µM dNTPs,
0.5 µM forward and reverse primer,
20 ng template DNA (plasmid DNA or PCR product),
1 unit Phusion DNA polymerase

Amplification of DNA fragments was typically performed in 30 cycles. Following an initial denaturation for 30 s at 98°C the first cycle started with 10 s at 98°C used for denaturation of amplified DNA fragments at the beginning of each cycle. The second step called annealing was performed for 30 s. The basic annealing temperature (T_{an}) was calculated according to the following formula:

$$T_{an} = 4x\ (G + C)\ +\ 2x\ (A + T)$$

G: number of guanine bases, C: number of cytosine bases, A: number of adenine bases, T: number of thymine bases.

For primers > 20 nucleotides a final annealing temperature of +3°C of the T_{an} of the lower primer was chosen; for primers < 20 nucleotides the calculated annealing temperature of the lower primer was chosen as final annealing temperature.
Following the annealing, elongation was performed at 72°C for 30 s/ kb of amplified product. After the last cycle 7 minutes elongation time for completion of incomplete DNA fragments were added. PCR amplification was analyzed by agarose gel electrophoresis.

3.5. Vectors

VASP constructs were cloned into pQE 30 (Qiagen) to get a 6x histidine-tag at the N-terminal end of the protein. This vector contains an ampicillin resistance. pGEX 6P2 (GE Healthcare) was used for expression of αII-Spectrin constructs. This vector contains the nucleotide sequence for GST (glutathione-S-transferase) at the N-terminal end of the insert and encodes also an ampicillin resistance. For expression of αII-Spectrin constructs in eukaryotic cells, pEGFP C1 (Clontech) was used, which encodes an EGFP (Enhanced Green Fluorescent Protein)-tag at the N-terminal end of the insert. This vector contains a kanamycin resistance.

3.6. Constructs

3.6.1. pQE 30 + His_6-VASP ΔTD

A mutant of His_6-VASP was generated which lacks the tetramerization domain of WT-VASP named His_6-VASP ΔTD. The tetramerization domain consists of the amino acids 336-380 of the human VASP [15, 16]. To delete these amino acids, primers were designed, which included the sequence of WT-VASP without the tetramerization domain. As template for the PCR, pQE30 + WT-VASP [16] was used. The product was cloned into pQE30 using the BamHI and SalI recognition sites as restriction sites (compare table 2).

3.6.2. pQE 30 + His_6-Mini-VASP

To get more information about the domains of WT-VASP involved in binding to αII-Spectrin, a construct was generated containing only the sequence of the proline-rich region and the tetramerization domain.

Figure 3: His_6-Mini-VASP. The His_6-Mini-VASP construct consists only of the proline-rich region (PRR) and the tetramerization domain (TD). Before and after the PRR some additional nucleotides according to the WT-VASP sequence were added (see also Figure 9).

At the N-terminus of this construct called His_6-Mini-VASP a BamHI restriction site was introduced, at the C-terminus an EcoRI (for subcloning into pGEX 6P1) followed by a SalI restriction site. The whole construct was synthesized by Eurofins MWG operon and cloned into the pCR2.1-Topo vector via TA cloning. The plasmid was shipped in lyophilized form in an amount of 4.3 µg and dissolved in distilled water at a final DNA concentration of 0.1 µg/ µl. The plasmid DNA was digested using BamHI and SalI and the resulting insert was subcloned into the digested pQE 30 vector.

Synthesized sequence:

5′GGATCCCAGCCCGGCCCGTCGGAGCACATAGAGCGCCGGGTCTCCAATGCAGGAGGCCCACCTGCTCC
CCCCGCTGGGGGTCCACCCCCACCACCAGGACCTCCCCCTCCTCCAGGTCCCCCCCCACCCCCAGGTTTG
CCCCCTTCGGGGGGTCCCAGCTGCAGCGCACGGAGCAGGGGGAGGACCACCCCCTGCACCCCCTCTCCCGG
CAGCACAGGGCCCTGGTGGTGGGGGAGCTGGGGCCCCACCCAGCTCCAGTGATTACTCGGACCTACAGAG
GGTGAAACAGGAGCTTCTGGAAGAGGTGAAGAAGGAATTGCAGAAAGTGAAAGAGGAAATCATTGAAGCC
TTCGTCCAGGAGCTGAGGAAGCGGGGTTCTCCCTGAGAATTCGTCGAC 3′

Yellow: BamHI; blue: EcoRI; violet: SalI; turquoise: S157-phosphorylation site; orange: proline-rich region; green: tetramerization domain. The non-colored areas are part of the normal WT-VASP sequence surrounding the proline-rich region.

3.6.3. PGEX 6P2 + His_6-Mena

The purification of Mena from *E. coli* is challenging [47]. Therefore, we used an approach with two tandem-tags: The murine Mena cDNA with an N-terminal 6x histidine (His_6)-tag was inserted into the pGEX 6P2 vector. So it was possible to purify GST-His_6-Mena via glutathione affinity purification. To get the His_6-Mena without GST, the GST-tag could be removed using the PreScission (MoBiTec) cleavage site of the pGEX 6P2 vector.

To generate the plasmid, primer for site directed mutagenesis were designed using pGEX 6P2 + WT-Mena as template (compare table 2).

3.6.4. pEGFP C1 + αII-Spectrin-α9A

To look at the αII-Spectrin – VASP/Mena interaction in the cell, the alternatively spliced repeat 9 of αII-Spectrin (including the 20 amino acids splice variant downstream of the SH3 domain) was cloned into the pEGFP C1 vector. As template pEGFP C1 + W1004R-α9A-mito (construct cloned during this work, data not shown) was used. The product was cloned into the pEGFP C1 vector using EcoRI and SalI as restriction enzymes (compare table 2).

3.6.5. pGEX 6P2 / pEGFP C1 + W1004R-αII-Spectrin-α9A

To get information about the importance of the Mena/VASP αII-Spectrin-α9A interaction in the cell, a construct with a point mutation in the SH3 domain was cloned. Because tryptophan 1004 is essential for SH3 mediated binding, mutation of this residue to an arginine should severely impair or abrogate interactions of the SH3 domain [42]. To get the point mutation, a site-directed

mutagenesis was performed either with the pGEX 6P2 + α9A construct as template or with the pEGFP C1 + αII-Spectrin-α9A + mito construct (construct cloned during this work, data not shown) (compare table 2).

3.6.6. Chaperones

Plasmids for expression of the chaperones described in table 5 were a friendly gift from Bernd Bukan [48]. The plasmids were directly used for transformation with Rotitransform (Roth, P043.1) according to the manufacturer's instruction.

Table 2: Constructs.

Restriction sites: underlined and indicated in colors (BamHI: orange, SalI: green, EcoRI: red). Start codons (ATG) are marked in green; Stop codons are marked in red. Nucleotides matching to the template sequence are indicated in blue. Nucleotides matching to the sequence of the GST-tag are indicated in magenta. The point mutation of the αII-Spectrin construct is shaded in violet. His_6-tag is indicated in lower case letters. Black capital letters mark an initial or final overhang or an additional nucleotide to ensure in frame amplification or to adjust the annealing temperature (T_{an}).

Construct	template	primer name	sequence (5'-3' direction)	T_{an} used in PCR
pQE 30+ His_6-VASP ΔTD	pQE 30+ WT-VASP	Forward: VASPdTD_Bam_F1	CACGGATCCGCCATGAGCG	62°C
pQE 30+ His_6-VASP ΔTD	pQE 30+ WT-VASP	Reverse: VASPdTD_Bam_R1	GGGGTCGACTCACGTGCAGGGTTGGGTCTCG	
pGEX 6P2+ His_6-Mena	pGEX 6P2+ Mena	Forward: Tandem-Mena F1	CTGGAAGTTCTGTTCCAGGGGCCCcatcaccatcaccatcacATGAGTGAACAGAGTATCTGTCAG	68°C
pGEX 6P2+ His_6-Mena	pGEX 6P2+ Mena	Reverse: Tandem-Mena R1	CTGACAGATACTCTGTTCACTCATgtgatggtgatggtgatgGGGCCCCTGGAACAGAACTTCCAG	
pGEX 6P2+ W1004R-αII-Spectrin-α9A	pGEX 6P2+ αII-Spectrin-α9A	Forward: SPCN W1004R_F	GCACAAACAAGGATAAGGTGGAAAGTGGAAG	63°C
pGEX 6P2 + W1004R-αII-Spectrin-α9A	pGEX 6P2+ αII-Spectrin-α9A	Reverse: SPCN W1004R_R	CTTCCACTTTCCACCTATCCTTGTTTGTGC	63°C
pEGFP C1+ αII-Spectrin-α9A	pEGFP C1 + αII-Spectrin-α9A-mito	Forward: EGFPa9i for	ACCGAATTCCCAGCAGTACTTTGCCGATGC	65°C
pEGFP C1+ αII-Spectrin-α9A	pEGFP C1 + αII-Spectrin-α9A-mito	Reverse: EGFPa9i rev_2	TTGGTCGACTCACTCCAACATGCCTTTTCTCTTC	
pEGFP C1+ W1004R-αII-Spectrin-α9A	pEGFP C1 + W1004R-αII-Spectrin-α9A-mito	Forward: EGFPa9i for	ACCGAATTCCCAGCAGTACTTTGCCGATGC	65°C
pEGFP C1+ W1004R-αII-Spectrin-α9A	pEGFP C1 + W1004R-αII-Spectrin-α9A-mito	Reverse: EGFPa9i rev_2	TTGGTCGACTCACTCCAACATGCCTTTTCTCTTC	

3.7. PCR purification

To clean up PCR fragments or to extract DNA from an agarose gel, the NucleoSpin Extract kit from Macherey-Nagel (Düren, Germany) was used. Because of the pore size in the silica membranes of the kit, small nucleotide fragments such as primers do not bind to the matrix allowing the purification of the PCR fragments alone. To purify DNA fragments from restriction enzyme digestions, agarose gel electrophoresis was performed followed by excision and purification of the DNA fragment as described above.

3.8. Cloning of PCR products

3.8.1. Cloning by introduction of restriction sites

PCR products, which were generated with primers that introduced new restriction sites, were digested with the corresponding restriction endonucleases. The corresponding vectors were treated in the same way. Vectors, which were digested with only one restriction endonuclease, were dephosphorylated using 1 µl shrimp alkaline phosphatase (SAP) to prevent re-ligation of the vector. After gel electrophoresis, digested inserts and vectors were excised and purified by gel extraction.

Ligation was performed according to the manufacturer's instruction of New England Biolabs T4 DNA ligase in a final volume of 20 µl. Ligation reactions contained 1 µl T4 DNA ligase, 2 µl 10x T4 DNA ligase buffer (Fermentas), and vector and insert DNA in a molar ratio of 1:3. Ligations were incubated for 30 minutes at room temperature.

3.8.2. Cloning by site-directed mutagenesis

Site-directed mutagenesis is used to generate point mutations, replace amino acids or delete/insert single or multiple amino acids [49]. In general, the double strand DNA of a vector with the insert of interest is used with two oligonucleotide primers, which contain the desired mutation. The primers are both complementary to the opposite strands of the vector. PCR amplification (performed as described above) leads to a mutated plasmid. Following the PCR reaction, treatment of the reaction with DpnI is necessary to digest the

methylated parental template DNA and to select for the mutated new synthesized DNA, which is not methylated.

Primers were designed as described above. For calculation of the annealing temperature only complementary nucleotides were considered. For digestion of the parental DNA, 1 µl of DpnI was added to the PCR reaction for one hour at 37°C. PCR amplification was analyzed by agarose gel electrophoresis.

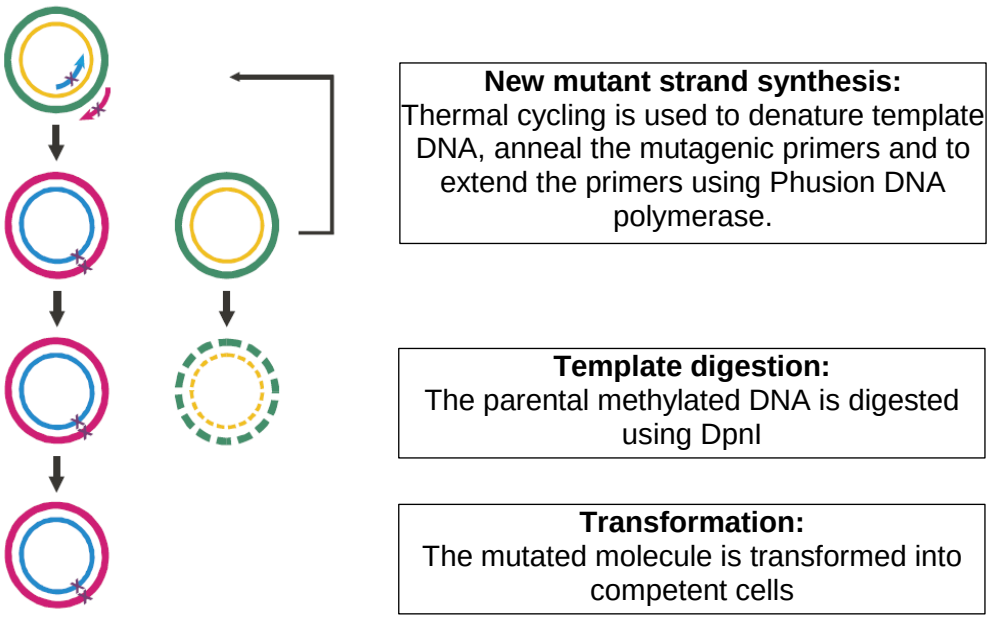


Figure 4: Site-directed mutagenesis. Further explanations are given in the text. Figure taken from QuickChange II Site-directed mutagenesis kit, instruction manual, Agilent Technologies.

3.9. Transformation and expression of plasmid DNA

About 50 ng of plasmid DNA or 10 µl of ligation reactions were transformed into XL10 ultracompetent cells (Stratagene, 200315) according to the manufacturer's instructions. After transformation, the bacteria were plated onto 1.5% LB agar plates containing an appropriate antibiotic resistance. The plates were incubated overnight at 37°C.

Table 3: Vectors and corresponding antibiotic resistances.

Vector	Antibiotic resistance
pGEX 6P2	Ampicillin
pQE 30	Ampicillin
pEGFP C1	Kanamycin

3.10. DNA Sequencing

DNA sequencing was performed by MWG using the chain-termination method by Sanger.

4. Bacteria strains

For DNA expression, XL10 ultracompetent cells (stratagene, 200315) were used as described above. For protein expression, plasmids were transformed into *Escherichia coli (E.coli)* Rosetta DE3 (Novagen, 70954-3). This BL21 derivative supplies tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and GGA on a compatible chloramphenicol-resistant plasmid pRARE. By supplying rare codons, the Rosetta strain allows the expression of eukaryotic proteins containing for example many prolines, which would otherwise be limited by the codon usage of *E.coli*. Only in case of chaperone expression, the parental *E. coli* BL21 strain was used. This strain has no antibiotic resistance itself. All bacteria were cultivated in 2x YT medium (Roth, X966.2) at 37°C. The medium contained 16 g/l trypton, 10 g/l yeast extract and 5 g/l NaCl. According to the bacteria and plasmid resistance, ampicillin (100 µg/ml final concentration), chloramphenicol (40 µg/ml final concentration) and/or spectinomycin (100 µg/ml final concentration) were added.

5. Transformation and expression of chaperones

The plasmids containing the different chaperone combinations (one chaperone with spectinomycin resistance, the other with chloramphenicol resistance) described in table 8 were transformed into *E.coli* BL21 (no antibiotic resistance) by Rotitransform (Roth, P043.1). Bacteria were plated onto 1.5% LB agar

dishes + spectinomycin + chloramphenicol overnight at 37°C. After transformation, the bacteria were inoculated in 2x YT+ spectinomycin (100 µg/ml final concentration) and chloramphenicol (40 µg/ml final concentration) and used for transformation of GST-Mena (see 2.7).

Subsequently, Rosetta DE3 (chloramphenicol resistance) were transformed with pBB 535 or pBB 542 (spectinomycin resistance). Bacteria were plated onto 1.5% LB agar dishes + spectinomycin overnight at 37°C. After transformation, the chaperone-expressing bacteria were used for transformation of GST-His_6-Mena.

6. Expression and purification of His_6-tagged VASP and GST fusion proteins

6.1. Transformation of bacteria strains

For overexpression of human His_6-VASP and three mutants of wild type VASP (His_6-VASP G181P, His_6-Mini-VASP, and His_6-VASP ΔTD) *E. coli* Rosetta DE3 were used and cultured as described above. For transformation, Rotitransform (Roth, P043.1) was used according to the manufacturer's instruction (about 1 µg of plasmid was used for transformation). The bacteria were plated on 1.5% LB agar plates + ampicillin.

For overexpression of the Mena protein, about 1 µg of the GST-Mena plasmid was transformed into *E.coli* BL21 already expressing a plasmid combination encoding further chaperones (see table 8); 1 µg of the GST-His_6-Mena plasmid was transformed either into the commercial Rosetta DE3 strain or into Rosetta DE3 already expressing a plasmid encoding further chaperones (pBB 535 or pBB 542; see table 8). The bacteria were plated on 1.5% LB agar plates + ampicillin + spectinomycin + chloramphenicol.

For overexpression of αII-Spectrin proteins, about 1 µg of plasmids encoding GST, A, SH3, α9, or α9A (all constructs are friendly gifts from Dr. Peter Benz) was transformed into Rosetta DE3. The bacteria were plated on 1.5% LB agar plates + ampicillin. All plates were incubated overnight at 37°C.

Table 4: Plasmids used for transformation of *E.coli.*

	Vector	Insert	Designer
His_6-VASP	pQE 30	Human VASP	[16]
His_6-VASP G181P	pQE 30	G181P VASP	Dr. Peter Benz
His_6-Mini-VASP	pQE 30	Mini-VASP	Kristin Offner
His_6-VASP ΔTD	pQE 30	VASP ΔTD	Kristin Offner
GST-Mena	pGEX 6P2	Mena	Dr. Peter Benz
GST-His_6-Mena	pGEX 6P2	Mena	Kristin Offner
GST-αII-Spectrin constructs	pGEX 6P2	αII-Spectrin constructs	Dr. Peter Benz

6.2. Expression of recombinant proteins

After transformation, one colony was inoculated in 5 ml medium containing ampicillin (100 µg/ml final concentration) and chloramphenicol (40 µg/ml final concentration) at 37°C and 220 rpm/min. In case of chaperone co-expression, the medium additionally contained spectinomycin at a final concentration of 100 µg/ml. When the cultures appeared cloudy, new medium was added. End volumes are given in table 5.

The cultures were shaken in flasks at 37°C and 220 rpm/min until OD_{600} reached about 1.0 (in case of chaperone co-expression: OD_{600} reached about 1.6). Then protein expression was induced by addition of isopropyl-β-D-thiogalactopyranosid (IPTG) (Roth, 2316.4). Final concentrations, overall expression times, and expression temperatures are given in table 5. After expression of the proteins at 220 rpm/min the bacteria were harvested by centrifugation for 20 minutes at 4600 rpm (Heraeus Sorvall, 75006445) and 4°C. The supernatant was discarded; the bacteria pellets were stored in 50 ml falcons at -20°C. Estimated pellet volumes are given in table 5.

Table 5: Expression parameters used for purification of proteins.

	Bacteria strain	Final volume	Final IPTG concentration	Temperature for protein expression	Expression time	Pellet volume	Size of construct	Additional chaperones
His_6-VASP	Rosetta DE3	800 ml	1 mM	37°C	40 hours	400 ml	46 kDa	-
His_6-VASP G181P							46 kDa	
His_6-Mini-VASP		400 ml	0.3 mM		5 hours	50 ml	15 kDa	
His_6-VASP ΔTD		2x 700 ml	1 mM		24 hours	300 ml	40 kDa	
GST-Mena	*E.coli* BL21	400 ml	0.3 mM	10°C	36 to 48 hours	100 to 200 ml	113 kDa	pBB 528/pBB 541
								pBB 530/pBB 535
								pBB 535/pBB 540
								pBB 540/pBB 542
								pBB 540/pBB 550
GST-His_6-Mena	Rosetta DE3	500 ml	0.3 mM	10°C	72 hours	600 ml	113 kDa	-
		400 ml			36 to 48 hours	100 to 200 ml		pBB 535
								pBB 542
GST-αII-Spectrin fusion proteins	Rosetta DE3	500 ml	0.3 mM	30°C	4 to 6 hours	50 to 100 ml	25- 49 kDa	-

6.3. Purification of His_6-VASP proteins

The bacteria pellets were thawed on ice and re-suspended in 15 ml binding buffer (see table 6) containing 1x protease inhibitor (complete, EDTA–free protease inhibitor cocktail tablets; Roche, 1 873 580) by overnight rotation at 4 °C. Then lysozym (Serva, 28262.03) was added to a final concentration of 1 mg/ml and the bacteria were incubated on ice for 30 min. Subsequently, sonication of the bacteria was performed with 70% intensity and 90% cycle interval (Bandelin, equipped with a M573 tip sonoplus SH 70G) for 3 minutes (His_6-Mini-VASP: 2 minutes; His_6-VASP ΔTD: 3.50 minutes). After lysis, the bacteria were centrifuged for 20 minutes at 4°C and 32500 rpm (Beckman TI-70). Each supernatant was combined with 5 ml HisPur cobalt resin (Pierce, 89964; binding capacity 10 mg/ml) (His_6-Mini-VASP: 150 µl; His_6-VASP ΔTD: 100 µl) in a 15 ml falcon and rotated for 30 to 60 minutes at 4°C. Afterwards, the cobalt sepharose from each falcon tube was collected using a 5 ml disposable PE-column (Pierce, 29922). For the washing steps, the beads were first re-suspended and then centrifuged at 4°C and 500 g for one minute. Buffers and buffer amounts used for washing are shown in table 6. For the elution steps, the beads were re-suspended in elution buffer (see table 6) and incubated for about one minute at room temperature before saving the elution fraction. The purification was verified by SDS-PAGE and coomassie staining.

Table 6: Buffers used for purification of His_6-VASP proteins (ordered chronologically).

	His_6-VASP/ G181P-VASP		**His_6-Mini-VASP**		**His_6-VASP ΔTD**	
	amount	buffer	amount	buffer	amount	buffer
1. Binding buffer	15 ml	50 mM Na-Phosphat pH 7.4; 1 M NaCl; 5 mM imidazole pH 7.4	15 ml	50 mM Na-Phosphat pH 7.4; 1 M NaCl; 5 mM imidazole pH 7.4	8 ml	50 mM Na-Phosphat pH 7.4; 1 M NaCl; 1 mM imidazole pH 7.4
2. Washing buffer 1	50 ml	50 mM Natrium-Phosphat pH 7.4; 1 M NaCl; 20 mM imidazole pH 7.4	15 ml	50 mM Natrium-Phosphat pH 7.4; 1 M NaCl; 20 mM imidazole pH 7.4	10 ml	50 mM Na-Phosphat e pH 7.4; 1 M NaCl; 7.5 mM imidazole pH 7.4
3. Washing buffer 2	50 ml	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl; 20 mM imidazole pH 7.4	20 ml	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl, 5 mM ATP and 10 mM $MgSO_4$; 40 mM imidazole pH 7.4	10 ml	50 mM Natrium-Phosphat e pH 7.4; 100 mM NaCl; 10 mM imidazole pH 7.4
4. Washing buffer 3	20 ml	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl, 5 mM ATP and 10 mM $MgSO_4$	20 ml	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl; 40 mM imidazole pH 7.4	10 ml	50 mM Natrium-Phosphat e pH 7.4; 100 mM NaCl, 15 mM imidazole pH 7.4
5. Washing buffer 2	20 ml	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl; 20 mM imidazole pH 7.4				
6. Elution buffer	5x 300 µl	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl, 150 mM imidazole	3x 200 µl	50 mM Natrium-Phosphat pH 7.4; 100 mM NaCl, 300 mM imidazole	4x 100 µl	50 mM Natrium-Phosphat e pH 7.4; 100 mM NaCl, 150 mM imidazole

6.4. Gelfiltration of His_6-VASP proteins

For pull-down assays, elution fractions were pooled and gel-filtrated according to the manufacturer's instruction of Econo-Pac® 10DG columns (biorad, 732-2010; size exclusion 6 kDa). Therefore, a buffer containing 40 mM Hepes-NaOH pH 7.4 and 75 mM NaCl was used. For gel-filtration of His_6-VASP ΔTD, ¼ columns were used so 750 µl protein sample were eluted with 1 ml filtration buffer. Gelfiltration was verified by SDS-PAGE and coomassie staining.

6.5. Purification of GST fusion proteins

One bacteria pellet was re-suspended in 10 ml ice–cold PBS containing 1x protease inhibitor (complete, EDTA–free protease inhibitor cocktail tablets; Roche, 1 873 580). Then lysozym (Serva, 28262.03) was added to a final concentration of 1 mg/ml and the bacteria were incubated on ice for 30 min. Afterwards, sonication of the bacteria was performed with 70% intensity and 90% cycle interval (Sonoplus SH 70G, Bandelin) for 2.50 to 3.50 minutes. After lysis, the bacteria were centrifuged for 20 minutes at 4°C and 32500 rpm (Beckman TI-70). The supernatant was transferred to a 15 ml falcon with 500 µl glutathione sepharose 4 fast flow slurry (GE–Healthcare, 17–5132–01) for GST-Spectrin and 120 µl slurry for GST-His_6-Mena (binding capacity: about 12 mg protein/ml settled beads). The falcon was rotated for 60 minutes at 4°C. After this, the beads were spun down at 500 g and 4°C and the supernatant was discarded. The beads were washed three times with 10 ml ice-cold PBS, then they were stored in 5 ml ice-cold PBS containing 1x protease inhibitor on ice or eluted in elution buffer (300 mM Tris-HCl pH 9.3, 100 mM reduced glutathione).

7. Cleavage of GST fusion proteins (pGEX 6P2)

The pGEX 6P2 vector contains a cleavage site for the PreScission protease. By adding the protease to the GST fusion proteins it is possible to separate the GST-tag and the fusion protein (GST gene fusion system, Amersham Biosciences). There are two possibilities of cleavage: One is to add the protease directly to the glutathione sepharose with the bound GST fusion

protein (on-column cleavage) and the other is to elute the GST fusion protein from the sepharose and perform the cleavage in solution.

7.1. Cleavage of GST-Spectrin proteins

Cleavage of GST-Spectrin fusion proteins was performed using the on-column method. To get a time kinetic, 200 µl cleavage buffer (50 mM Tris-HCl pH 7.4; 50 mM NaCl; 1 mM DTT; 1 mM EDTA) were added to 100 µg GSH sepharose with GST-Spectrin. The beads were re-suspended and centrifuged in a table-top centrifuge. 20 µl of the supernatant were saved as control (t=0) with 10 µl SDS sample buffer. Then, 5 units HRV3C (MoBiTec; PR-ETA20010), which recognizes the same site as the PreScission protease, were added to the remaining slurry. After 2.5 hours and 25 hours at 4°C, 20 µl were saved as mentioned above. After this, the remaining supernatant was discarded and 200 µl of the loading dye were added to the beads to remove all the remaining protein from the beads.

7.2. Cleavage of GST-His_6-Mena

Cleavage of GST-His_6-Mena was performed after eluting the protein from the GSH sepharose. Therefore, 5x 100 µl elution buffer were added to the beads with the fusion protein. After pooling of the elution fractions, 10 µl were saved for SDS-PAGE. To the remaining elution 40 units HRV3C were added. The reaction was incubated for 16 hours at 4°C. After gel filtration and incubation of the filtrated reaction with GSH sepharose to get rid of the cleaved GST and the HRV3C protease, which also contains a GST-tag, 10 µl of the supernatant were saved for SDS-PAGE to analyze the cleavage reaction.

8. Purification of Mena from murine heart

Mena was purified from sacrificed murine wild type mice using an antibody column (binding capacity about 500 µg proteins). For the column, Mena antibody (α-Mena 438) was coupled to Affigel-10 (biorad, 153-6099) due to the isoelectric point of immunoglobines according to the manufacturer’s instruction. Elution of the protein was performed by pH-shift.

8.1. Antibody buffer change

Because primary amines bind to Affigel-10 spontaneously, it was necessary to change the normal Tris-buffer, in which the α-Mena 438 antibody was supplied, into a buffer without amino groups (100 mM MOPS pH 7.5). Therefore a gel filtration (Econo-Pac 10 DG, biorad) was performed according to manufacturer's instruction.

8.2. Coupling of the antibody to Affigel-10

The antibody was mixed with pre-equilibrated 400 µl Affigel-10 slurry and rotated overnight at 4 °C. Then the supernatant was discarded and the column was washed with 5 ml 100 mM MOPS pH 7.5. Remaining reactive sites were saturated using 5 ml 100 mM MOPS pH 7.5 + 400 µl 1 M ethanolamine pH 8.0. Afterwards, the Affigel-10 was transferred to a small column (Pierce, 29922) and washed with 10 ml 10 mM Tris-HCl pH 7.4, 500 mM NaCl, 5 ml 100 mM Glycine-HCl pH 2.5; 10 ml 10 mM Tris-HCl pH 9.3; and 10 ml 10 mM Tris-HCl pH 7.4.

8.3. Purification of Mena from murine heart

Mena was purified from 8 wild type murine hearts. The hearts were lysed by ultraturrax (Polyton PT 3100) in 1 ml lysis buffer per heart (lysis buffer: 40 mM Hepes-NaOH pH 7.4, 75 mM NaCl, 1% Igepal CA-630, 1x protease inhibitor). After centrifugation for 10 minutes at 17000 g, the supernatant was sterile-filtrated and transferred to the antibody column. After incubation for 1 to 3 hours at 4°C, the column was washed with 10 ml 40 mM Hepes-NaOH pH 7.4, 1% Igepal CA-630 and 500 mM or 1 M NaCl and afterwards with 10 ml aqua dest. Elution of the protein was performed using pH-shift. Therefore, 5x 300 µl 100 mM glycine-HCl pH 2.5 were added to the column and each flow through was directly added to 33 µl 1 M Tris-HCl pH 9.3. The pooled elutions were gel filtrated (Econo-Pac 10 DG, biorad) and analyzed by SDS-PAGE.
For re-usage of the antibody column, the column was washed with 5 ml 100 mM glycine-HCl pH 2.5 to get rid of all remaining protein, and then with 2 ml 100

mM Tris-HCl pH 8.5 to neutralize the pH. The column was stored at 4°C in 1x PBS and 1x protease inhibitor.

9. GST-pull-down assays

For GST-pull-down assays with heart lysate, two hearts of sacrificed wild type mice were lysed in 1 ml lysis buffer each (40 mM Hepes-NaOH pH 7.4, 75 mM NaCl, 1% Igepal CA 630, 1x protease inhibitor) by ultraturrax (Polyton PT 3100). The lysate was cleared by centrifugation at 17000 g and 4°C for 10 minutes and 400 µl of the lysate were then incubated with 2 µg GST coupled to GSH sepharose or equimolar amounts of the coupled GST fusion proteins for one hour at 4°C.

For pull-down assays with purified Mena/VASP protein, VASP or Mena (0.5 µg to 2 µg) were first transferred into a buffer containing 40 mM Hepes-NaOH and 75 mM NaCl by gel filtration (Econo-Pac 10 DG, Biorad) and then incubated with 1-5 µg coupled GST or corresponding amounts of the GST fusion proteins in a buffer containing 40 mM Hepes-NaOH pH 7.4, 75 mM NaCl, 1% Igepal CA-630, 1x protease inhibitor and 1% BSA. The final volume of all pull-down experiments was between 200 µl and 500 µl. After one hour incubation at 4°C the precipitates were washed with pull-down buffer and analyzed by SDS-PAGE and Western blotting.

10. Isothermal titration calorimetry (ITC)

ITC is a convenient method to study SH3 domain interactions with up to 20 amino acid long peptides by measurement of thermodynamic parameters of the biochemical binding process. ITC can be used for the quantitative measurement of the binding affinity (K_D), binding enthalpy changes (ΔH), and stoichiometry (n). Using these parameters it is possible to calculate the entropy changes (ΔS) and Gibbs energy changes (ΔG).

An isothermal titration calorimeter is composed of two identic thermal conductive materials like gold or hastelloy, which are surrounded by an adiabatic jacket. Highly sensitive thermoelements determine the temperature of

the liquid in these cells precisely. One of the cells is used as reference (containing water or buffer solution), the other cell contains the molecule of interest in the same solution as in the reference cell. Prior to the addition of the ligand, a constant power is applied to both cells. A feedback loop regulates the power application in the sample cell in accordance to the temperature in the cell. During the measurement, the ligand is added in a precisely known amount. This leads to an increase of the temperature in the sample cell in case of an exothermic reaction, whereas the temperature decreases in case of an endothermic reaction. ITC measures the time-dependent application of power that is needed to adjust the temperature of the sample cell in accordance to the reference cell. The observations are plotted as the power that is needed to maintain the same temperatures in both cells per second (µcal/sec). The diagram of the raw data shows so-called spikes, each of them corresponds to a ligand injection and the consecutive temperature change and feedback regulation in the sample cell. Integration of the spikes with respect to time gives the total heat exchanged per injection. The pattern of these effects can then be analyzed as a function of the molar ratio of ligand/macromolecule, allowing the determination of the thermodynamic parameters of the interaction.

In case of investigation of the interaction between SH3 domains and the up to 20-fold larger Mena/VASP, ITC would require protein amounts in the high milligram range, which are challenging to obtain as described above. Therefore, a truncated Mena peptide consisting of the 22 amino acids QASVALPPPPGPPPPPPLPSTG was used in the experiments. This protein partially contains the proline-rich region but is missing the tetramerization domain leading to a 22 amino acid monomeric protein. The experiment was performed in collaboration with Dr. Stephan Feller, University of Oxford. The experiments were performed with 0.1 mM truncated Mena and GST-αII-Spectrin-SH3, -α9, and α9A (2 mM for the first two peptides, 4 mM for the latter).

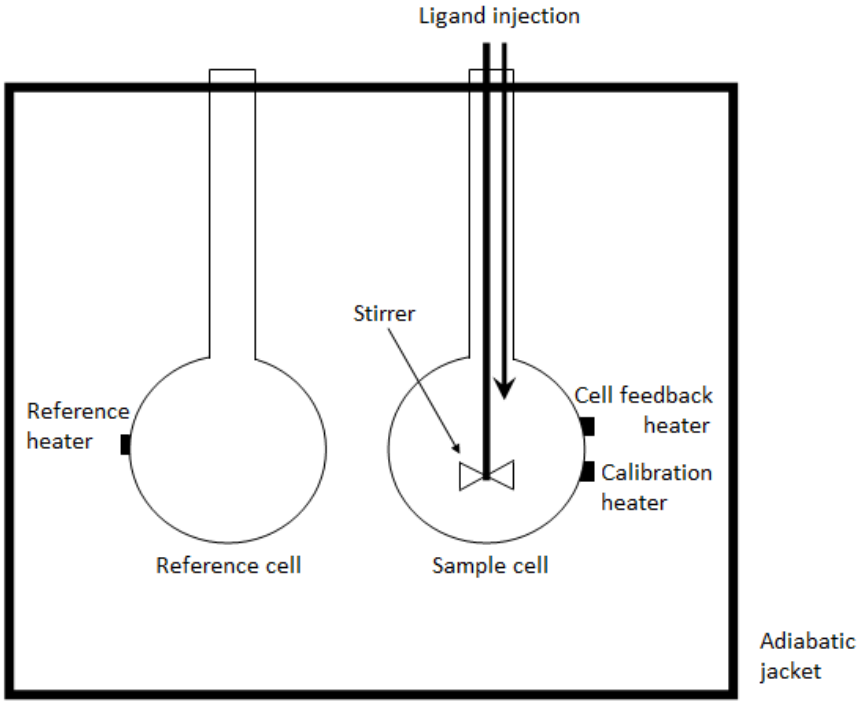


Figure 5: Schematic Figure of the ITC instrument. The two cells of the instrument, consisting of a highly heat conducting material like gold, are surrounded by an adiabatic jacket. The reference cell contains a reference solution like water or buffer and is heated by a reference heater. The sample cell contains the same liquid as the reference cell, but with the macromolecule of interest. Prior to the addition of the ligand, the same power is applied to both cells. After addition of the ligand the feedback heater registers the heat changes that are caused by the reaction and regulates the application of power to the sample cell in accordance to the reference cell. Figure taken from https://en.wikipedia.org/wiki/Isothermal_titration_calorimetry#/media/File:ITC1.png

11. Cell culture and transfection of the cells

Endothelial cells (ECV 304, [50]) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) F12 (Invitrogen, 11320-074) containing 4.5 g/l glucose, 1 mM sodium pyruvate, 10% FCS and 1x L-glutamine at 37°C and 5% CO_2. Cell passages were usually performed twice a week when cells had reached confluence. Transfection was performed with Lipofectamin 2000 (Invitrogen, 11668-027) according to the manufacturer's instruction.

12. Immunofluorescence staining of ECV 304

For immunofluorescence microscopy of ECV 304 endothelial cells two different approaches were performed: In one approach, the cells were cultivated on chamber slides (Lab Tek II, 2-well chamber slides, 154461, thermoscientific) until they reached an adherent state. Then transfection was performed and the cells were incubated another 10 hours. In the other approach, the cells were first cultivated in 6 cm cell culture dishes until they reached confluence, then transfected and incubated for another 10 hours, but then they were rinsed and re-cultivated for several hours on chamber slides to fix them in a migrating, not fully adherent state. In detail, the cells from the second approach were rinsed and detached with trypsin-EDTA and collected in complete growth medium. After centrifugation for 4 minutes at room temperature and 240 g the supernatant was discarded and the cells were collected in new medium. The cells were then plated for 5 hours on chamber slides.

The cells on the chamber slides (both approaches) were then washed with 37°C warm 1x PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and then fixed using 4% paraformaldehyde (PFA) in 1x CB (10 mM PIPES pH 6.8, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$) for 8 minutes at room temperature. The surplus PFA was quenched using 50 mM NH_4Cl in 1x CB, permeabilized using 0.2% TritonX-100 in 1x CB, and blocked in 10% NGS in 1x CB for one hour. After overnight incubation with primary antibodies, the cells were washed with 1x CB and incubated with fluorescence conjugated secondary antibodies or phalloidin (visualizes F-actin) for 3 hours. After three rounds of washing, the cells were mounted onto glass slides using MOWIOL DABCO (Sigma). All steps were performed with sterile filtered solutions.

Table 7: Antibodies and fluorescent probes used for immunofluorescence microscopy.

Antibody	**Concentration**
Anti-VASP (19728)	1:750
Anti-Mena (438)	1:250
Anti-GFP (G 6539, Sigma)	1:200
TRITC-Phalloidin (P1951, Sigma)	1:350
Alexa 633 goat anti-rabbit (Molecular Probes, A21070)	1:800
Alexa 488 goat anti-mouse (Molecular Probes, A11001)	1:800

3 RESULTS

1. Purification of GST-αII-Spectrin proteins

For usage in all interaction experiments, different GST-αII-Spectrin constructs were designed. As described in 1.4, αII-Spectrins consist of 20 triple-helix motives. In this work, the focus of interest was the function of the SH3 domain in repeat 9 of αII-Spectrin and the alternative splice variant SH3*i*, which contains a 20 amino acid insertion C-terminal to the SH3 domain [2, 35]. Therefore, the following GST-αII-Spectrin proteins were purified (Figure 6):

A) GST alone: Serving as negative control
B) GST+A: GST with the additional 20 amino acid of the SH3*i* splice variant
C) GST+SH3: GST with the SH3 domain of repeat 9
D) GST+α9: GST with the whole α9 repeat
E) GST+α9A: GST with the whole α9 repeat of SH3*i* including the alternatively spliced 20 amino acids
F) GST+W1004R-α9A: GST with a point-mutated α9A repeat in position 1004 (tryptophan to arginine), leading to a non-functional SH3 domain

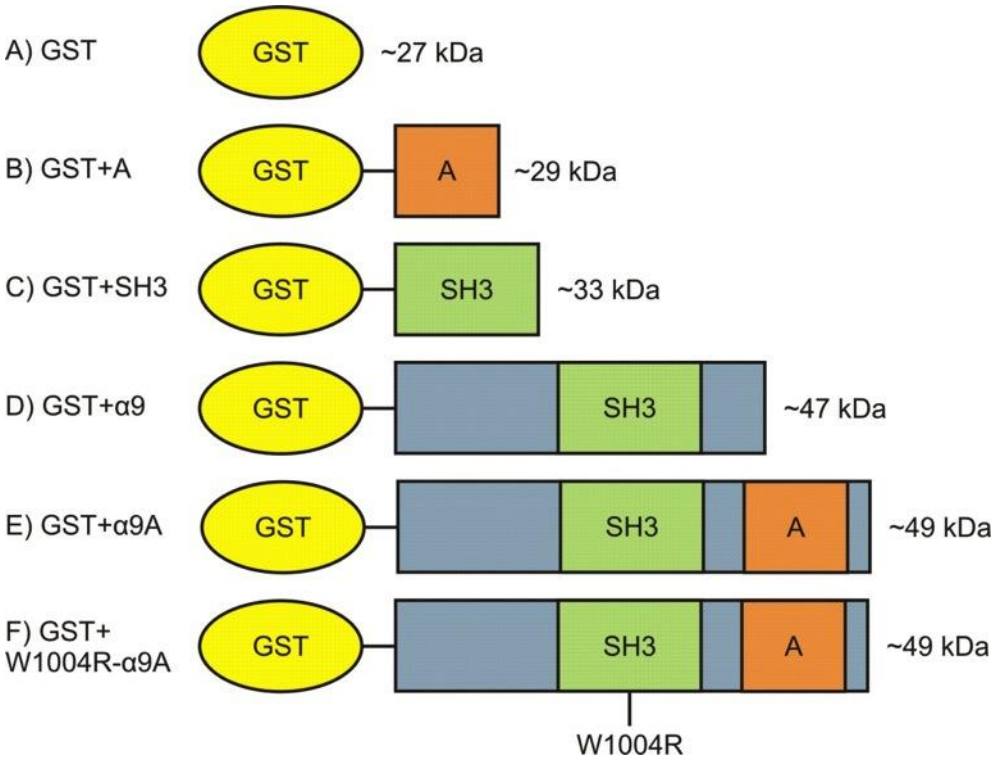


Figure 6: GST-αII-Spectrin fusion proteins. A) GST alone was used as negative control; B) GST+A consists of GST+ the 20 amino acid insert of the SH3*i* splice variant (A indicated in red); C) GST+SH3 consists of GST+ the SH3 domain of αII-Spectrin repeat 9 (SH3 indicated in green); D) GST+α9 consists of GST+ repeat 9 of αII-Spectrin (α9 repeat indicated in blue); E) GST+α9A consists of GST+ repeat 9 of SH3*i* including the alternatively spliced 20 amino acids; F) GST+W1004R-α9A consists of the GST+ repeat 9 of αII-Spectrin SH3*i* with the alternatively spliced 20 amino acids and is mutated in position 1004 (W→R) resulting in an inactive SH3 domain.

The proteins were purified using glutathione affinity chromatography as described in 2.6. Purification was performed using 50 ml bacteria culture for GST; GST+A; GST+SH3 and GST+α9 whereas 100 ml bacteria culture for GST+α9A and GST+W1004R-α9A were used. After purification, the GSH sepharose-bound protein was diluted in 5 ml 1x PBS. Because of high protein amounts, another 1:10 dilution was necessary to analyze the purification by SDS-Page and coomassie staining. Therefore, 10 µl of 5x SDS loading buffer were added to 10 µl of the dilution and the samples were loaded onto a 12% SDS gel (Figure 7). Loaded protein amounts equal 2‰ of the total protein amount.

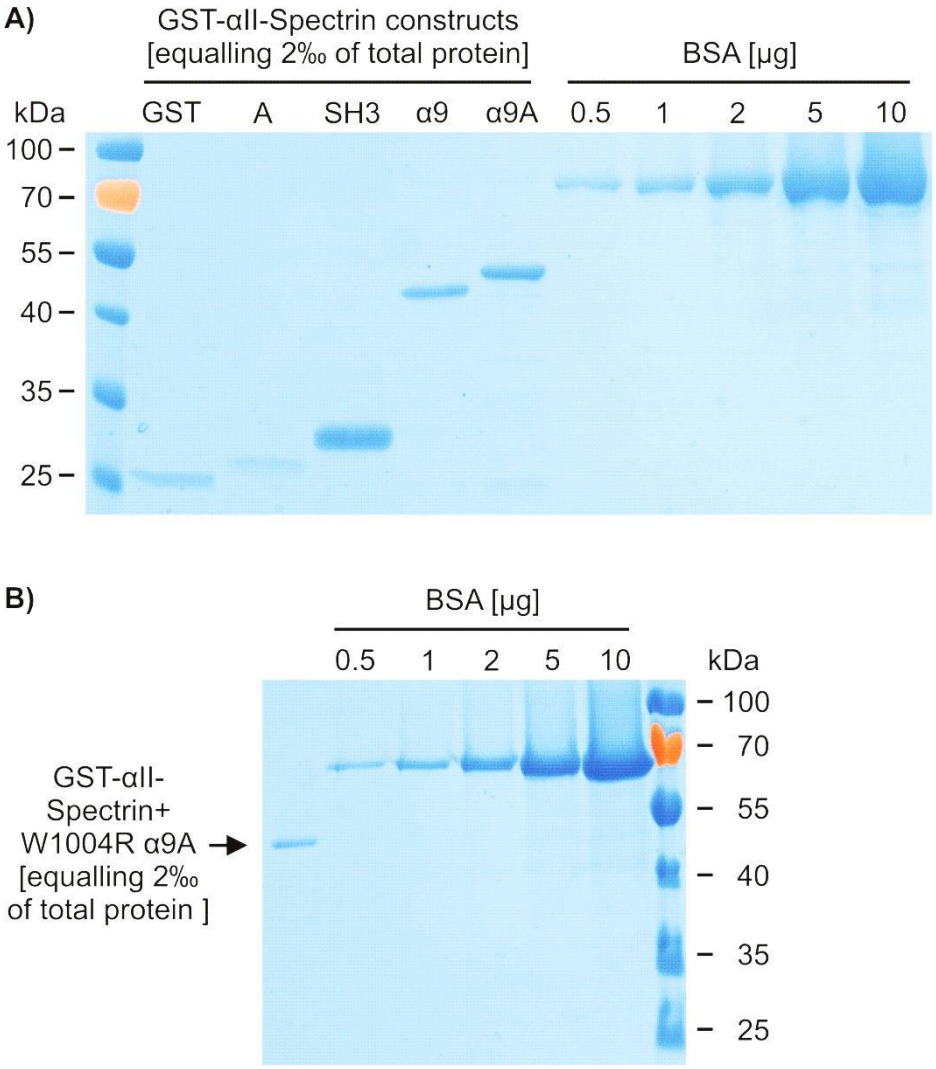


Figure 7: Purification of GST-αII-Spectrin constructs (12% SDS-PAGE).
A) Purification of GST, GST+A, GST+SH3, GST+α9, GST+α9A. Protein sizes and explanation of the constructs are given in Figure 6.
B) Purification of GST+ W1004R-α9A.
Protein sizes and explanation of the constructs are given in Figure 6. The GSH sepharose-bound protein was originally diluted in 5 ml 1x PBS. Because of the high protein amounts, another 1:10 dilution was necessary, loaded protein amounts equal 2‰ of the total protein amount. Protein amounts were estimated by comparison with the BSA standard. Purification amounts varied from 2.5 mg/50 ml bacteria culture for GST+A; to 15 mg/50 ml for GST+SH3. Amounts of the other GST-αII-Spectrin constructs were in between these amounts.

The protein purification of the GST-αII-Spectrin fusion proteins showed single bands in good agreement with the calculated molecular weight and no degradation products, indicating excellent purity. Even for the point-mutated GST+W1004R-α9A the purification showed a single band without degradation,

indicating correct protein folding. Comparison of the protein amounts with the BSA standard showed that GST and GST+α9 could be purified in an amount of about 100 mg/l bacteria culture. The amount of GST+A and GST+α9A was little lower with about 50 mg/l bacteria culture regarding GST+A; 60 mg/l bacteria culture regarding GST+α9A and 40 mg/l bacteria culture regarding GST+W1004R-α9A. GST-SH3 could be purified in highest protein amounts up to 300 mg/l.

2. Peptide scan arrays

Because of the divergent proline-rich regions (PRR) of Ena/VASP proteins, binding of the αII-Spectrin-SH3 domain to all human Ena/VASP proteins was compared in peptide spot arrays as described in 2.2. In brief, 30 amino acid long peptides spanning the whole Mena, VASP or EVL sequence were synthesized onto a cellulose membrane and then incubated with the GST-SH3 fusion protein or GST alone as control. Detection was performed by Western blotting using GST specific antibodies and ECL. The arrays were performed in collaboration with Dr. Stephan Feller, University of Oxford.

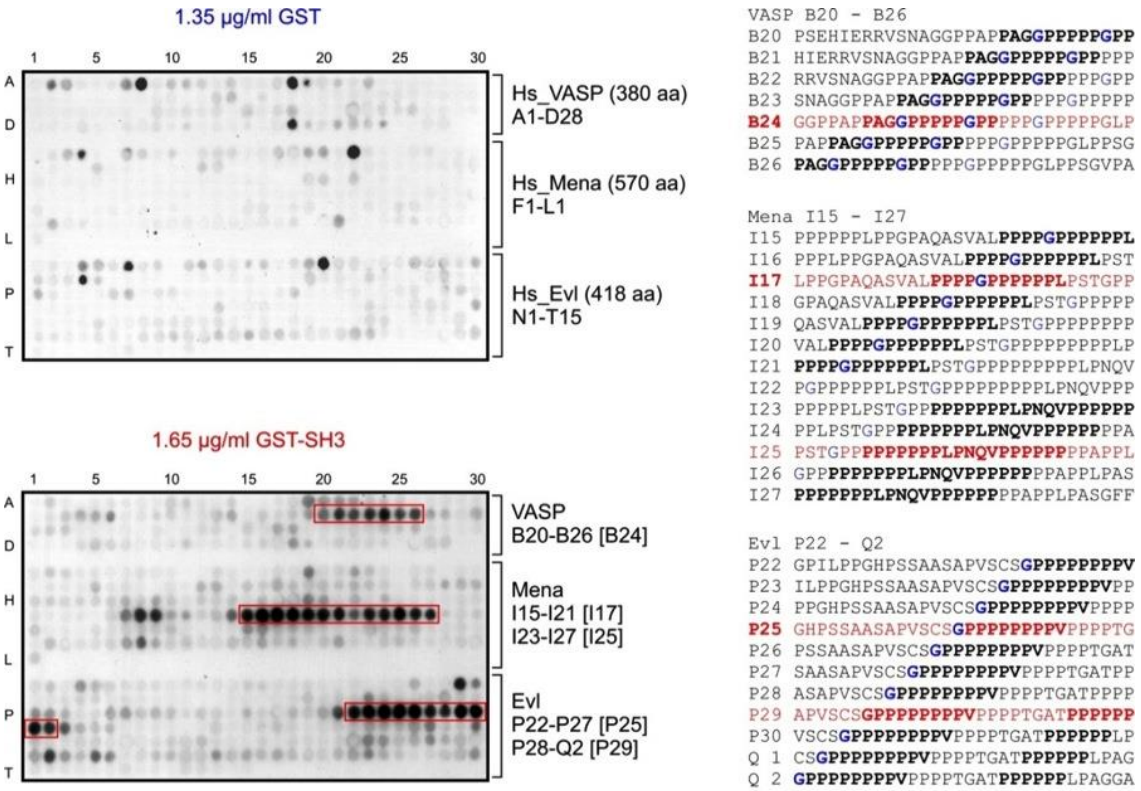


Figure 8: Human Ena/VASP peptide spot arrays probed with GST or GST-SH3. 30-mer peptides with 27 (Mena and VASP) or 28 (EVL) amino acid (aa) overlap, spanning the entire Mena (570 aa), VASP (380 aa), or EVL (418 aa) protein sequence, were synthesized in an array of 30 x 20 spots on a cellulose membrane and incubated with GST protein at a final concentration of 1.35 µg/ml (upper panel) or an equimolar concentration of GST-αII-Spectrin-SH3 fusion protein (1.65 µg/ml, lower panel). Bound protein was visualized with GST-specific antibodies followed by incubation with HRP-conjugated secondary antibodies. X-ray films with equal exposure times are shown. Peptides that bound to GST-SH3 are highlighted in the lower panel and corresponding sequences are shown on the right. VASP peptide B24, Mena peptides I17 and I25, and EVL peptides P25 and P29 displayed the strongest binding to GST-SH3 and are highlighted in red in the right panel. Bold sequences correspond to motives, which contribute to the interaction with the αII-Spectrin-SH3 domain. Figure corresponds to EMBO short-term fellowship report by Dr. Peter Benz.

Binding of the control (GST) to the immobilized peptides was detectable only in small amounts. In contrast, signals of bound GST-SH3 fusion protein could be detected with a series of VASP, Mena, and EVL peptides, which all overlapped with the PRR of the corresponding proteins. In these semi-quantitative arrays, signals of the αII-Spectrin-SH3 domain after incubation with Mena and EVL peptides were comparable and substantially stronger than signals after incubation of the αII-Spectrin-SH3 domain with VASP peptides. Within the PRR of a single protein, the SH3 domain bound best to those peptides that contain a

glycine followed by a proline-stretch (GP_x). Comparing the signal intensities between Mena, VASP, and EVL peptides, motives with a stretch of at least six prolines (GP_6), as they appear in Mena and EVL peptides, seem to be beneficial for the stronger SH3 binding (VASP peptides only contain GP_5 motives). Moreover, binding to Mena and EVL peptides occurred in a "biphasic" manner, i.e. two sets of high spot signals separated by spots with low signal intensities. This could indicate that, in contrast to VASP, the αII-Spectrin-SH3 domain interacts with two or more motives in the proline-rich region of Mena and EVL.

3. Purification of Mena and VASP proteins

Because of the results of the peptide scan arrays that showed different binding of Mena to αII-Spectrin-SH3 compared to VASP and the resulting assumption that the differences in the PRR of the proteins could be responsible for that finding (compare 3.2.), binding studies with the entire Mena/VASP proteins instead of the short peptides used in peptide scan arrays were performed. In these experiments, also the influence of tetramerization on the binding capability of Mena/VASP should be investigated. Therefore, different VASP proteins based on the protein sequence of human VASP were cloned into the pQE 30 vector and purified by affinity chromatography via the His_6-tag.

A) Human wild type VASP: This protein was used to investigate the physiological binding capacity of VASP.
B) G181P-VASP: The point mutation of the glycine in position 181 of human VASP to proline leads to a GP_6 motive in the PRR. This mutant was designed to imitate the PRR of the Mena protein and to investigate if the GP_6 motives are critical for binding to the αII-Spectrin-SH3 domain.
C) VASP ΔTD: To show the influence of tetramerization on the binding capability of VASP, this mutant was designed lacking the tetramerization domain.
D) Mini-VASP: Due to the finding that the PRR as well as the tetramerization domain are crucial for sufficient protein binding of VASP,

another mutant was designed consisting only of the PRR and the tetramerization domain. It was expected that this shortened mutant would lead to better purification amounts and equal binding capability to αII-Spectrin-SH3 compared to the full-length VASP and therefore could be used in ITC experiments eventually.

E) GST-His_6-Mena: Because of difficulties in expression and purification of His_6-tagged Mena in bacteria [47], the murine His_6-Mena protein was cloned into pGEX 6P2 and purified by affinity chromatography. The large GST-tag provides better protein solubility in the bacteria cell compared to His_6-tag. For comparison of the binding of Mena and VASP to GST-αII-Spectrin proteins in pull-down assays, both proteins were needed only His_6-tagged to use an α-His-antibody for equal detection of both proteins in Western blotting. Therefore, cleavage of the purified GST-His_6-Mena protein with the PreScission protease was needed to be performed to get rid of the GST-tag and to receive the desired His_6-tagged Mena.

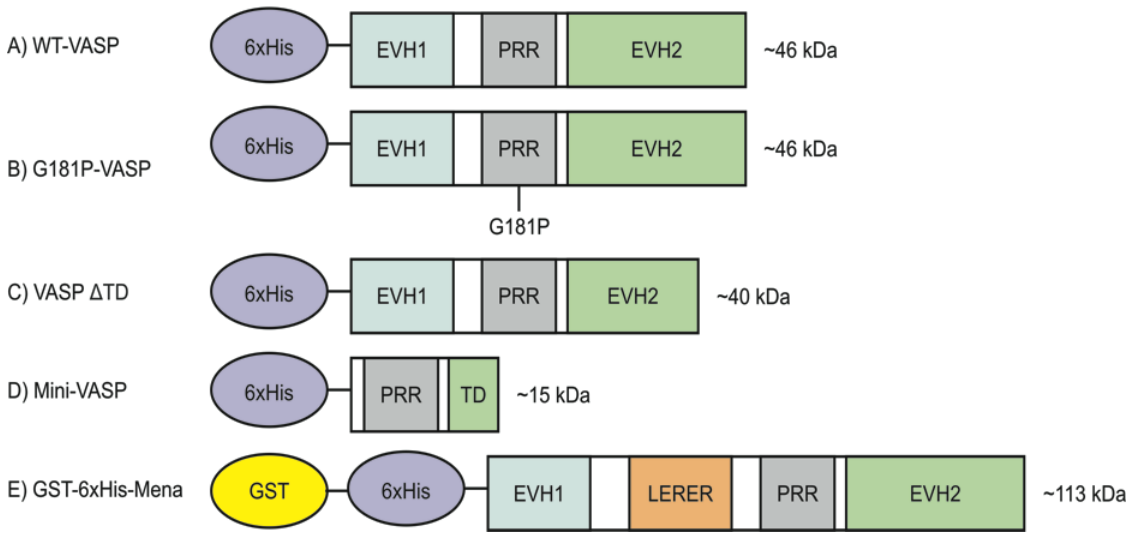


Figure 9: Mena/VASP constructs. A) Human wild type VASP; B) G181P mutated VASP leading to a GP_6 motive in the PRR; C) VASP ΔTD lacking the tetramerization domain in the EVH2 region; D) Mini-VASP consisting only of the PRR and the tetramerization domain; All proteins are tagged with 6x histidine (His) encoded by the pQE 30 vector at the N-terminus. E) Tandem-Mena consisting of the murine Mena N-terminally-tagged with GST (encoded by the pGEX 6P2 vector) and 6x His. Further information about the constructs is given in the text. Tags and domains are indicated in colors, estimated molecular mass is indicated behind the protein structure.

3.1. Purification of human His_6-VASP (wild type, G181P-mutant)

In addition to the human wild type (WT) His_6-VASP protein, one point-mutated form of VASP was purified. In this protein, the glycine of the second GP_5 motive was mutated to proline (G181P, generated by Dr. Peter Benz). The proteins were purified as described in 2.6. In brief, the proteins were expressed at 37°C for 40 hours and a final IPTG concentration of 1 mM. Purification was performed via cobalt affinity chromatography. The eluted VASP proteins were analyzed via SDS-PAGE and coomassie staining.

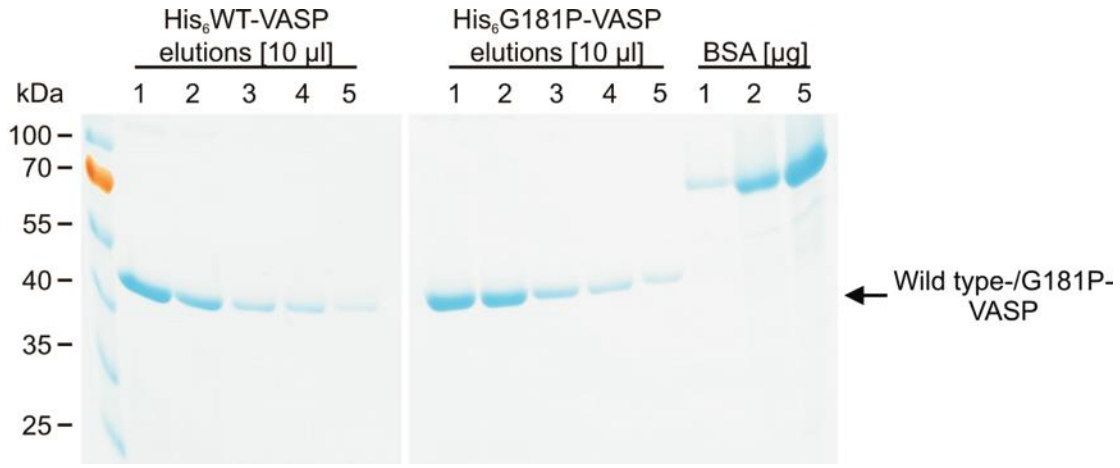


Figure 10: Purification of His_6-VASP (human wild type (WT), G181P; 9% SDS-PAGE). Both proteins were purified by affinity chromatography as described in 2.5. The proteins were eluted each in 5x 200 µl elution buffer. The coomassie gel shows 10 µl of each elution fraction. Total protein amount was estimated by comparison of the elution fractions with the BSA standard to an amount of about 500 µg protein per liter bacteria culture in high purity.

The His_6-VASP proteins (46 kDa) were obtained in excellent purity (Figure 10). Comparison with the BSA standard indicated that about 500 µg wild type VASP protein per liter bacteria culture could be purified. The amount of the point-mutated G181P-VASP was always about 25% higher and ranging from 600 to 700 µg per liter.

3.2. Purification of His_6-VASP ΔTD

Early purification attempts of His_6-VASP ΔTD showed that the purification conditions of the other His_6-VASP proteins were not suitable for the purification of this mutant, which lacks the tetramerization motive of the EVH2 domain. Therefore, different expression and purification conditions were tested. The best possible conditions are described in 2.6. In brief, expression temperature (37°C) and final IPTG concentration (1 mM) were the same as used for wild type VASP, but the expression time was shortened from 40 hours to 24 hours. Purification was performed via cobalt affinity chromatography, but in contrast to the imidazole concentration in the binding buffer and washing buffers used for wild type VASP (5 mM and up to 20 mM imidazole respectively), the binding buffer used for purification of VASP ΔTD contained only 1 mM imidazole and the washing buffers up to 15 mM imidazole. Without this reduction of the imidazole concentration nearly no VASP ΔTD protein could be purified, indicating a weaker binding capability of the mutated protein in comparison to wild type VASP. The eluted protein was analyzed by SDS-PAGE and coomassie staining.

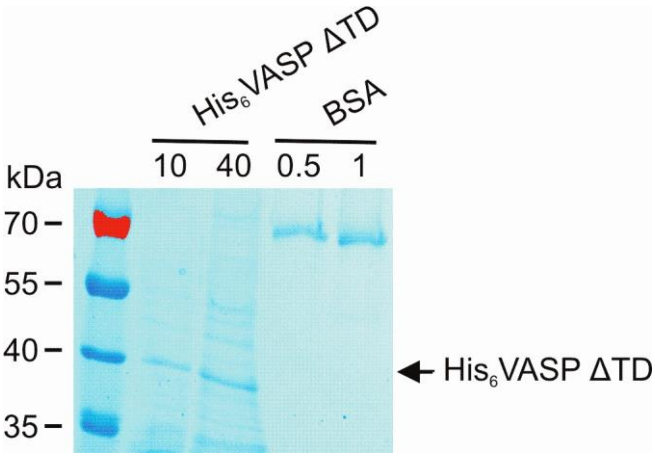


Figure 11: Purification of His_6-VASP ΔTD (9% SDS-PAGE). Two amounts of the protein elution were loaded (10 µl and 40 µl). Comparison of the protein (40 kDa) with the BSA standard showed a protein amount of 1 µg in 40 µl elution and therefore in total purification amounts of about 7 µg/liter bacteria culture.

His_6-VASP ΔTD (~ 40 kDa) was purified in little amounts. Comparison with the BSA standard showed an amount of only 1 µg in 40 µl gel filtrated elution (total amount: 7 µg per liter bacteria culture). The poor purification yield was in agreement with earlier studies [16], where the same problems with the purification of this VASP mutant had been described (detection of the protein in

their experiments was only possible in Western blotting). Furthermore, the purification showed low protein purity reflected by the multiple protein bands (Figure 11). Unfortunately, it was not possible to find expression conditions that led to better protein purity or quantity (data not shown).

3.3. Purification of His_6-Mini-VASP

Purification of His_6-Mini-VASP did not work with the same conditions as used for purification of WT-His_6-VASP or G181P-VASP. After testing different expression and purification conditions (for example protein expression at 30°C and at 37°C), the conditions described in 2.6. were chosen for purification of Mini-VASP due to the best purity and quantity. In brief, expression of the protein was performed at 37°C for 5 hours with a final IPTG concentration of 0.3 mM. Purification was performed via cobalt affinity chromatography. For purification of Mini-VASP, it was possible to use the same binding buffer as for the wild type VASP (5 mM imidazole), and the washing steps could be performed with up to 40 mM imidazole (wild type VASP: max. 20 mM imidazole). Following the washing steps, elution was performed with 3x 200 µl elution buffer containing twice as much imidazole than the elution buffer used for elution of wild type VASP (300 mM imidazole instead of 150 mM imidazole). 40 µl of the pooled washing steps with 40 mM imidazole and 20 µl of the elution fractions were saved for SDS-PAGE and coomassie staining. Figure 12 shows the purification of His_6-Mini-VASP expressed at 30°C versus expression at 37°C.

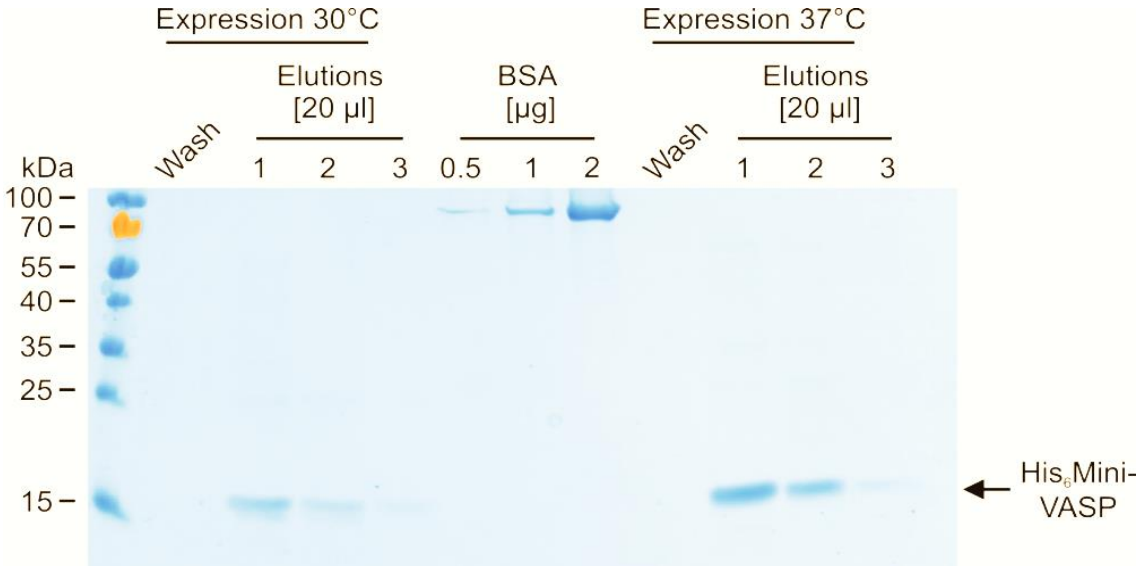


Figure 12: Purification of His_6-Mini-VASP at 30°C versus 37°C (15% SDS-PAGE). Purification details are described in 2.5. Elution fractions (1-3) after protein expression at 30°C and 37°C are visualized by coomassie staining. As control, 40 µl of the pooled washing steps were also loaded. To compare purification amounts, 0.5 µg, 1 µg and 2 µg BSA were loaded onto the gel. Protein amounts were estimated to 20 µg/50 ml bacteria culture when expression was performed at 30°C. Protein amounts were threefold higher when the protein expression temperature was 37°C leading to an amount of 60 µg/50 ml bacteria culture.

As described in Figure 12, purification of His_6-Mini-VASP showed better quantity and quality when expressed at 37°C than when expressed at 30°C. The clear bands at a size of 15 kDa corresponding to the calculated protein size of His_6-Mini-VASP indicated about threefold higher protein amounts as when expressed at 30°C. In total, approximately 60 µg protein per 50 ml bacteria pellet could be purified in excellent purity and without losing protein while washing.

3.4. Purification of GST-Mena after chaperone co-expression

Overproduction of recombinant proteins sometimes leads to misfolding and aggregation especially when expressing big proteins in prokaryote cells like *Escherichia coli (E.coli).* In the cell, various heat-shock proteins called chaperones assist the folding of newly synthesized proteins. In case of misfolded or aggregated proteins refolding is supported by the chaperones [51].

In the cytosol, there are two major systems called DnaK (plus the co-chaperones DnaJ and GroE; KJE), and GroEL (plus the co-chaperone GroES; ELS). Moreover, the different chaperones prevent aggregation of overproduced protein or provide solubility of aggregated proteins in cooperation with ClpB. The last group of chaperones consists of the small heat-shock proteins of *E.coli* called IbpA and IbpB, which also support disaggregation and refolding of proteins [48]. Because of the slightly different mechanisms and functions of the chaperones, there are some chaperone combinations, which were found to work best. Transformation of host cells with special plasmids (called pBB 528, 530, 535, 540, 541, 542, 550) encoding the chaperone or the chaperone combination under the control of the IPTG-regulated promotor PA1/lacO-1 (strong) or Plac (weak) and additional transformation with the recombinant protein of interest showed considerably better expression of non-aggregated and correct folded protein [48]. Table 8 shows the different plasmids and their corresponding chaperones as well as possible plasmid combinations.

Because the expression and purification of Mena in bacteria is challenging [52], the different chaperone combinations described in table 8 were co-expressed with GST-Mena in BL21 and pBB 535 and pBB 542 (single plasmids) were co-expressed with GST-His_6-Mena in Rosetta DE3. Purification conditions were equal for all chaperone-protein combinations and are described in 2.6. As control the GST-Mena construct was expressed without chaperones in Rosetta DE3. Protein elutions were saved for 8% SDS-PAGE and coomassie staining (see Figure 13).

Table 8: Chaperones. Explanations to the chaperones mentioned in this table are given in the text (3.3.4.). The chaperones and chaperone combinations were used to improve the expression of Mena in *E.coli*.

Plasmid	Chaperone(s)	Resistance
pBB 528	(-);$LacI^Q$-Repressor	CHL
pBB 530	GrpE	CHL
pBB 535	DnaK, DnaJ	Spec
pBB 540	GrpE, ClpB	CHL
pBB 541	GroESL	Spec
pBB 542	DnaK, DnaJ, GroESL	Spec
pBB 550	DnaK, DnaJ, GroESL	Spec

Plasmid combinations	Chaperone expression
a) pBB 528; pBB 541	ELS
b) pBB 530; pBB 535	KJE
c) pBB 535; pBB 540	KJE, ClpB
d) pBB 540; pBB 542	KJE, ClpB, high amounts of ELS
e) pBB 540; pBB 550	KJE, ClpB, low amounts of ELS

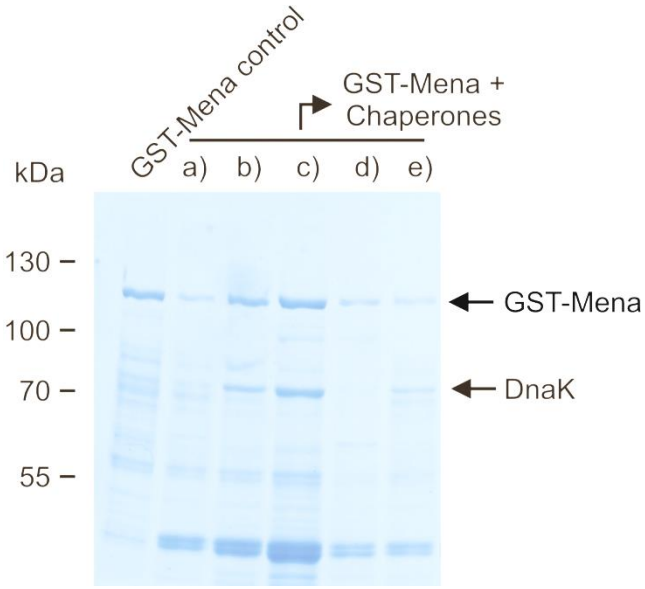


Figure 13: Purification of GST-Mena co-expressed with different chaperone combinations (8% SDS-PAGE). The different chaperone combinations explained in 3.3.4. were tested by co-expression with GST-Mena in BL21. Expression of GST-Mena in Rosetta DE3 without further chaperones served as control. Co-expression of GST-Mena with pBB 528/541 (a), pBB 540/542 (d) and pBB 540/550 (e) showed worse purification results than expression of the protein in Rosetta BL21 without further chaperones (control). Co-expression of GST-Mena with pBB 530/535 (b) and especially pBB 535/540 (c) was beneficial and increased the amount of GST-Mena by almost 100%. Protein bands at 70 kDa likely correspond to protein size of the over-expressed chaperone DnaK.

Purification of the GST-Mena protein expressed at 10°C for 36 hours in Rosetta DE3 without further chaperones showed little degradation. Co-expression of GST-Mena with one of the five chaperone combinations in BL21 resulted in different amounts and purity of the protein for each chaperone combination.
Expression of GST-Mena with the chaperone combinations pBB 528/541 (a), pBB 540/542 (d) and pBB 540/550 (e) did not improve the yield and purity of GST-Mena as compared to the expression in Rosetta DE3 without chaperones (control). In contrast, co-expression of GST-Mena with pBB 530/535 (b) and pBB 535/540 (c) doubled the yield of purified GST-Mena. Protein bands at 70 kDa likely correspond to protein sizes of the over-expressed chaperone DnaK.
Because of these results, another experiment was performed using Rosetta DE3 instead of BL21 bacteria for transformation of pBB 535 or pBB 542 (due to their spectinomycin resistance) and pGEX 6P2 + His_6-Mena. The chaperone combinations used above could not be tested because of their chloramphenicol resistance, which corresponds to the resistance of the Rosetta DE3 bacteria.
In final experiments, purification of GST-His_6-Mena (see below) expressed in Rosetta with or without chaperones showed no differences in protein purity or amounts. Therefore, in further experiments GST-His_6-Mena was expressed in Rosetta DE3 without chaperones.

3.5. Purification of GST-His_6-Mena

GST-His_6-Mena was designed to compare the binding capability of Mena and VASP to αII-Spectrin in pull-downs. Analysis of the pull-downs should be performed by Western blotting. Using an α-His-antibody, it should be possible to directly compare the binding capability of Mena and VASP to αII-Spectrin. Therefore, it was necessary to purify Mena with a His-tag. Because of the good purification results when expressing the Mena protein with a GST-tag, a double-tagged Mena fusion protein was designed.
Purification of GST-His_6-Mena was performed by glutathione affinity chromatography as described in 2.6. In brief, the proteins were expressed at 10°C for 36 to 72 hours and a final IPTG concentration of 0.3 mM. Because expression amounts were not equal to the amounts of the GST-Spectrin

constructs, 400 ml to 500 ml bacteria culture were used for purification of the protein. After purification, 10 µl of each elution fraction were saved for SDS-PAGE. Afterwards, 100 µl 5x SDS sample buffer were added to the eluted GSH sepharose to control the success of the elution. The elution samples and 10 µl of the control were loaded onto a 12% SDS gel and stained by coomassie.

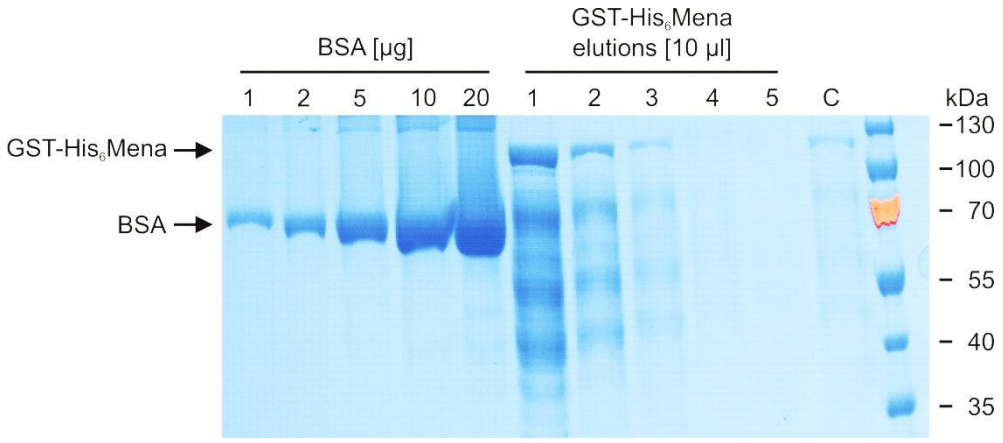


Figure 14: Purification of GST-His$_6$-Mena (12% SDS-PAGE). 600 ml of GST-His$_6$-Mena bacteria culture were purified by GST affinity chromatography. The GSH-bound protein was eluted in 5x 100 µl elution buffer. 10 µl of the elution fraction were loaded onto the gel after adding loading buffer. As elution control (C), the remaining GSH sepharose was incubated with 100 µl of SDS sample buffer and 10 µl were loaded onto the gel. The GST-His$_6$-Mena protein could be localized at the estimated size of about 113 kDa. The total protein amount was estimated to 40 µg/ 600 ml bacteria culture after comparison with the BSA standard. Unfortunately, protein purity was very low and multiple bands below 100 kDa can be seen after staining. The remaining weak protein band in the control shows that elution conditions were not sufficient to elute all the protein.

Protein expression and purification showed a clear signal at the expected size of about 113 kDa. Comparison of the protein amounts in all elution fractions with the BSA standard revealed a total protein amount of about 40 µg/ 600 ml bacteria culture. The weak protein signal at 113 kDa in the control indicates that it was not possible to elute all of the protein from the GSH sepharose with the chosen conditions resulting in a loss of available protein. Therefore in further purification experiments the incubation time or the concentration of the reduced glutathione had to be increased.

4. Cleavage of GST-αII-Spectrin constructs and of GST-His_6-Mena

As detailed in 3.3.5., cleavage of GST-His_6-Mena was necessary to perform Western blotting with an α-His-antibody to compare the resulting signal intensities with the signals of the His_6-VASP proteins later on. To perform pull-down assays with GST-His_6-Mena, it was necessary to cleave the GST-tag from the Spectrin proteins. Both GST-αII-Spectrin constructs and GST-His_6-Mena were cloned into the pGEX 6P2 vector. Therefore a specific restriction site for the PreScission protease between the GST and the protein sequence is present in the fusion protein. This protease recognizes the core amino acid sequence Leu-Phe-Gln-Gly-Pro. The cleavage occurs between the Gln and Gly residues. Cleavage of the fusion proteins will separate the GST-tag from the Spectrin proteins or His_6-Mena, respectively. Because the protease itself binds to GSH sepharose, the cleaved GST and the protease can be separated from the Spectrin constructs or His_6-Mena by affinity chromatography with GSH sepharose.

Different cleavage options are described in the manufacturer's instruction of PreScission. Cleavage is possible with eluted proteins as well as "on-column". For each fusion protein, it is necessary to find out the best cleavage conditions by testing different incubation times, temperatures and amounts of protease. In general, one unit of PreScission will cleave 90% of 100 µg fusion protein in cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0 at 25°C) in 16 hours at 5°C.

After extensive testing, two different cleavage conditions were chosen for GST-αII-Spectrin constructs and GST-His_6-Mena as detailed in 2.7.

4.1. Cleavage of GST-αII-Spectrin constructs

In brief, 5 units of HRV3C (equal to PreScission protease) were added to 100 µg of GSH sepharose-bound GST-αII-Spectrin fusion proteins in a volume of 200 µl of cleavage buffer. The reaction was performed at 4°C to protect the proteins from degradation. As control, 20 µl of the supernatant of each reaction (GST; GST+A; GST+SH3; GST+α9; GST+α9A) were saved for SDS-PAGE directly before addition of the protease (t=0). A GST fusion protein named

GST+Spred2 EVH1 (cloned and purified by Dr. Peter Benz) was used as positive control, because successful cleavage of this fusion protein had already been shown. Two and a half hours (t=2.5 h) and 25 hours (t=25 h) after addition of the protease another 20 µl of the supernatant of each reaction were saved for SDS-PAGE analysis. To visualize un-cleaved protein, 200 µl SDS loading buffer were added to the remaining sepharose after removal of the supernatant and 20 µl were loaded onto a 12% gel. Analysis was performed by SDS-PAGE and coomassie staining (Figure 15).

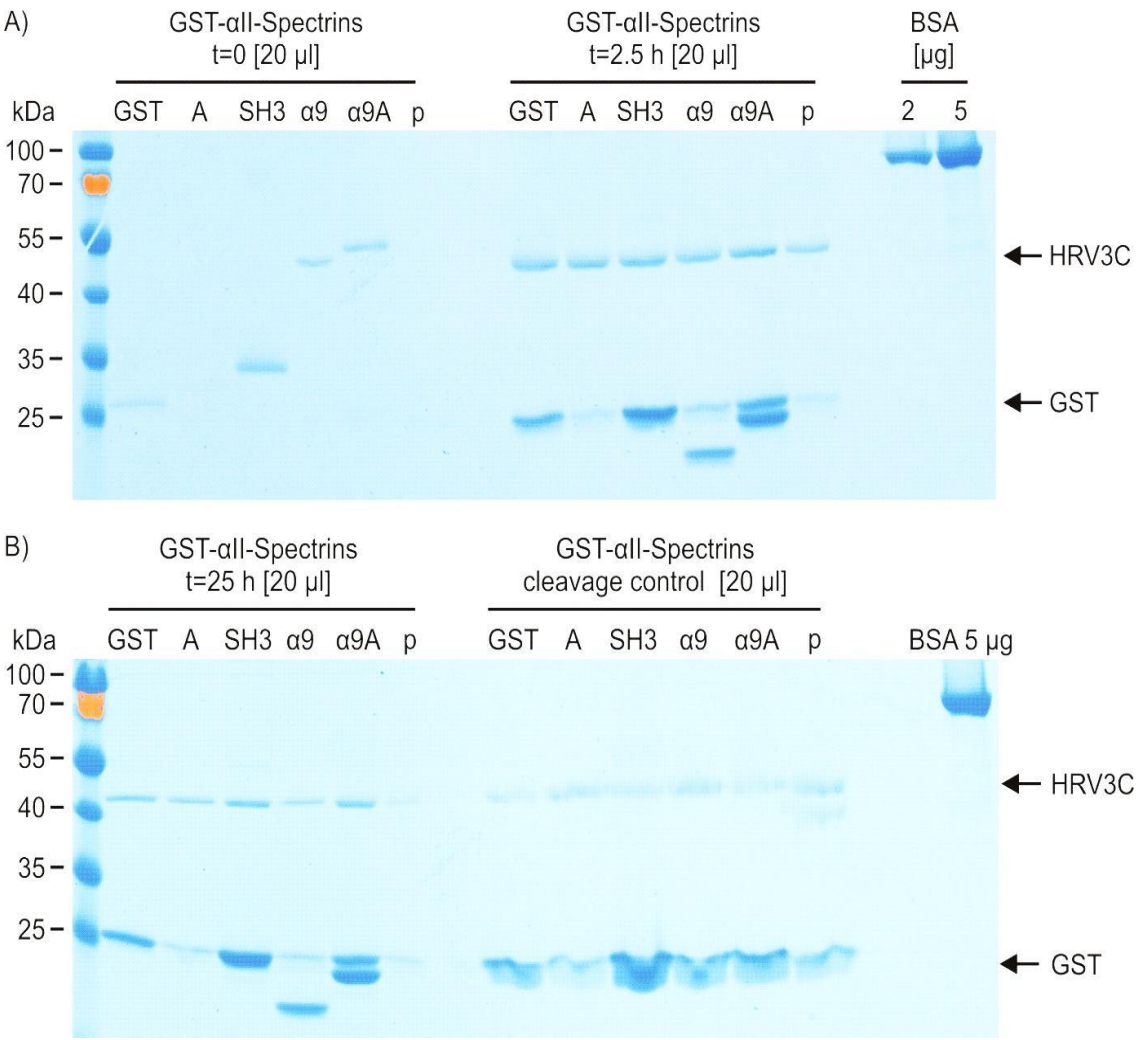


Figure 15: Cleavage of GST-αII-Spectrin fusion proteins (12% SDS-PAGE). A) Proteins before starting the cleavage reaction (t=0) and 2.5 hours after addition of the protease (t=2.5). B) Proteins 25 hours (t=25 h) after addition of the protease and cleavage control. The GST fusion proteins are described in the text (see also Figure 4). As positive control (p) a known cleavable GST fusion protein was used. Successful cleavage reduces the apparent molecular weight of the proteins by 25 kDa (corresponding to the molecular weight of GST). Because of the low molecular weight of GST+A (29 kDa) and GST+SH3 (33 kDa) the cleaved Spectrin peptides are not visible on the gel. However the cleaved α9 and α9A (47 kDa and 49 kDa) can be clearly seen at 22 kDa and 24 kDa. GST signals can be seen at 25 kDa and protease signals at 46 kDa. The protein bands of the t=0 reactions show the molecular weights of the whole GST fusion proteins caused by leakage of small amounts of the protein from the column. Protein bands after t=2.5 hours indicate that cleavage has almost been completed at this time, because only little signal increase can be found after 25 hours. Protein bands in the cleavage control also indicate that almost complete cleavage could be achieved after 25 hours. In summary, 2.5 hours reaction time seems to be sufficient to cleave most of the on-column protein.

Figure 15 shows the kinetic of the cleavage reaction. Surprisingly, samples that were taken just before addition of the protease showed some fusion protein with

the normal calculated molecular weights, probably because little amounts of protein leaked from the GSH sepharose (Figure 15A). After 2.5 hours of incubation the Spectrin proteins migrated with an apparent molecular weight that was 25 kDa below their original sizes (A and SH3 cannot be seen due to the percentage of the SDS gel and their molecular weights). Furthermore, the GST-tag appeared at 25 kDa in each lane confirming successful cleavage, but also meaning that the amounts of the GSH sepharose or the binding capacity were not sufficient to bind the cleaved GST. The protein signal that can be seen at 46 kDa is caused by the protease. 25 hours after addition of the protease, the intensity of the protein bands hardly increased indicating that cleavage of GST-αII-proteins was already complete after 2.5 hours incubation (Figure 15B). This finding was confirmed by the control samples, which showed signals at 25 kDa and 46 kDa for GST and the protease but only very weak to no signal for the original fusion proteins. In summary, the cleavage results showed that cleavage of the GST-αII-Spectrin fusion proteins by PreScission protease works with the on-column method using 5 units of protease for 100 µg protein for 2.5 hours at 4°C. To remove the unbound GST-tag, it would be necessary to incubate the reactions with GSH sepharose to obtain the cleaved Spectrin proteins GST-free.

4.2. Cleavage of GST-His_6-Mena

Cleavage of GST-His_6-Mena was performed in solution. Cleavage of the eluted protein was started by adding 40 units of the HRV3C protease to 40 µg purified GST- His_6-Mena. Because the cleavage was performed in normal elution buffer and not in the cleavage buffer mentioned above, the protease was added in a 100x higher concentration than described in the manufacturer's description. The cleavage reaction was incubated for 16 hours at 4°C. To analyze the cleavage reaction, 10 µl of the elution prior to addition of the protease and after incubation with the protease were analyzed by 12% SDS-PAGE and coomassie staining (Figure 16).

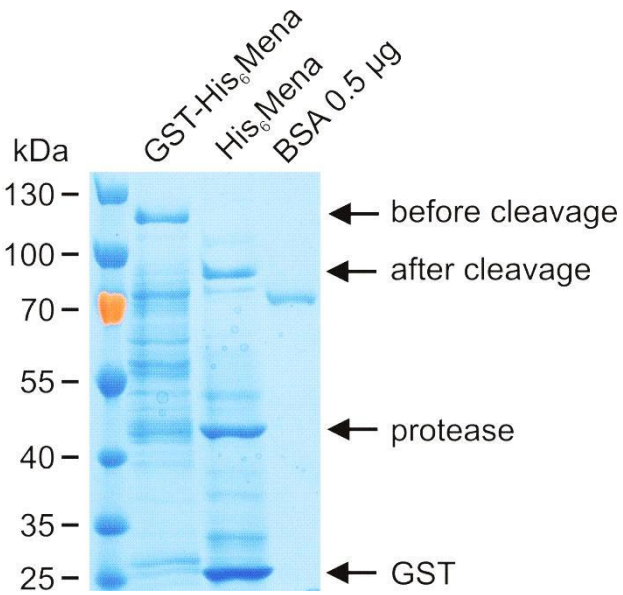


Figure 16: Cleavage of GST-His_6-Mena (12% SDS-PAGE). Cleavage was performed with eluted GST-His_6-Mena in normal elution buffer instead of cleavage buffer. Therefore, 1 unit of HRV3C protease (equal to the PreScission protease) was added per µg Mena protein. Before and after cleavage, 10 µl of the protein in solution was saved for SDS-PAGE. Cleavage led to an expected shift from 113 kDa to about 88 kDa and to a signal of GST at 25 kDa. The full-length fusion protein at 113 kDa was undetectable after cleavage indicating successful cleavage of the whole fusion protein. Cleavage also resulted in good protein purity.

Cleavage of GST-His_6-Mena is demonstrated in Figure 16. Before cleavage, GST-His_6-Mena has a molecular weight of about 113 kDa. Because of the loss of the GST-tag, the protein mass after the cleavage is decreased to about 88 kDa. Furthermore, at 25 kDa the signal for GST could be detected. The cleavage reaction seems to be complete, because no full-length fusion protein could be detected after cleavage. For further experiments, the cleaved protein was transferred into 40 mM Hepes-NaOH pH 7.4 and 75 mM NaCl using gel filtration (Econo-Pac® 10DG Columns). Then 200 µl of equilibrated GSH sepharose slurry were added to remove the GST-tag and the protease. After one hour incubation at 4°C and centrifugation for one minute, the supernatant with the single His_6-Mena was transferred into a new vial. SDS-PAGE and coomassie analysis showed that amounts of protease as well as of the GST-tag could be strongly reduced (data not shown).

In contrast to the GST-αII-Spectrin fusion proteins, cleavage of GST-His_6-Mena did not work with the on-column method (data not shown).

5. Isothermal titration calorimetry (ITC)

As described in detail in 2.10., ITC is a convenient method to study SH3 domain interactions with up to 20 amino acid long peptides by measurement of thermodynamic parameters of the biochemical binding process. ITC can be used for the quantitative measurement of the binding affinity (K_D), binding enthalpy changes (ΔH), and stoichiometry (n). Using these parameters, it is possible to calculate the entropy changes (ΔS) and Gibbs energy changes (ΔG).

As already mentioned, investigation of the interaction between SH3 domains and the up to 20 fold larger Mena/VASP, ITC would require protein amounts in the high milligram range. Because these amounts are challenging to obtain, a truncated Mena peptide (QASVALPPPPGPPPPPPLPSTG) was used in the experiments. This protein partially contains the proline-rich region but is missing the tetramerization domain leading to a 22 amino acid monomeric protein. The experiment was performed in collaboration with Dr. Stephan Feller, University of Oxford.

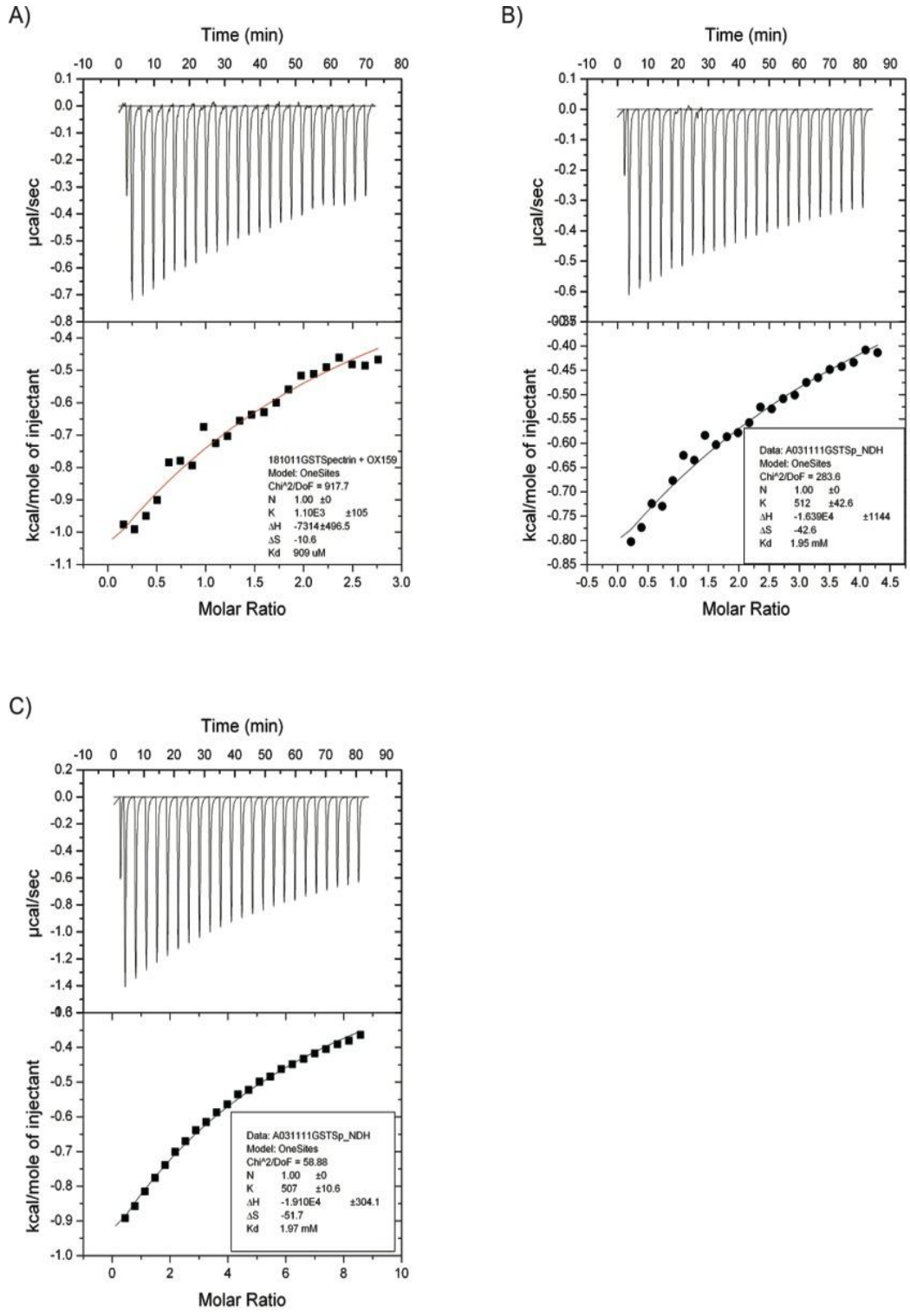


Figure 17: ITC data. In ITC experiments, a 22 amino acid long Mena partially containing the proline-rich region but lacking the tetramerization domain was used to measure the binding affinity when exposed to GST-αII-Spectrin constructs (A: SH3, B: α9, C: α9A). The dissociation constant K_D indicates the binding strength. Binding of the Mena protein to the SH3-Spectrin construct shows twofold stronger binding (K_D 909 µM) than binding to α9- or α9A-Spectrin construct (K_D 1.95 µM or 1.97 µM).

For the experiment, 0.1 mM Mena peptide and 2 mM peptide for GST-αII-Spectrin-SH3 and GST-αII-Spectrin-α9 were used. For GST-αII-Spectrin-α9A the peptide was doubled to 4 mM, which still gave the same affinity as for the GST-α9 construct. Both dissociation constants were two fold weaker than that of the GST-αII-Spectrin SH3.

These data show that *in vitro* binding between the monomeric Mena peptide and the Spectrin constructs is weak. One reason for the weak binding could be caused by the physiological role of the protein interaction. Mena/VASP are important regulators of the actin cytoskeleton, which undergoes highly dynamic changes [1]. Weak binding therefore seems to be necessary for a dynamic regulation. Also, the weak affinity could be caused by the absence of the tetramerization domain of the Mena protein (see 1.3). As shown by Ahern-Djamali et al., lack of this domain abrogates the binding between VASP and SH3 [53]. Second, the data indicate that binding of the GST-SH3 construct is about two fold stronger than binding of the GST-α9 and GST-α9A constructs. One possible reason for these findings could be that an additional protein is serving as an adaptor in the cell, which is lacking in ITC.

6. GST-pull-down assays

GST-pull-down assays were performed using the GST-αII-Spectrin constructs described in Figure 6 to study the interaction with endogenous or recombinant Mena/VASP proteins. Pull-down assays were performed as detailed in 2.9. In brief, pull-downs were performed either with heart lysate of two sacrificed wild type mice, or with 0.5 µg to 2 µg recombinant Mena/VASP protein. After addition of 1 µg to 5 µg of GST and equimolar amounts of the other GST-αII-Spectrin proteins the reaction was incubated for one hour at 4°C. After several washing steps analysis of the pull-downs was performed by SDS-PAGE and Western blotting.

6.1. GST-αII-Spectrin-pull-down assays with cleaved GST-His_6-Mena

To look at the αII-Spectrin Mena interaction *in vitro*, GST-pull-down assays were performed with GST (control), GST+A, GST+SH3, GST+α9, and GST+α9A αII-

Spectrin constructs (see Figure 6). In this experiment, purified and cleaved GST-His_6-Mena expressed in *E.coli* was used (see 2.6.). As described detailed in 3.3., expression of the Mena protein in bacteria is challenging [52]. Knowing the good purification results of GST-tagged proteins, therefore a GST- and His_6-tagged Mena was designed. The GST-tag should provide good purification results, whereas the His_6-tag should be used for comparison of the binding capability of Mena and VASP in Western blotting. Therefore, the GST-tag must be cleaved by a specific protease first (see 3.4.). The pull-down experiment was performed as described above using 1.17 µg GST and equimolar amounts of the other constructs and 3 µg Mena. Analysis of the reaction was visualized in Western blots using an anti-Mena antibody. Detailed experiment conditions are described in 2.9.

Figure 18: GST-αII-Spectrin pull-down assay with recombinant Mena (9% SDS-PAGE). GST-pull-down experiment was performed using 1.17 µg GST (control) and equimolar amounts of the other GST-αII-Spectrin constructs. Mena was purified as GST-His_6-tagged protein. After cleavage of the GST-tag, 3 µg His_6-Mena were used for pull-down. Binding was visualized by Western blotting using a specific antibody against Mena and an HRP-conjugated secondary antibody.

Western blotting analysis of the pull-down experiments showed minor signals of the Mena protein in pull-down reactions with GST and the GST+A αII-Spectrin construct compared to the other three constructs (Figure 18). Analysis of these pull-down reactions showed substantially stronger signals, especially regarding the pull-down with the GST-α9A construct. This indicates first a direct interaction between the GST-αII-Spectrin constructs and the recombinant Mena *in vitro*. Moreover, the interaction between the proteins seems to be dependent on the SH3 domain with strongest signals for the alternatively spliced SH3 domain of repeat 9 of αII-Spectrin.

6.2. GST-αII-Spectrin-pull-down assays: Comparison of recombinant human wild type (WT) VASP with G181P-mutated VASP and VASP ΔTD

GST-pull-down assays with the different GST-αII-Spectrin constructs and different variants of recombinant VASP (WT-VASP, point-mutated G181P-VASP and VASP lacking the tetramerization domain, ΔTD; see also Figure 9) were used to get further information about the residues of VASP that are involved in binding to αII-Spectrin. Pull-down assays were performed as described above using 1 µg GST-α9 and equimolar amounts of the other GST-Spectrin constructs and about 500 ng VASP proteins. Pull-downs were analyzed by SDS-PAGE and Western blotting using an antibody against the His_6-tag of the VASP proteins, so that comparison of the signals was possible.

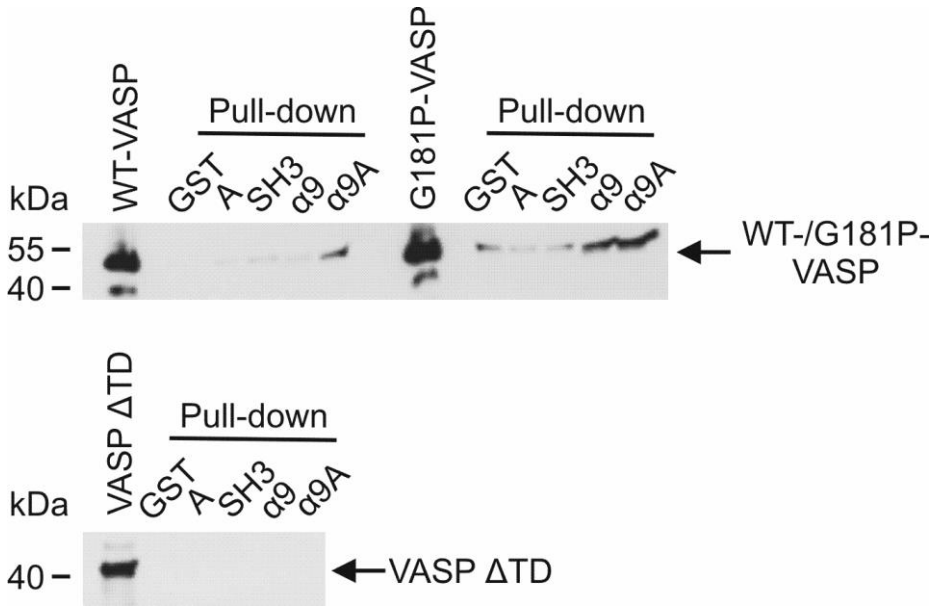


Figure 19: Pull-down with GST-αII-Spectrin constructs and wild type (WT)-, G181P- and ΔTD-VASP (12% SDS-PAGE).
a) GST; b) GST+A; c) GST+SH3, d) GST+α9, e) GST+α9A (see Figure 4). All different VASP variants were detected using an antibody against the His_6-tag of the proteins followed by an HRP-conjugated secondary antibody (WT- and G181P-VASP: 46 kDa; VASP ΔTD: 40 kDa).

As shown in Figure 19, signal intensity in pull-down reactions with the point-mutated G181P-VASP seems to be stronger than signal intensity in pull-down reaction with the human WT-VASP. Moreover, no signal at all could be observed when performing pull-down assays with VASP ΔTD. Regarding the

different GST-αII-Spectrin constructs, strongest signals could be observed in pull-downs with GST-α9A but in case of G181P-VASP also with GST-α9. These findings in general indicate stronger binding of the G181P-point-mutated variant of VASP compared to WT-VASP, whereas pull-down assays with VASP ΔTD indicate that there is no binding at all. This finding demonstrates the importance of tetramerization for the binding functionality of Mena/VASP-proteins as previously observed for the SH3 interactions of EVL [19].

In case of the point-mutated VASP mutant the stronger binding possibly could indicate a positive effect of GP_6-motives compared to GP_5-motives on binding of Mena/VASP proteins to αII-Spectrins as already observed in peptide scan arrays. Due to the higher protein amounts in pull-down experiments with the G181P-mutated VASP compared to the amounts in pull-downs with WT-VASP, the stronger binding of the mutant has to be proved in further experiments. Also the loss of VASP ΔTD binding needs to be confirmed by more experiments, because abrogated binding could also be explained by technical problems.

The pull-down experiment was also performed with recombinant His_6-Mini-VASP (see Figure 9). This construct consisting of only the proline-rich region and the tetramerization domain (see Figure 3, 2.3.6) was designed to analyze the binding domains that are crucial for binding of Mena/VASP to αII-Spectrin, and also because it was thought that purification of this small protein should result in higher protein amounts. But as described for VASP ΔTD, no signals at all could be observed in Western blotting analysis of the pull-down reactions (data not shown). It still remains unclear if this finding is caused by technical problems or if this construct really is not sufficient to bind to αII-Spectrin. Because the protein is severely changed (consisting only of the PRR and the TD domain) compared to the wild type protein, it is also possible that the protein functionality is impaired.

6.3. GST-αII-Spectrin-SH3 competition pull-down assay with recombinant Mena vs. wild type VASP

This competition experiment was performed due to the results of the peptide scan arrays described in 3.2. and the results of the pull-down assay described above. In brief, both experiments indicated that Mena binding to αII-Spectrin is significantly stronger than binding of VASP, possibly because of its GP_6 motive in the PRR, which is lacking VASP. To further test this hypothesis, a competition pull-down assay with equimolar amounts of GST-αII-Spectrin-SH3 (385 ng) and both recombinant His_6-Mena after removal of the GST-tag (780 ng) and of recombinant His_6-VASP (550 ng) was performed. The SH3 construct was used in this small amount so that Mena and VASP competed for binding to the construct. The assay was analyzed by SDS-PAGE and Western blotting using an anti-His_6-antibody, so comparison of the binding intensity between both proteins was possible.

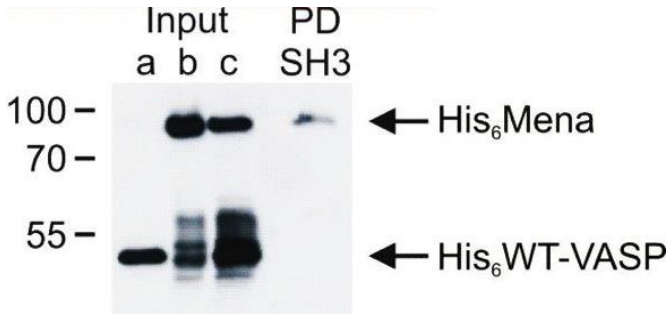


Figure 20: Competition pull-down with GST-αII-Spectrin-SH3 and recombinant His_6-Mena and His_6-VASP (9% SDS-PAGE). a: 50 ng His_6-VASP; b: 50 ng His_6-Mena (GST-His_6-Mena after cleavage); c: 50 ng His_6-Mena and 50 ng His_6-VASP combined; PD: pull-down assay with equimolar amounts of GST-αII-Spectrin-SH3 (385 ng) and His_6-Mena/His_6-VASP (780 ng and 550 ng, respectively). Because of the small amount of GST-SH3, Mena and VASP had to compete against each other for binding to the SH3 domain. Western blotting analysis shows a signal for Mena, but not for VASP in pull-down reaction with GST-SH3, indicating a stronger binding of Mena compared to VASP. This result matches the hypothesis that Mena binding is stronger possibly due to its GP_6 motive in its PRR (see also 3.2.).

Western blotting analysis of the pull-down reaction with GST-SH3 showed a signal only for Mena, but not for VASP when performing the experiment with equimolar amounts of the recombinant His_6-Mena and His_6-VASP. Due to the N-terminal His_6-tag of both Mena and VASP, Western blotting analysis with an

anti-His_6-antibody followed by an HRP-conjugated secondary antibody allowed the precise quantitation of the proteins. The signal intensities indicate stronger binding of Mena compared to VASP. This matches the results of the peptide scan array described in 3.2. as well as the finding that pull-down reactions with the point-mutated G181P-VASP showed possibly stronger signal intensities compared to reactions with WT-VASP.

6.4. GST-αII-Spectrin pull-down assays with heart lysate

Because of possible differences between bacteria and mammalian cells concerning posttranslational modification of Mena/VASP proteins, pull-down experiments were performed using murine heart lysate. In brief, the lysate of two murine wild type hearts was divided into 5 parts (400 µl each). Then 2 µg GST or equimolar amounts of the other GST-αII-Spectrin constructs were added. After incubation for one hour at 4°C the precipitates were washed with pull-down buffer and saved for two-step gradient SDS-PAGE (12% and 7%) and coomassie staining or Western blotting. Mena was visualized by a specific α-Mena-antibody followed by HRP-conjugated secondary antibody.

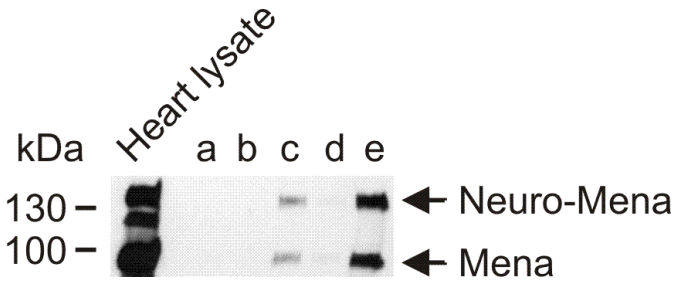


Figure 21: Pull-down with GST-αII-Spectrin constructs and murine heart lysate (12% / 7% SDS-PAGE). a) GST, b) GST+A, c) GST+SH3, d) GST+α9, e) GST+α9A. GST-pull-down assays were performed with the different GST-αII-Spectrin constructs (see Figure 4) and murine heart lysate. Mena was detected using a specific antibody followed by an HRP-conjugated secondary antibody. Due to the different splice variants of Mena, characteristic signals at 80/88 kDa and 140 kDa (neuronal splice variant) could be detected in the first lane (input: heart lysate without GST fusion proteins). The strongest signal was found in pull-down reaction with GST-α9A. Pull-downs with heart lysate showed only weak signal with GST-SH3 and nearly undetectable signal with GST-α9.

As shown in Figure 21, GST-pull-down assays were performed with the heart lysate of sacrificed mice and the different GST-αII-Spectrin constructs. The two characteristic signals of the Mena protein at 80/88 kDa and 140 kDa (neuronal splice variant) could be detected strongest in pull-down reactions with GST-α9A, corresponding to pull-downs with recombinant Mena protein described in 3.6.1. Concerning the ratio of the signal intensities between the two Mena splice variants, the signal of the ubiquitous Mena was about three times stronger compared to the neuronal variant in the input lane. In a pull-down reaction with GST-α9A the ratio was about 1:1, indicating a stronger binding of the neuronal splice variant in comparison to the ubiquitous variant. In pull-down reactions with GST-SH3 a weak signal could be detected, whereas in pull-downs with GST-α9 signal detection was nearly impossible. This finding was in contrast to the pull-downs with recombinant Mena, as well as that signal intensity of endogenous Mena in pull-downs with GST-α9A was about 20fold stronger than in pull-downs with GST-SH3. Different explanations for this clear positive effect of GST-α9A on binding to Mena are imaginable. First, the findings could be caused by differences between endogenous protein and recombinant protein, like posttranslational modification. Also the binding *in vivo* could be influenced by additional proteins, like adaptor proteins. To further investigate this hypothesis, pull-down assays with endogenous Mena purified from murine heart were performed.

6.5. GST-αII-Spectrin pull-down assays with Mena purified from heart

Due to the finding, that pull-down experiments with heart lysate showed a significantly stronger binding signal with the GST-α9A construct compared to the other GST-constructs, pull-down assays were performed using endogenous Mena purified from murine hearts by an antibody column. This experiment should clarify, if there are any signal differences when performing pull-downs with heart lysate or with the purified endogenous mammalian protein.

As detailed in 2.8 for purification of the endogenous Mena protein Mena antibody (α-Mena 438) was covalently coupled to a sepharose matrix; remaining reactive binding sites were saturated with ethanolamine and after

extensive washing mouse heart lysates were added to the column. To get rid of any bound adaptor proteins, a washing was performed using 500 mM to 1 M NaCl prior to elution of the endogenous Mena protein from the column by a pH-shift. The low pH of the elution fractions was directly neutralized by appropriate amounts of Tris-HCl pH 9.3. Then the eluted protein was directly used for GST-pull-down assays. Analysis of the purified Mena by SDS-PAGE and coomassie staining showed no apparent signals at 80/88 kDa or 140 kDa indicating protein amounts below the detection limit of coomassie staining (Figure 21A). Incubation of the Western blotting membrane with specific antibody against Mena showed clear signals for the protein at 80/88 kDa and 140 kDa according to the Mena splice variants (see Figure 21B, first lane).

Pull-down assays were directly performed with the purified Mena protein without knowing exact amounts of the protein. Each pull-down reaction contained 2 µg GST or an equimolar amount of the GST-αII-Spectrin constructs (A, SH3, α9, α9A). The experiment was performed in 200 µl final volume (buffer described in 2.9.). After incubation, washing of the precipitates, and addition of SDS sample buffer, precipitated proteins were analyzed by coomassie staining (12% SDS-PAGE) or Western blotting (10% SDS-PAGE) as shown in Figure 21.

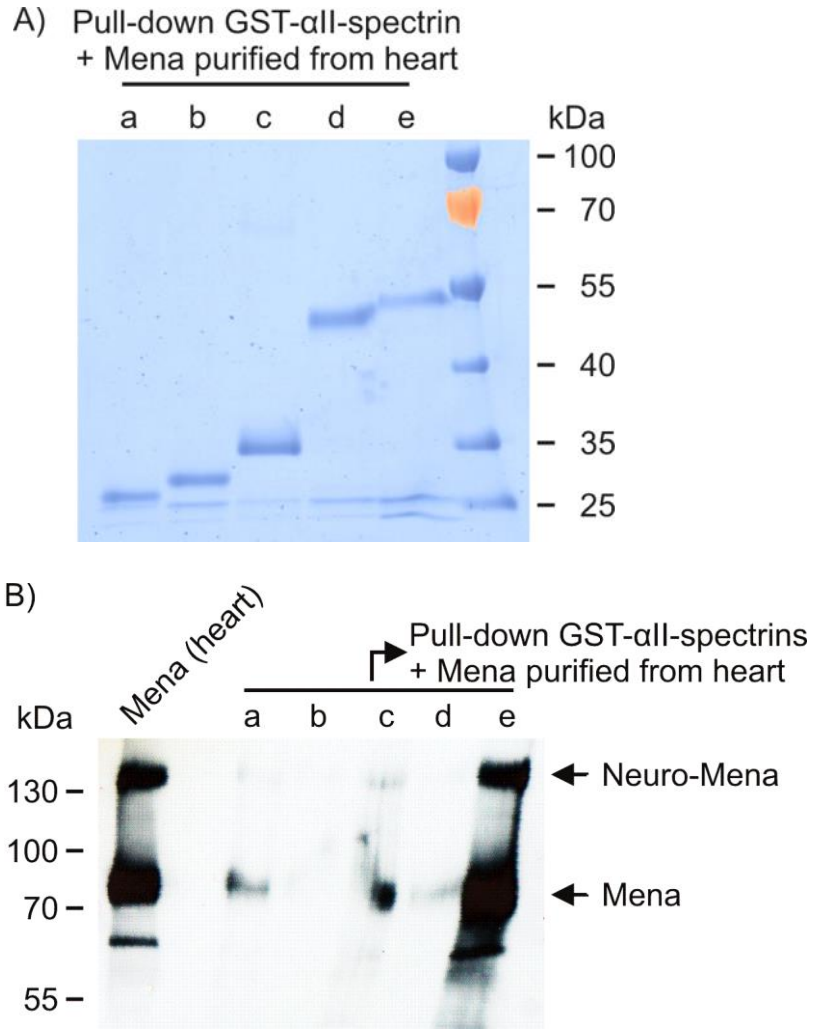


Figure 22: Pull-down GST-αII-Spectrin constructs with Mena purified from murine heart (A: 12% SDS-PAGE; B: 10% SDS-PAGE). a: GST; b: GST+A; c: GST+SH3; d: GST+α9; e: GST+α9A. One half of the pull-down experiment was loaded onto the 12% gel (A; Coomassie staining), the other onto the 10% gel (B; Western blotting membrane). A) Coomassie staining shows the GST-αII-Spectrin protein amounts used in pull-down experiments. Protein bands of the GST fusion proteins showed comparable intensities indicating similar protein amounts. The amounts of Mena in the pull-down experiments were too low to be detected by coomassie staining. B) As expected, Western blotting analysis using a specific Mena antibody showed signals at 80/88 kDa and 140 kDa as expected (first line, input control). The weak signal at about 65 kDa can be found in each Western blotting with endogenous Mena, but has yet not been identified. The amount of precipitated Mena when performing pull-down with GST-αII-Spectrin-α9A was substantially stronger than with GST or the other constructs, indicating about 20x stronger binding of Mena to this peptide than to the other peptides.

Coomassie staining of the 12% SDS-PAGE gel showed that the GST-αII-Spectrin constructs were used in equimolar amounts as expected (Figure 22A). However, the Mena protein used in pull-down experiments could not be detected by coomassie staining meaning that amounts of Mena were below

detection limit of this staining method. Therefore, detection of Mena binding was analyzed by Western blotting analysis (Figure 22B). As shown in the input control (purified Mena from the mouse heart, lane 1, Figure 22B), detection of Mena using a specific Mena-antibody showed clear protein bands at 80/88 kDa and 140 kDa corresponding to the endogenous splice variants. Pull-down experiments of GST-αII-Spectrin with Mena purified from heart showed a substantially stronger signal when performing the pull-downs with the GST-α9A construct than when performing the pull-downs with the other constructs. As expected, pull-downs with GST alone (control) showed only minor signal, just as pull-downs with GST-A or GST-α9. Concerning the pull-down with GST-SH3, stronger signal intensity compared to GST, GST-A, and GST-α9 could be detected, but not as strong as the signal in pull-down experiments with GST-α9A.

In summary, these experiments showed that the GST-α9A construct is the construct that is most potent to pull down Mena. Moreover, the experiment showed that binding of endogenous Mena to the GST-αII-Spectrin constructs is different than when performing the experiment with the recombinant Mena/VASP proteins purified from *E.coli*, indicating a significant difference between the proteins.

6.6. GST-pull-down assay with GST-αII-Spectrin-α9A versus GST-W1004R-αII-Spectrin-α9A and murine heart lysate

To get more information about the function and importance of a sufficient Mena/VASP αII-Spectrin-α9A interaction, a construct with a point mutation in the alternatively spliced SH3 domain was cloned either into the pGEX 6P2 vector for pull-down experiments or into the pEGFP C1 vector for immunofluorescence staining of endothelial cells (see 3.7.). Because tryptophan 1004 is essential for SH3 mediated binding, mutation of this residue to arginine should severely impair or abrogate interactions of the SH3 domain [42]. Pull-down assays with this point-mutated variant (GST+W1004R-α9A) and the non-mutated construct (GST+α9A) were performed to verify the inhibitory effect of the mutation on binding to Mena. The pull-down assays were performed with

heart lysate from sacrificed mice. In brief, 3 µg GST and equimolar amounts of GST-α9A or GST-W1004R-α9A were incubated with heart lysate for one hour. After washing and addition of loading buffer, the pull-downs were analyzed by 8% SDS-PAGE and Western blotting (Figure 23).

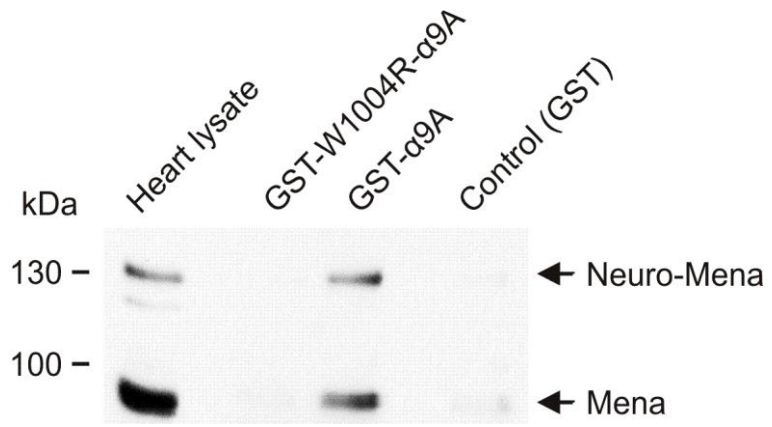


Figure 23: GST-pull-down assay with the point-mutated GST-W1004R-α9A vs. normal GST-α9A and heart lysate (8% SDS-PAGE). Both splice variants of Mena can be detected (80/88 kDa and 140 kDa) in the first lane (input control: Heart lysate without GST-αII-Spectrin constructs) using a specific α-Mena-antibody followed by an HRP-conjugated secondary antibody. Signals with the normal GST-α9A indicate an interaction between the protein and Mena, while little to no signal in pull-down reaction with the control (GST) or the mutated GST-W1004R-α9A could be detected, showing that the mutated binding domain is strongly impaired. Whereas the signal for the ubiquitous Mena is about 10fold stronger in the input control compared to the neuronal splice variant, the signals are equal in pull-down with GST-α9A, indicating a stronger binding of the neuronal splice variant. Figure published by Benz et al. [2]

Consistent with the previous findings (Figure 21) GST-αII-Spectrin-α9A efficiently pulls down the neuronal- and general Mena splice variant from heart lysate. In sharp contrast, little to no signal could be detected when performing the experiment with the mutated construct. Even the negative control (GST alone) showed stronger signals in Western blotting than the Spectrin mutant, indicating that the W1004R-point mutation was sufficient to strongly impair or to completely disturb the SH3 binding domain of the α9A construct.

7. Immunofluorescence of Mena/VASP and Spectrin in ECV

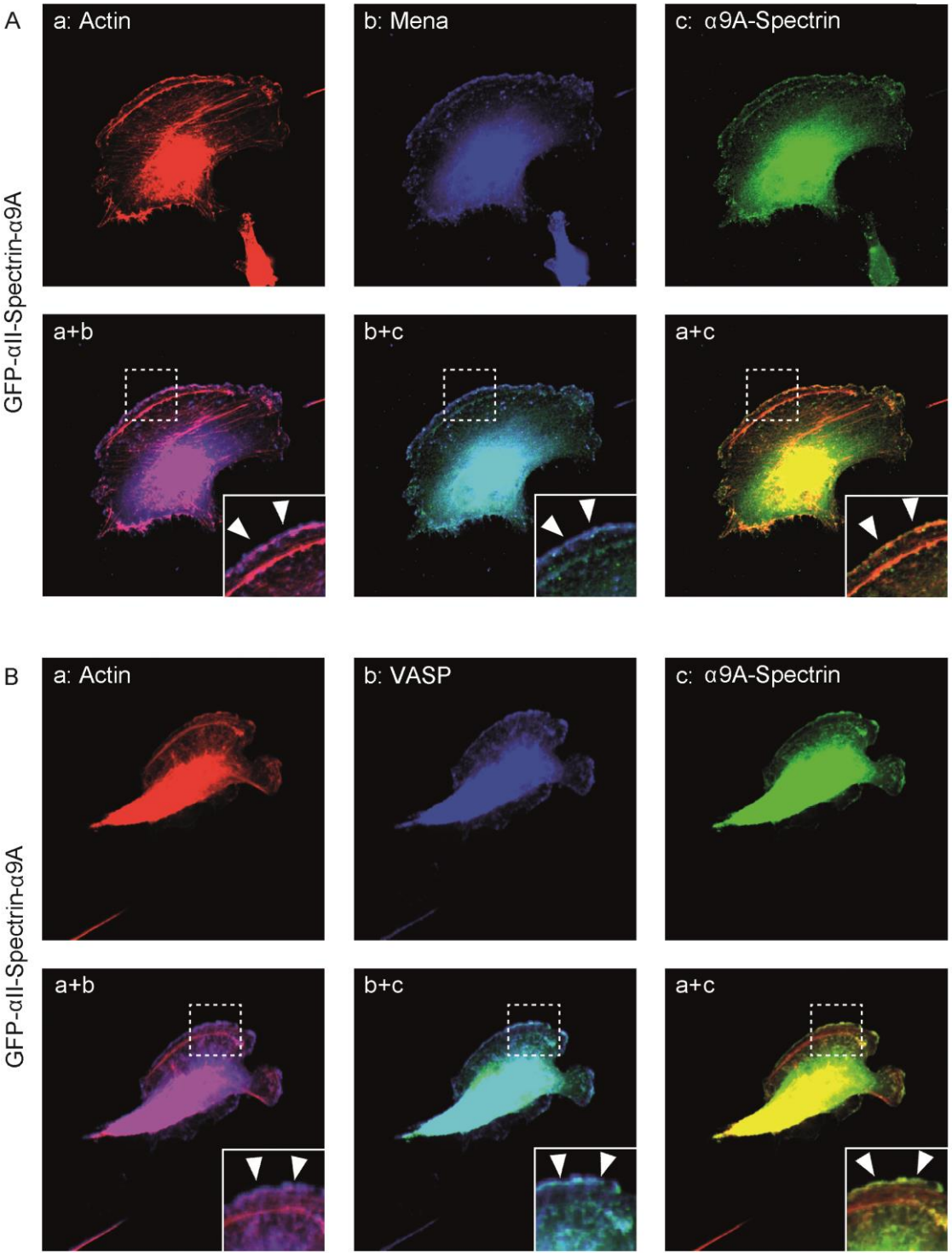
As detailed in 3.6., the findings of the pull-down experiments strongly indicate a direct interaction between Mena/VASP and αII-Spectrin. Moreover, binding of the alternative splice variant αII-Spectrin-α9A seems to be much more efficient

than binding of the other αII-Spectrin constructs. To further investigate the function and intracellular localization of the interaction of Mena/VASP and αII-Spectrin in the cell, immunofluorescence staining of endothelial cells (ECV 304) was performed after transfection of the cells with the αII-Spectrin constructs.

Endothelial cells show different morphology when they adhere to the dish surface as explained in 1.1. In this work, staining of the cells was performed when the cells were in a dynamic state and especially when lamellipodia could be observed at the edge of the endothelial cells in phase contrast light microscopy.

As described in detail in 2.12., in one approach the cells were cultivated on chamber slides until they reached an adherent state prior to transfection. In the other approach, transfection was performed after cultivation of the cells in 6 cm cell culture dishes until they reached confluence. After transfection, these cells were rinsed and re-cultivated for several hours on chamber slides to fix them in a migrating, not fully adherent state.

Staining was performed after transfection of the wild type GFP-αII-Spectrin-α9A DNA or the point-mutated GFP-αII-Spectrin-W1004R-α9A. The vector used for cloning of the constructs encodes the green fluorescent protein (GFP) N-terminally. To ensure fluorescence after fixation of the cell, the αII-Spectrins were stained additionally with an antibody against GFP, endogenous Mena/VASP and actin were stained with specific antibodies against the proteins and secondary antibodies, tagged with different fluorescent markers as described detailed in 2.12.


A
a: Actin
b: Mena
c: α9A-Spectrin
GFP-αII-Spectrin-α9A
a+b
b+c
a+c
B
a: Actin
b: VASP
c: α9A-Spectrin
GFP-αII-Spectrin-α9A
a+b
b+c
a+c

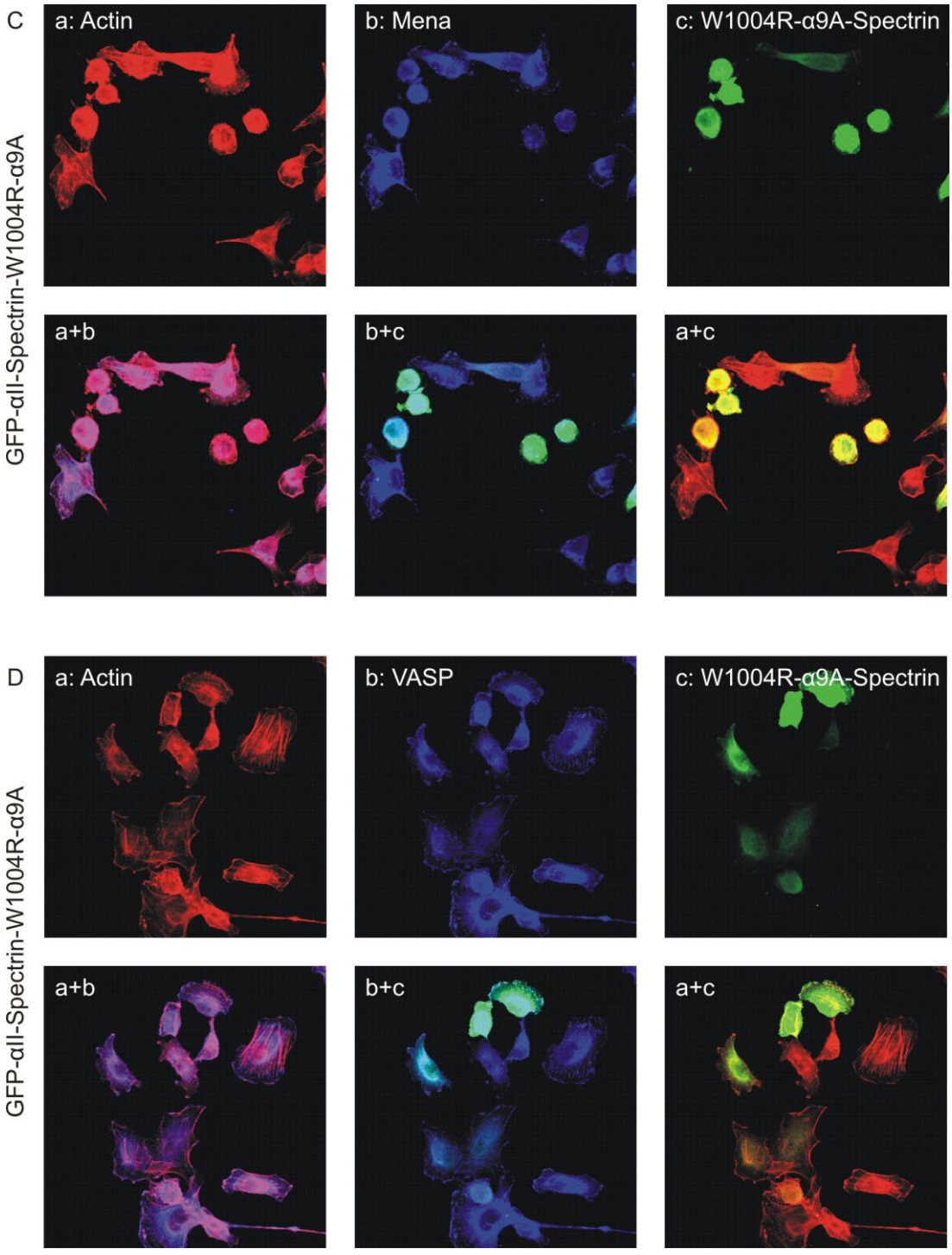


Figure 24: Immunofluorescence staining of endothelial cells. Confocal microscopy images of endothelial cells stained for actin (a, red), Mena (A+C) and VASP (B+D) (b, blue), and αII-Spectrin-α9A (c, green). In Figures A+B the endothelial cells were transfected with GFP-αII-Spectrin-α9A; in Figures C + D the endothelial cells were transfected with GFP-αII-Spectrin-W1004R-α9A. Staining with the wild type α9A-Spectrin construct shows significant signals of all three proteins at the leading edge of the endothelial cells (lamellipodia, marked with arrow heads, Figure A+B), indicating co-localization of the proteins. In contrast, immunofluorescence of the cells transfected with the W1004R-α9A-Spectrin mutant hardly shows cells with an intact cytoskeleton whereas surrounding non-transfected cells seem more or less healthy (Figure C+D).

Immunofluorescence experiments with the wild type GFP-α9A-Spectrin construct showed co-localization of Mena and VASP with the construct as well as co-localization of both proteins with actin at lamellipodia (Figure 24, A and B, lamellipodia are marked with arrowheads). This finding is in agreement with the results of the pull-down experiments, which indicated a direct interaction of these proteins. Because lamellipodia are important for cell motility, cell membrane stability, and shape change, co-localization of Mena/VASP and the αII-Spectrin-α9A indicates that both proteins could be involved in these processes. To get further information about the interaction of Mena/VASP proteins and the alternatively spliced αII-Spectrin construct, the point-mutated W1004R-α9A-Spectrin construct was also used in immunofluorescence. As shown in Figure 24, C and D, almost all transfected cells failed to show typical cell shape - like lamellipodia or filopodia. They rather showed a spherical conformation, which probably means that the cells died or failed to adhere. Different explanations for this finding are imaginable, most likely the construct itself or the purified DNA have a toxic effect on the cells. It is also imaginable that the impaired interaction of Mena/VASP and αII-Spectrin could cause the cytoskeleton dysregulation. Due to the remaining endogenous αII-Spectrin such a strong effect is rather unlikely, because the endogenous αII-Spectrin should compensate for this effect.

In summary, wild type alternatively spliced repeat 9 of αII-Spectrin seems to co-localize strongly with endogenous Mena/VASP proteins and actin, indicating that all proteins share the same physiological role in the cell and could be involved in formation of cell shape, cell motility, cell membrane stability and cell adhesion.

4 DISCUSSION

As described in several publications, interactions of Ena/VASP proteins with different SH3 domains were identified. For example, interactions with the SH3 domain of the tyrosine kinases src and abl have been investigated [54, 55]. Recently, also a SH3-mediated interaction of αII-Spectrins with Ena/VASP proteins has been described [14]. As described by Ursitti et al., several splice variants of αII-Spectrin exist in heart tissue [35]. The characterization of the differences in binding between Ena/VASP proteins and these splice variants were part of this work. To analyze possible physiological effects of the interaction, the subcellular localization of the proteins was investigated by immunofluorescence staining of endothelial cells.

1. Peptide scan arrays

To characterize the exact binding domains involved in binding of Ena-/VASP-proteins to αII-Spectrin-SH3 first a peptide scan array was performed. Peptide scan arrays in general are used to study kinetics, functionality and binding properties of protein-protein interactions. Therefore, various peptides, which are displayed on a surface, are incubated with antibodies or proteins and the interaction is visualized by secondary antibodies and Western blotting. This method allows to map antibody epitopes or to find the key residues of a protein-protein interaction, respectively.

As described in 3.2., the results of the peptide scan arrays indicate that binding of the Spectrin-SH3 domain to Ena-/VASP-proteins is mediated by the proline-rich region (PRR) of the proteins. In general, SH3 domains bind to proline-rich ligands with the consensus sequences + xΦPxΦP or ΦPxΦPx + (class I or class II ligands, where x is any, Φ is a hydrophobic, and + is a positively charged residue, respectively; [56]). Because Ena-/VASP-proteins do not contain positively charged amino acids, the SH3 domain of Spectrin seems to belong to the atypical SH3 domains. These domains prefer ligands with hydrophobic amino acids that contact the specificity pocket [57]. Moreover, the

known VASP binding partner abl [24] also contains an atypical SH3 domain [44].

With the peptide scan arrays it was possible to get detailed information about the binding motive involved in binding of Ena-/VASP-proteins to Spectrin-SH3. Therefore, motives with a glycine followed by a proline-stretch (GP_x) seem to be crucial for binding strength, as it has already been shown for the VASP protein [14]. In contrast to the finding that Mena does not interact with Spectrin-SH3 [58] the results of the peptide scan arrays showed that binding of Spectrin-SH3 to the GP_{6+} motives of Mena and EVL is substantially stronger than to the GP_5 motive of VASP. Additionally, binding of Spectrin-SH3 to Mena and EVL peptides occurred in a "biphasic" manner, i.e. two sets of high spot signals were separated by spots with low signal intensities. This finding suggests that the Spectrin-SH3 domain could interact with two or more motives in the PRR of Mena and EVL.

2. Purification of recombinant proteins from *E.coli*

All recombinant proteins used in the experiments were purified from the bacteria strain *Escherichia coli.* This bacteria strain is a model organism for protein expression and purification (for example because of its high cell division rate (doubling time 20 minutes)). Excluding chaperone co-expression experiments (expression in *E.coli* BL21 because of chaperone resistance) all proteins were expressed in a sub-strain from *E.coli* called Rosetta DE3 (BL21 derivate). Rosetta DE3 bacteria are protease deficient and are designed for enhanced expression of eukaryotic proteins containing codons that are rarely used in *E.coli*. Rosetta DE3 supply tRNAs for codons that are important especially for VASP and Mena expression due to their high content of prolines in their PRR.

All proteins were cloned with an N-terminal affinity-tag, which allows easy purification of the tagged protein by affinity chromatography without changing the protein functionality. In case of VASP proteins, affinity purification was performed via a His_6-tag while Spectrin proteins were purified via a GST-tag (tags are encoded by the vector used for cloning). Because expression of the Mena protein turned out to be very difficult, purification of this protein was

performed using a two-step procedure including affinity chromatography with GST and cleavage of this tag to get the remaining GST-less protein (His_6-Mena).

In general, the His_6-tag is much smaller than the GST-tag and purification is performed with a cobalt sepharose column. This method provides high specificity because bacteria rarely express proteins containing histidine stretches. GST-tagged proteins can be purified using GSH sepharose columns. One advantage of GST-tags is better protein solubility in the bacteria cell that is provided by the large GST as well as increased expression levels.

Purification varied largely in the level of expression, purity and yields. While expression of all different αII-Spectrin constructs at 30°C for 4-6 hours and a final concentration of 0.3 mM IPTG (induces protein over-expression) led to protein amounts up to 10-15 mg/ 50 ml bacteria culture in excellent purity, expression of the VASP and Mena constructs was more difficult. Because of the planned pull-down experiments, both Mena and VASP had to be tagged with 6x histidine to get comparable signals in Western blotting analysis using an anti-His-antibody. First, VASP expression and purification were optimized. Purification of human WT-VASP showed best results when expression was performed at 37°C for 40 hours and a final concentration of 1 mM IPTG. It turned out that these conditions fit best also for the G181P-mutant. In total, 500 µg protein per liter bacteria culture could be purified in case of WT-VASP, while purification amounts for the point-mutated VASP were always about 25% higher. This could mean that mutation of the GP_5 motive to GP_6 motive stabilizes the three dimensional structure of the protein so that expression is improved.

As compared to the full-length protein, Mini-VASP and VASP ΔTD were a lot more difficult to express in *E.coli*. Various conditions including temperature variations from 6 to 37°C, IPTG concentrations from 0.1 mM to 1 mM, and expression times from 3 hours to few days were tested. Purification of Mini-VASP showed best results when expression was performed at 37°C for 5 hours and a final concentration of 0.3 mM IPTG, whereas purification results of VASP ΔTD were best when performing the protein expression at 37°C for 24 hours

and a final concentration of 1 mM IPTG. While yields in case of Mini-VASP could be improved to about 120 µg per 100 ml bacteria culture, yields for VASP ΔTD stayed quite low (about 7 µg per liter culture), so it remained difficult to get enough protein for pull-down experiments. Similar problems were described by Bachmann et al. [16]. In their purification attempts VASP ΔTD was only detectable in Western blotting analysis, indicating protein amounts below 50 ng.
Finally, it was tried to express and purify the murine Mena protein in bacteria. Because this protein is even bigger than VASP, expression in bacteria is very difficult [47]. Therefore, some attempts were performed to express Mena in eukaryote cells for example by Suzuki et al. [52]. This group developed chinese hamster ovary (CHO) cells with stable integration of GST-Mena by multiple retroviral transfections. As mentioned in the publication, a thrombin cleavage site between the GST-tag and Mena could be used to get rid of the GST-tag. This system was tested in my bachelor thesis and showed multiple problems. First, purification amounts were not as good as described in the publication; second, purity level was very low and finally it turned out that Mena itself harbors the sequence for cleavage by thrombin. Therefore this system was not suitable to obtain the full-length protein without tag, and expression in bacteria was tested.
In initial experiments, His_6-tagged Mena was expressed in *E.coli* Rosetta DE3 under all conditions mentioned above. However, these attempts failed to show any protein band after purification, neither in coomassie staining nor in Western blotting analysis. Because of the protein length, the three dimensional structure, and the high content of amino acids, which are hardly expressed in bacteria, it is likely that Mena was not soluble and therefore part of inclusion bodies. Given the good purification results when performing expression of proteins with GST-tag, Mena was cloned into the pGEX 6P2 vector and was then expressed in *E.coli* BL21 in combination with different chaperones or in Rosetta DE3 without further chaperones. Chaperones are small heat shock proteins that can improve protein folding and expression in bacteria [51]. To summarize, GST-Mena expression and purification with chaperone co-expression were successful when expression was performed at 10°C for 36 to 48 hours and a final

concentration of 0.3 mM IPTG. Especially two of the chaperone combinations (named pBB 535/540 and pBB 530/535, encoding DnaK, DnaJ, GrpE and ClpB; compare table 8) were beneficial for GST-Mena expression/purification. Because simultaneous comparison of Western blotting signals from Mena and VASP should be possible, a Mena construct with a His_6-tag following the GST-tag was cloned (GST-His_6-Mena). So, expression and purification could be performed using the GST-tag and after cleavage of the tag by PreScission protease (restriction site encoded by the pGEX 6P2 vector) it was planned to do pull-down experiments with the remaining GST-less His_6-Mena. The double-tagged protein therefore was expressed in *E.coli* Rosetta DE3 (10°C, 72 hours, 0.3 mM IPTG) or in *E.coli* Rosetta DE3 with either pBB 535 (DnaK, DnaJ) or pBB 542 (DnaK, DnaJ, GroESL) (10°C, 36 to 48 hours, 0.3 mM IPTG). Because the plasmid of the Rosetta DE3 strain encodes a resistance against chloramphenicol, two chaperone plasmids containing a spectinomycin resistance (pBB 535 and pBB 542, compare table 8) were chosen for co-expression. Purification was analyzed by coomassie staining. No substantial differences concerning protein purity or amounts could be detected when GST-His_6-Mena was expressed in the presence or absence of pBB 535 or pBB 542. For the sake of simplicity further experiments were performed without the additional chaperones. In case of different resistances, the best combination would have been co-expression of both pBB 535/540 and GST-His_6-Mena. To further test this hypothesis cloning attempts could be performed to change the resistances of the chaperone combinations.

Another problem was the cleavage of the GST-tag using HR3VC, a protease that recognizes the same sequence as the PreScission protease [59]. Different conditions were tested to allow a complete cleavage without significant protein degradation. It turned out that performing the reaction in solution with a 100x higher concentration of protease than suggested in the manufacturer's description at 4°C was sufficient to achieve complete cleavage after 16 hours. This could be confirmed by coomassie staining and Western blotting, where the expected 25 kDa decrease in the apparent molecular weight, corresponding to the molecular weight of GST, could be observed. In contrast to Mena, cleavage

of the GST-Spectrin constructs was performed under different conditions. Almost complete cleavage could be observed after only 2.5 hours incubation of 100 µg protein with 5 units of the protease at 4°C. Therefore, these promising attempts were transmitted to pull-down experiments, where cleavage of the Mena/VASP GST-αII-Spectrin precipitates should help to analyze the pull-down results (see 4.4).

Future experiments should be performed to increase the yields of Mena/VASP purification and to ensure that purified Mena/VASP are equal to the endogenous proteins. Some possible attempts are described in 4.4.

3. Purification of Mena/VASP proteins

To get information about the absolute binding strength of the αII-Spectrin-Mena interaction, microcalorimetry with peptides of Mena and αII-Spectrin-SH3 was performed. Microcalorimetry is a method that takes advantage of energy changes that are involved in all chemical reactions [60]. When reactions lead to release of energy the reactions are called exothermic reactions. In case of heat absorption the corresponding reactions are called endothermic reactions. Microcalorimetry is a method that allows measurement of even very small heat changes in a defined reaction. Therefore, this method can be used for example to study interactions between molecules or proteins [60]. Using isothermal titration calorimetry, measurement of binding parameters, which are caused by the heat changes accompanying the binding between two molecules or proteins, can be achieved in an in-solution experiment. These parameters include the binding affinity (K_D), reaction stoichiometry (n), enthalpy (ΔH), and entropy (ΔS) [61].

ITC is a convenient method for binding studies of SH3 domains, especially for measurement of interactions with up to 20 amino acid long peptides. Due to the fact that VASP and Mena are up to 20-fold larger, ITC would require protein amounts in a high milligram range. As described earlier, purification of such amounts is very challenging. Therefore, a Mena peptide consisting of the 22 amino acids QASVALPPPPGPPPPPPLPSTG was used in these experiments.

This monomeric protein is missing the tetramerization domain but partially contains the proline-rich region that seems to be important for binding.

ITC experiments with GST-αII-Spectrin-SH3, -α9 or -α9A and the Mena peptide indicated several things. First, binding affinity was strongest with the GST-SH3 construct (K_D 909 µM) whereas binding of GST-α9 and GST-α9A showed similar and two-fold weaker affinities (K_D 1.95 and 1.97 mM). These results indicate that binding of the normal SH3 domain within α9 is most potent to interact with Mena, whereas the whole α9 *in vitro* repeat and the alternatively spliced α9 (α9A) *in vitro* repeat show weaker binding potency.

As described in 3.6.4. this finding is in contrast to the results of the pull-down experiment with heart lysate. One reason could be that the truncated Mena peptide used in ITC is not sufficient to reflect the binding of the full-length Mena protein. This could be caused by the missing tetramerization domain of the Mena protein that was not included in the Mena peptide [16]. Another reason for the findings could be that in the cell additional proteins are serving as adaptors leading to a multi-protein complex that improves and mediates binding of Mena and VASP to αII-Spectrin variants [2]. We recently showed that potential adaptors could be actin, sarcomeric α-actinin, and connexin 43 (Cx43) [2]. It was reported that gap junctions are stabilized by interactions of connexins with the actin cytoskeleton and associated proteins and that αII-Spectrin co-precipitates Cx43 [62]. Due to impaired Cx43-positive gap junction morphology in hearts of Mena/VASP double-deficiency mice a complex formation with Cx43, Mena/VASP and αII-Spectrin could be crucial for the stabilization and intercellular communication of gap junctions [2]. Also complex formation with Mena/VASP, sarcomeric α-actinin, and αII-Spectrin is imaginable. α-actinin attaches actin filaments to the Z-disks and therefore is involved in coordinated contractions of the sarcomeres [63]. Because both αII-Spectrin and Mena could be localized at Z-disks [2], a complex formation with α-actinin seems to be likely.

Moreover, the weak binding affinities found in ITC could partially be caused by the explanations above. Nonetheless, the weak binding affinity of the proteins

and their interaction surely is needed for fast changes of binding and non-binding that are needed for the cytoskeletal remodelling [1].

To get a better view on the situation *in vivo*, future ITC experiments should be performed with the full-length Mena or VASP tetramers to determine the binding parameters and binding differences between the two proteins and the GST-αII-Spectrin constructs. The high amounts that are needed for such experiments could possibly be achieved i.e. by transfection of eukaryote cells like SF9 cells with Mena/VASP DNA, as also described in 4.4. [64]. Usage of eukaryotic cells for expression of Mena and VASP should lead to correct protein translation, folding and posttranslational modification. Another important factor seems to be the protein tag; as described in 4.2. the GST-tag seems to be most beneficial for high yields in excellent purity.

4. GST-αII-Spectrin pull-down assays

Because of the findings of the peptide scan arrays and to further characterize the interaction between αII-Spectrins and proteins of the Ena/VASP family (Mena and VASP), pull-down assays with the different GST-αII-Spectrin-SH3 constructs and different variants of Mena and VASP were performed (compare Figure 6 and 9).

Pull-down assay is a convenient and frequently used method to detect protein-protein interactions or to identify new protein-protein interactions [65]. In brief, a so-called bait protein tagged for example with GST and recombinant protein or cell lysate are incubated. In case of interaction, analysis of washed precipitate (affinity purification of the bait protein with interacting proteins) by Western blotting should show signals for the recombinant protein. When pull-down is performed to find new interaction partners, SDS-PAGE and coomassie staining can be used to detect bands of interacting proteins. These protein bands can be used to characterize the underlying protein by mass spectrometry [65]. It has to be mentioned that proven interaction in pull-down assay does not necessarily reflect the situation *in vivo*, because of lacking cell specificity and cell compartmentalization [66]. Nonetheless, proven interaction in pull-down assay can give first hints at protein-protein interactions and involved protein domains.

Optimization of technical pull-down features

In initial experiments, pull-down assays with recombinant Mena/VASP protein purified from *E.coli* were performed. First, several technical problems had to be solved. To reduce background signals, three different methods were tested: Addition of glycerol, BSA (final concentration 1%), or elution from the column after the assay without further agents (data not shown). Analysis of these three methods showed weakest background signals in pull-downs with BSA, so this agent was added to all further pull-down experiments. A remaining problem was the circumstance, that human recombinant VASP and the GST-αII-Spectrin constructs α9 and α9A have similar molecular weights (VASP: 46 kDa; GST-α9: 47 kDa; GST-α9A: 49 kDa). First, it was tried to analyze the VASP-Spectrin interaction by SDS-PAGE and coomassie staining without proceeding with Western blotting. Due to the molecular weights of the three proteins, it was impossible to distinguish between the GST-fusion proteins and VASP. Therefore, further experiments were analyzed by SDS-PAGE and Western blotting. However, it turned out that even the specific detection of VASP by antibodies was impaired by GST-α9/α9A (data not shown). The GST fusion proteins produced a weak background signal, but enough to impair the results. Different conditions were tested to reduce this effect. The easiest and best method was to run a 7% SDS-PAGE gel as far as possible without losing the VASP protein, so proteins were separated as much as possible. The method was sufficient to get rid of GST-α9A background signals (3 kDa difference in the molecular weight of VASP and GST-α9A); unfortunately, the background signal for GST-α9 still remained. Therefore, it was tried to cleave the GST-tag from the GST-Spectrin-VASP precipitates after the pull-down experiments using the internal cleavage site of the GST fusion proteins. In accordance to the results of the cleavage of the GST-Spectrin constructs (compare 3.4.1.), PreScission protease was added to the pull-down precipitates after the normal pull-down experiment in accordance to the on-column method (4°C, one hour, 10fold excess of protease; data not shown). Then the supernatant was analyzed by coomassie staining and Western blotting. However, it turned out that cleavage differed between the different GST-fusion-proteins leading to different cleaved

protein amounts in the analysed supernatant. Therefore, differences in binding of the GST-αII-Spectrin constructs to VASP could be an effect of the constructs as well as this could be an effect of different precipitate amounts caused by insufficient cleavage. The attempt to get rid of the GST-tag by cleavage was stopped; in consequence, when performing pull-down assays with GST-α9 and VASP, VASP signals still could partially be an effect of the Spectrin construct.

Pull-downs with recombinant His_6-Mena/His_6-VASP: Strongest binding signals with α9A

After optimization of the pull-down assay itself, the experiments with recombinant His_6-Mena and His_6-VASP showed conflicting results. First, very weak to no background signal in pull-downs with GST alone (control) could be observed, indicating that signals occurring in pull-down reactions with the GST-αII-Spectrin constructs should not be due to unspecific binding. The assays also had in common that pull-downs with the GST+A construct (GST+ 20 amino acid (aa) insert of the alternatively spliced SH3 domain of αII-Spectrin) only showed very weak to no signals, indicating that the 20 aa insert of the splice variant alone is not sufficient for binding to Mena/VASP proteins. The known interaction of Mena/VASP and the SH3 domain of repeat 9 of αII-Spectrin (GST+SH3) could also be observed in all pull-down assays [14].

Interestingly, binding of Mena and VASP to the alternatively spliced repeat 9 (GST+α9A) seemed to be significantly stronger than binding to GST-SH3 or GST-α9 (normal repeat 9 of αII-Spectrin). When performing the experiment with cleaved GST-His_6-Mena, the Mena signal intensity was strongest with GST-α9A, followed by GST-SH3 and then GST-α9 (Figure 18). In experiments with wild type VASP, a signal in pull-down reactions with GST-α9A could only be observed (Figure 19).

Pull-downs with point-mutated G181P-VASP: Doubtful comparable binding to α9 and α9A

Because of the results of peptide scan arrays another experiment was performed, using a point-mutated VASP protein with a GP_6 motive in the

proline-rich region instead of the normal GP_5 motive of wild type VASP. It was thought that the GP_6 motive should imitate the Mena proline-rich region, which inherits this motive as well. However, pull-down experiments showed almost the same signal intensities for the mutated VASP in Western blotting analysis of pull-downs with GST-α9 and GST-α9A (Figure 19). However, as detailed above, it remains unclear if the strong VASP signal in GST-α9 precipitates may be an artefact. Also, it remains unclear if the binding of Mena to αII-Spectrin is really stronger than that of VASP because of the GP_6 motive, because in all performed experiments the Mena-imitating mutated VASP showed higher protein amounts meaning that in consequence the signal intensities in pull-downs were stronger.

Competition pull-downs: Mena binding to SH3 is substantially stronger as compared to VASP binding

In further pull-down experiments the binding of Mena and VASP to αII-Spectrin should be compared. In initial experiments it was not possible to detect cleaved GST-His_6-Mena with an α-His_6-antibody so a new antibody was ordered. Then a so-called competition pull-down assay was performed using equimolar amounts of GST-SH3 and His_6-Mena/His_6-VASP in one pull-down reaction. Therefore, a 2:1 ratio of Mena/VASP compared to GST-SH3 was added to the reaction. In consequence, Mena and VASP competed for binding to GST-SH3. Experiment analysis showed that only Mena signals in pull-down experiment could be observed, while no VASP signal could be detected. This finding strongly indicates that Mena binding to αII-Spectrin is stronger than VASP binding, as already observed in peptide scan arrays.

Pull-downs with VASP ΔTD: No visible binding to Spectrin constructs

Because of the known important effect of the tetramerization domain of Mena and VASP on binding to other proteins, a mutant was designed lacking the tetramerization domain (VASP ΔTD). As mentioned in 4.2., purification of His_6-VASP ΔTD turned out to be really difficult indicating low protein stability and possibly low protein functionality. In pull-down experiments VASP ΔTD showed

no protein binding to any of the GST-Spectrin fusion proteins indicating that indeed the tetramerization domain is important for binding. This is in agreement with a previous study, where binding of a truncated Ena to the abl SH3 domain was severely impaired [53]. To get more information about the importance of the tetramerization domain for binding of Mena and VASP to other proteins, further expression and pull-down attempts should be performed.

Assuming that the proline-rich region and the tetramerization domain are the most important structural motives of Mena and VASP, a new mutant was designed consisting only of these two elements (Mini-VASP, 15 kDa). No binding could be observed in pull-down assays (data not shown). It has to be further investigated if the protein is not functional or if technical problems in pull-down experiments are responsible for this result. Because of the artificial protein structure, the first explanation seems to be more likely.

Pull-downs with murine heart lysates/ endogenous Mena purified from mouse hearts: Strongest binding signals with α9A

Because the importance of the differences in binding of Mena/VASP to the different Spectrin-SH3 splice variants still had to be investigated in living cells, pull-down experiments with mouse heart lysates were performed. Therefore, the lysates were incubated with the GST-αII-Spectrin constructs. The precipitates were then analyzed by Western blotting using an α-Mena antibody. Due to the fact that VASP is weakly expressed in murine heart tissue, VASP could not be detected in this assay. Whereas the signals for murine Mena in pull-downs with SH3 and α9 showed minor intensities as in the previous performed pull-down assays, the signal intensities in pull-down experiments with GST-α9A were significantly stronger, indicating a preferred binding of Mena to this splice variant *in vivo*. Compared to pull-downs with GST-SH3, the signal intensity was about 20-fold stronger in pull-downs with GST-α9A. The initially observed strong binding of GST-α9 could not be confirmed in pull-downs with heart lysate, suggesting an artificial background signal of the GST-α9 construct in pull-downs with VASP. Because this clear difference in binding strength could not be observed in pull-downs with recombinant Mena and VASP protein, we purified

endogenous Mena protein from heart lysates and compared the binding of the protein with Mena from heart lysates or recombinant protein purified from *E.coli* in pull-down assays.

Purification of murine Mena from heart lysates by an antibody column included several washing steps, one with 1 M NaCl to remove any Mena interacting proteins. The Mena protein was eluted from the column by pH-shift and was immediately neutralized and used for pull-down experiments. Analysis of the pull-downs by SDS-PAGE and coomassie staining showed that all αII-Spectrin constructs were used in equal amounts. Mena could not be detected, meaning that amounts of purified protein were below the limit of this method (detection limit about 100 ng). Therefore, Western blotting was performed, showing very similar results as pull-downs with heart lysate. Mena signals in pull-downs with GST-α9A were strongest, as before about 20-fold higher than in pull-down with GST-SH3. Clear protein bands in the input lane at 140 kDa and 80/88 kDa indicated a successful purification of the protein. Nonetheless, it still remains possible that the similar results when performing the pull-downs with lysate and purified endogenous protein are based on additional proteins bound to Mena. One possible explanation could be formation of a multi-protein complex with adaptor proteins, i.e. connexin 43 or α-actinin that enhance or influence the binding of Mena and VASP to αII-Spectrin as already mentioned. Another explanation for the differences between endogenous and recombinant protein could be that bacteria are not suitable to synthesize and express the large Mena/VASP correctly. Another problem could be the different codon usage of bacteria compared to the mammalian codon usage. When transforming mammalian DNA into bacteria, it is possible that the bacteria use some codons only rare. Therefore, we used the Rosetta DE3 strain that supplies these codons. Due to the fact that posttranslational modifications differ between bacteria and eukaryote cells, it is possible that these differences, i.e. in phosphorylation of serine, could also be responsible for the observed differences between endogenous mammalian and recombinant protein [67].

In further experiments, purification of recombinant protein expressed in mammalian cells should be investigated, for example in SF9 moth cells that are

very easy to transfect with the low pathogenic baculovirus [64]. Usage of mammalian cells, i.e. stable HEK cells, for expression of Mena and VASP should lead to correct protein translation, folding and posttranslational modification. Pull-down experiments with these proteins should give evidence, if the enhanced binding of GST-αII-Spectrin-α9A to Mena/VASP is reproducible.

5. Immunofluorescence

Because of the finding that Mena binding to GST-α9A *in vivo* seems to be much stronger than binding to all other GST-αII-Spectrin constructs (see pull-down experiments), localization of Mena/VASP proteins in endothelial cells, that express these proteins endogenously, and GST-α9A was visualized by immunofluorescence.

Immunofluorescence is a convenient method to visualize protein localization in mammalian cells. Therefore, cells are cultivated on glass dishes called chamber slides and fixed at a defined time point. Then antibody staining of the proteins of interest is performed, followed by secondary antibodies tagged with defined fluorophores. Subsequent fluorescence microscopy finally allows visualization of the stained proteins as well as localization of the proteins in the cell. Because antibodies cannot penetrate the cell membrane of living cells, this method is limited to fixed cells, so no live time imaging is possible.

To look at the co-localization of Mena/VASP and the alternatively spliced repeat 9 of αII-Spectrin, plated endothelial cells had to be transfected with the GFP-αII-Spectrin-α9A construct. Endothelial cells show different physiological forms when they adhere at a surface. Cells show highest mobility and shape change when they adhere at the surface, whereas these dynamics decrease when cells grow to confluence [6]. In this work, cells were fixed when especially lamellipodia could be observed in light microscopy either prior to transfection in an adherent, but dynamic state or after transfection and subsequent rinse with partial re-adherence (compare 1.1.). After fixation of the cells, Mena or VASP, actin and GFP-α9A were stained as described in 2.12. Evaluation then was performed by confocal microscopy.

As expected, immunofluorescence of endothelial cells showed localization of the Mena/VASP proteins and actin at the leading edge of the cells, especially at lamellipodia. Moreover, the transfected GFP-α9A as well could be localized at the lamellipodia, strongly indicating co-localization of all three proteins (see Figure 24, A and B). Together with the pull-down results, direct interaction can strongly be assumed. The physiological significance of the co-localization at lamellipodia still remains to be shown. Today it is known that actin dynamics and lamellipodia formation are important for cell motility and shape change indicating that the protein-protein interaction could be involved in these processes [6].

To get information about the effects of a disrupted Mena/VASP-αII-Spectrin interaction, point-mutated GST-W1004R-α9A and GFP-W1004R-α9A constructs were designed. Point mutation of the tryptophan in position 1004 to arginine should abrogate the binding ability of the SH3 domain [42]. First, the binding loss was proved with GST-W1004R-α9A in pull-down assay (see 3.6.). Mena binding to this mutant was strongly reduced versus binding to the non-mutated GST-α9A, the signal intensity was comparable to the background-binding signal to the control (GST). Therefore, sufficient disruption of the SH3 domain was assumed and immunofluorescence experiments with the mutated GFP-α9A were performed as described above.

As shown in Figure 24, C and D, nearly all transfected ECV 304 cells did not show typical cell shape or formation of lamellipodia. Most cells rather showed a spherical conformation, which could indicate that the cells died before fixation or, in case of rinse prior to fixation, that adhesion of the cells could be impaired. Several explanations for this finding are imaginable. One possibility is a toxic effect of the construct itself. Another explanation could be that the cells cannot build up their normal and physiological shape when interaction of Mena/VASP and αII-Spectrin-α9A is impaired. Due to the endogenous αII-Spectrin protein that still is expressed in the cells and should compensate for the effect of the mutated Spectrin this hypothesis seems to be unlikely.

In summary, the non-mutated αII-Spectrin-α9A seems to co-localize strongly with endogenous Mena/VASP proteins and actin, indicating that all proteins join

in the same physiological role in the cell and could be involved in formation of cell shape, motility, cell membrane stability and cell adhesion. Further experiments should be performed with newly purified DNA of the mutated GFP-α9A to get more information about a possible toxic effect of the construct. Also experiments are imaginable using cells without endogenous Mena/VASP protein. Transfection of the proteins would allow monitoring the effects of an intact binding in contrast to a non-interacting situation. Therefore, it has to be investigated if Spectrin recruits Mena/VASP proteins to the leading edge of the endothelial cells or if recruitment is *vice versa.* Besides, the importance of the interaction for physiological processes like cell movement and migration remains to be defined.

5 SUMMARY AND FUTURE PROSPECTS

Regulation of actin cytoskeletal turnover is necessary to coordinate cell movement and cell adhesion. Proteins of the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family are important mediators in cytoskeleton control, linking cyclic nucleotide signaling pathways to actin assembly. In mammals, the Ena/VASP family consists of mammalian Enabled (Mena), VASP, and Ena-VASP-like (EVL). The family members share a tripartite domain organization, consisting of an N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline-rich region (PRR), and a C-terminal EVH2 domain. The EVH1 domain mediates binding to the focal adhesion proteins vinculin and zyxin, the PRR interacts with the actin-binding protein profilin and with Src homology 3 (SH3) domains, and the EVH2 domain mediates tetramerization and actin binding.

Endothelial cells line vessel walls and form a semipermeable barrier between blood and the underlying tissue. Endothelial barrier function depends on the integrity of cell-cell junctions and defective sealing of cell-cell contacts results in vascular leakage and edema formation. In a previous study, we could identify a novel interaction of the PRR of VASP with αII-spectrin. VASP-targeting to endothelial cell-cell contacts by interaction with the αII-spectrin SH3 domain is sufficient to initiate perijunctional actin filament assembly, which in turn stabilizes cell-cell contacts and decreases endothelial permeability. Conversely, barrier function of VASP-deficient endothelial cells and microvessels of VASP-null mice is defective, demonstrating that αII-spectrin/VASP complexes regulate endothelial barrier function *in vivo.*

The aim of the present study was to characterize the structural aspects of the binding of Ena/VASP proteins to αII-spectrin in more detail. These data are highly relevant to understand the cardiovascular function of VASP and its subcellular targeting. In the present study, the following points were experimentally addressed:

1. Comparison of the interaction between αII-spectrin and Mena, VASP, or EVL

In contrast to the highly conserved EVH1/EVH2 domains, the PRR is the most divergent part within the Ena/VASP proteins and may differ in binding modes and mechanisms of regulation. More specifically, VASP contains a triple GP_5 motif, whereas EVL and Mena contain one or more GP_6 motifs or even longer proline stretches. In the present study, we used peptide scans and competitive αII-spectrin SH3 pull-down assays with the recombinant Mena, VASP, and VASP mutants to investigate the relative binding efficiency. Our results indicate that binding of the αII-spectrin SH3 domain to GP_6 motifs is superior to GP_5 motifs, giving a rationale for a stronger interaction of αII-spectrin with EVL and Mena than with VASP.

2. Interaction of SH3i with Ena/VASP proteins

In the mammalian heart, an αII-spectrin splice variant exists (SH3*i*), which contains a 20 amino acid insertion C-terminal to the SH3 domain. We used GST-fusion proteins of αII-spectrin, comprising the SH3 domain with or without the alternatively spliced amino acids, to pull-down recombinant Mena, VASP or VASP mutants. The results demonstrate a substantially increased binding of the C-terminal extended SH3 domain as compared to the general αII-spectrin isoform without the 20 amino acid insertion. These findings were also confirmed in pull-down experiments with heart lysates and purified Mena from heart muscle. The increased binding was not due to an alternative, SH3-independent binding interface because a pointmutation of the SH3 domain (W1004R) in the alternatively spliced αII-spectrin isoform completely abrogated the interaction. To analyze the interaction of SH3*i* and Ena/VASP proteins in living cells, we expressed the extended SH3 domain as GFP fusion proteins in endothelial cells. Here, we observed an extensive co-localization with Mena and VASP at the leading edge of lamellipodia confirming the in vivo relevance of the interaction with potential impact on cell migration and angiogenesis.

3. Binding affinity and influence of the Ena/VASP tetramerization domain

We also determined the binding affinity of the general and the alternatively spliced αII-spectrin SH3 with Ena/VASP proteins by isothermal titration calorimetry (ITC) using a peptide from the PRR of Mena (collaboration with Dr. Stephan Feller, University of Oxford). Surprisingly, the binding affinity of the general SH3 domain was low (~900 µM) as compared to other SH3 domain-mediated interactions, which commonly display binding constants in the low micromolar range. Furthermore and in contrast to the pull-down assays, we could not detect an increased binding affinity of the C-terminally extended SH3 domain. This could be either explained by the existence of a third protein, which "bridges" the Mena/αII-spectrin complex in the pull-down assays, or, more likely, by the small size of the Mena peptide, which lacks major parts of the Mena protein, including the tetramerization domain. Indeed, it has been previously shown that the tetramerization of Ena is crucial for the interaction with the Abl-SH3 domain, although no SH3 binding sites are found in the tetramerization domain. To address this point experimentally, we used a VASP mutant that lacks the tetramerization domain in pull-down assays. Neither the general nor the alternatively spliced SH3 domain bound to the monomeric VASP, demonstrating the crucial (indirect) impact of Ena/VASP tetramerization on the interaction with αII-spectrin.

In summary, we conclude that the αII-spectrin SH3 domain binds to the proline-rich region of all Ena/VASP proteins. However, binding to EVL and Mena, which both possess one or more GP_6 motifs, is substantially more efficient than VASP, which only contains GP_5 motifs. The C-terminally extended SH3 domain, which is present in the αII-spectrin splice variant SH3i, binds stronger to the Ena/VASP proteins than the general isoform and expression of the isolated domain is sufficient for co-localization with Ena/VASP in living endothelial cells. Finally, the tetramerization of the Ena/VASP proteins is indispensable for the interaction with either isoform of αII-spectrin.

6 ZUSAMMENFASSUNG

Die Regulation des Umbaus des Aktinzytoskeletts ist für die Fortbewegung sowie die Adhäsion von Zellen essentiell. Proteine der Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) Familie sind wichtige Mediatoren bei diesem Prozess, indem sie zyklische Nukleotidprotein-Signalwege mit dem Aktinzytoskelett-Aufbau verknüpfen. In Säugern besteht die Ena/VASP-Protein Familie aus mammalian Enabled (Mena), VASP und Ena-VASP-like (EVL). Diese Proteine teilen sich einen gemeinsamen strukturellen Aufbau: N-terminal befindet sich die Ena/VASP homology 1 (EVH1) Domäne, zentral liegt eine prolinreiche Region (PRR) und C-terminal befindet sich eine EVH2 Domäne. Die EVH1 Domäne vermittelt eine Interaktion mit den fokalen Adhäsionsproteinen Vinculin und Zyxin, die PRR interagiert mit dem aktinbindenden Protein Profilin sowie mit Src homology 3 (SH3) Domänen und die EVH2 Domäne vermittelt die Tetramerisierung der Proteine sowie die Interaktion mit Aktin.

Endothelzellen kleiden die Gefäßwand aus und bilden eine semipermeable Barriere zwischen Blut und dem umgebenden Gewebe. Die Funktion des Endothels hängt dabei von der Integrität der Zell-Zell-Kontakte ab. Die Zerstörung dieser Kontakte führt zu vaskulärer Leckage sowie zur Ausbildung von Ödemen. In einer vorausgehenden Arbeit konnten wir eine neue Interaktion zwischen der PRR von VASP und αII-Spektrin zeigen. Durch die Interaktion mit der SH3 Domäne von αII-Spektrin gelangt VASP an Zell-Zell-Kontakte von Endothelzellen und ist dort in der Lage die Aktinverknüpfung in der Umgebung der Zell-Zell-Kontakte zu initiieren, was wiederum die Zell-Zell-Kontakte stabilisiert und die vaskuläre Permeabilität reduziert. Umgekehrt konnten wir beobachten, dass die Barrierefunktion von Endothelzellen und Mikrogefäßen von VASP-defizienten Mäusen gestört ist, was darauf hindeutet, dass VASP/αII-Spektrin-Komplexe an der Regulation der endothelialen Barrierefunktion *in vivo* beteiligt sind.

Das Ziel dieser Arbeit war die detaillierte Charakterisierung der Strukturen von Ena/VASP Proteinen und αII-Spektrin, die an der Interaktion zwischen diesen

Proteinen beteiligt sind. Diese Daten sind äußerst wichtig um die genaue Funktion von VASP im kardiovaskulären System zu verstehen. In dieser Arbeit wurden die folgenden Punkte genauer analysiert:

1. Vergleich der Interaktion zwischen αII-Spektrin und Mena, VASP oder EVL

Verglichen mit den hochkonservierten EVH1/2 Domänen besitzt die zentrale PRR der Ena/VASP Proteinfamilie die größte Diversität und könnte sich bezüglich der Binde- und Regulationsmechanismen zwischen den einzelnen Proteinen unterscheiden. Im Detail besitzt VASP ein sich dreifach wiederholendes GP_5 Motiv wohingegen Mena und EVL ein sich ein- oder mehrfach wiederholendes GP_6 Motiv oder noch längere Prolinsequenzen aufweisen. In dieser Arbeit nutzten wir peptide scan arrays und kompetitive αII-Spektrin SH3 pull-down Versuche mit rekombinantem Mena, VASP und VASP Mutanten um die relative Bindungsstärke der Interaktion zu untersuchen. Die Ergebnisse zeigten, dass die Bindung der αII-Spektrin SH3 Domäne an GP_6 Motive der Bindung an GP_5 Motive überlegen ist, was darauf hindeutet, dass die Interaktion zwischen αII-Spektrin und EVL und Mena stärker ist als die Interaktion mit VASP.

2. Interaktion von SH3i mit Ena/VASP Proteinen

Im Säugerherzen kommt eine αII-Spektrin Splicevariante (SH3*i*) vor, die eine Insertion aus 20 Aminosäuren C-terminal der SH3 Domäne enthält. Wir nutzten GST-αII-Spektrin Fusionsproteine, die die SH3 Domäne mit oder ohne Aminosäuren-Insertion beinhalteten, im pull-down Versuch mit rekombinantem Mena, VASP oder mit VASP Mutanten. Die Ergebnisse zeigten eine deutlich ansteigende Bindungsrate bei der C-terminal verlängerten SH3 Domäne verglichen zu der üblichen αII-Spektrin Isoform ohne Aminosäureninsertion. Diese Ergebnisse konnten auch durch pull-down Versuche mit Herzlysat und aufgereinigtem Mena aus Herzmuskulatur bestätigt werden. Dieser Effekt auf die Bindung war dabei nicht bedingt durch ein alternatives, SH3-unabhängiges Bindungsmuster, da eine Punktmutation (W1004R) in der Sequenz der SH3

Domäne der alternativ gespleißten αII-Spektrin Isoform die Interaktion komplett aufhob. Um die Interaktion von SH3*i* und Ena/VASP Proteinen in lebenden Zellen zu untersuchen, exprimierten wir die verlängerte SH3 Domäne als GFP-Fusionsprotein in Endothelzellen. Hierbei konnten wir eine ausgeprägte Kolokalisation mit Mena und VASP an Lamellipodien beobachten, was die Relevanz der Interaktion im lebenden Organismus bestätigt und auf einen potentiellen Effekt auf Zellmigration und Angiogenese schließen lässt.

3. Bindungsaffinität und Einfluss der Ena/VASP Tetramerisierungsdomäne

Zusätzlich wurde die Bindungsaffinität der üblichen sowie der alternativ gespleißten αII-Spektrin SH3 Domäne mit den Ena/VASP Proteinen mittels isothermal titration calorimetry (ITC) bestimmt, wobei ein Peptid bestehend aus der PRR von Mena genutzt wurde (Kollaboration mit Dr. Stephan Feller, Universität von Oxford). Überraschenderweise war die Bindungsaffinität der üblichen SH3 Domäne verglichen mit anderen SH3 Interaktionen, die normalerweise Bindungskonstanten im unteren mikromolaren Bereich aufweisen, niedrig (~900 µM). Außerdem und im Kontrast zu den Ergebnissen der pull-down Versuche konnten wir keine gesteigerte Bindungsaffinität der C-terminal verlängerten SH3 Domäne feststellen. Dies könnte entweder durch die Beteiligung eines dritten Proteins erklärt werden, das den Mena/αII-Spektrin-Komplex in den pull-down Versuchen überbrückt, oder aber, was wahrscheinlicher ist, durch die geringe Größe des Mena Peptids, dem wichtige Bestandteile des Mena Proteins inklusive der Tetramerisierungsdomäne fehlen. Tatsächlich wurde erst kürzlich gezeigt, dass die Tetramerisierung von Ena essentiell für eine Interaktion mit der Abl SH3 Domäne ist, obwohl keine SH3 Bindestellen in der Tetramerisierungsdomäne gefunden wurden. Um dieser Beobachtung nachzugehen, nutzten wir eine VASP Mutante in pull-down Versuchen, der die Tetramerisierungsdomäne fehlt. Weder die übliche noch die alternativ gespleißte SH3 Domäne interagierte mit dem monomeren VASP, was einen essentiellen (indirekten) Einfluss der Tetramerisierung von Ena/VASP Proteinen auf die Interaktion mit αII-Spektrin nahelegt.

Zusammengefasst können wir sagen, dass die αII-Spektrin SH3 Domäne an die prolinreiche Region aller Ena/VASP Proteine bindet. Jedoch ist festzuhalten, dass die Bindung an EVL und Mena, die beide ein oder mehrere GP_6 Motive enthalten, deutlich effizienter ist als die Bindung an VASP, das ausschließlich GP_5 Motive beinhaltet. Die C-terminal verlängerte SH3 Domäne, die in der alternativ gespleißten αII-Spektrin Variante SH3*i* vorkommt, bindet stärker an Ena/VASP Proteine als die übliche αII-Spektrin Isoform und bereits die Expression der isolierten Domäne alleine ist für eine Kolokalisation mit Ena/VASP Proteinen in lebenden Endothelzellen ausreichend. Letztlich konnten wir zeigen, dass die Tetramerisierung der Ena/VASP Proteine unentbehrlich für die Interaktion mit allen untersuchten αII-Spektrin Isoformen ist.

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