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Teresia Magnuson Osborn

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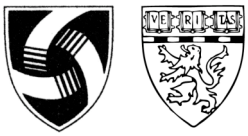

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This thesis is dedicated to *Eric* for all his love and support and to my parents, *Anders* and *Ingrid* for always encouraging me to do what I love.

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Abstract

Gelsolin (GSN) and filamin A (FLNa) are two actin-binding proteins discovered in our laboratory over 30 years ago. GSN is a calcium-activated actin severing and barbed end capping protein that is expressed as both intracellular and extracellular (plasma gelsolin, pGSN) isoforms. pGSN is present at relatively high concentrations (~ 200 μg/ml) in blood, but its extracellular functions have not been determined. pGSN levels decrease during acute inflammation and low levels correlate negatively with survival. Re-administration of pGSN to severely injured animals can rescue them from death, although the mechanism for this is unknown. pGSN levels during chronic inflammation have not been reported. FLNa is an important architectural component of three-dimensional actin networks in cells. It is an elongated homo-dimer that efficiently crosslinks F-actin into a gel in contrast to the gel-solating properties of GSN. Each subunit has an N-terminal "actin-binding domain" (ABD) followed by two rod-like domains and a C-terminal self-association domain. FLNa mediates actin-membrane connections, serves as a scaffold for >50 different binding partners, and FLNa-F-actin crosslinks accommodate cell shape changes and motility. However, as of yet there have not been sufficient details concerning FLNa's structure to fully explain its multiplicity of functions.

pGSN has lipid-binding sites and has been shown to bind to lysophosphatidic acid (LPA), a potent cell-activating phospholipid. Based on this, a new hypothesis positing pGSN as an anti-inflammatory protein was formed. Using platelets and neutrophils isolated from human blood, the effects of recombinant pGSN on platelet P-selectin exposure and neutrophil oxygen radical production induced by LPA and another structurally related phospholipid, platelet-activating factor (PAF), were investigated. Results showed that pGSN modulated cellular activation induced by both of these inflammatory phospholipids. In order to investigate pGSN levels during chronic inflammation, plasma and synovial fluids from patients with rheumatoid arthritis were analyzed. pGSN levels were lower in plasma from patients than age and gender matched healthy controls, and further reduced in synovial fluid.

To examine the mechanism behind FLNa's potency as a F-actin crosslinker, the FLNa-F-actin interaction was investigated by binding and gel-point assays, electron microscopy, and real-time video microscopy using full-length and truncated FLNa molecules. A new F-actin binding site was identified, which functions in conjunction with dimerization, long flexible subunits, and the previously identified ABD, to explain high avidity binding to F-actin. The results also show that crosslinks are rigid structures and that the self-association domains determine high angle branching. The C-T domain of FLNa, which binds many partners, has a compact structure compared to the elongated N-T two-thirds of the protein, does not associate with F-actin and can bind partners while FLNa is bound to F-actin.

In conclusion, these findings demonstrate a novel function of pGSN as a modulator of phospholipids, a finding that may be important for inflammation, and that pGSN levels are decreased during chronic inflammation in addition to previously documented acute conditions. The mechanism of FLNa crosslinking of F-actin can be explained by the intrinsic structure and properties of the FLNa molecule.

Keywords: Cytoskeleton, crosslinking, F-actin, filamin, gelsolin, inflammation, plasma, platelet-activating factor, lysophosphatidic acid, rheumatoid arthritis.

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List of publications

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

I. **Modifications of cellular responses to lysophosphatidic acid and platelet-activating factor by plasma gelsolin.**
Teresia M. Osborn, Claes Dahlgren, John H. Hartwig, Thomas P. Stossel
Am J Physiol Cell Physiol 292:1323-1330, 2007.[§]

II. **Decreased plasma gelsolin levels in rheumatoid arthritis.**
Teresia M. Osborn, Margareta Verdrengh, Thomas P. Stossel, Andrej Tarkowski, Maria Bokarewa
Submitted manuscript, 2007

III. **Structural basis of filamin A functions.**
Fumihiko Nakamura*, Teresia M. Osborn*, Christopher A. Hartemink, John H. Hartwig, Thomas P. Stossel
Submitted manuscript, 2007
* contributed equally to this work

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Contents

Abbreviations

aa	amino acids
Aβ	amyloid β
ABD	actin binding domain
ABP	actin binding protein
ABS	actin binding site
AD	Alzheimer's disease
ADP	adenosine diphosphate
Ap3A	diadenosine 5',5"'-P1,P3-triphosphate
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
BAL	broncho-alveolar lavage
BSA	bovine serum albumin
C_c	critical concentration
CC	coiled coil
cGSN	cytoplasmic gelsolin
CH	calponin homology
CLP	cecal ligation and puncture
CRP	C-reactive protein
C-T	carboxy-terminal
CSF	cerebrospinal fluid
ddFLN	Dictyostelium discoideum FLN
EASS	extracellular actin scavenger system
EM	electron microscope/microscopy
F-actin	filamentous actin
FAF	familial amyloidosis of Finnish type
FLNa	filamin A
FLNb	filamin B
FLNc	filamin C
fMLF	formyl-methionyl-leucyl-phenylalanine
FN	fibronectin
G1-G6	gelsolin-like domains 1-6
G-actin	globular actin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GP1bα	glycoprotein 1bα
GSN	gelsolin
H1	hinge 1
H2	hinge 2
HSCT	hematopoietic stem cell transplantation
IL	interleukin
IPS	idiopathic pneumonia syndrome
Ig-like	immunoglobulin-like
IgFLNa	immunoglobulin-like FLNa domains
KO	knockout
LDL	low-density lipoprotein
LPA	lysophosphatidic acid
LPA_{1-5}	lysophosphatidic acid receptor 1-5

LPC	lysophosphatidyl choline
LPS	lipopolysaccharide
Lyso-PAF	lyso-platelet-activating factor
MMP	matrix metalloproteinases
MP	microparticle
mox-LDL	mildly oxidized LDL
NF-κB	nuclear factor-κB
N-T	amino-terminal
OPD	otopalatodigital
P1	phosphoinositide binding site 1
P2	phosphoinositide binding site 2
P3	phosphoinositide binding site 3
PA	phosphatidic acid
PAF	platelet-activating factor
PAF-AH	platelet-activating factor-acetylhydrolase
PAFR	platelet-activating factor receptor
PC	phosphatidyl choline
pGSN	plasma gelsolin
PIP_2	phosphatidylinositol 4,5-bisphosphate
PH	pleckstrin homology
PMA	phorbol 12-myristate 13-acetate
PNH	periventricular nodular heterotopia
PPARγ	peroxisome proliferator-activated receptor γ
PPI	phosphoinositides
RA	rheumatoid arthritis
rhpGSN	recombinant human plasma gelsolin
SF	synovial fluid
TRAP	thrombin receptor activating peptide
WT	wildtype
VWF	von Willebrand factor

1. INTRODUCTION

1.1 The eukaryote actin cytoskeleton

The cytoskeleton is one important feature that separates eukaryotic and prokaryotic organisms. As the name implies the cytoskeleton provides cells with structure and shape. However, it is not a skeleton in the same sense as our bodily bone skeleton; instead it is a structure undergoing constant structural changes where the "bones", which are made of polymerized proteins (filaments), break and reform in different dimensions, giving rise to shape changes, coordinated and directed movement, organelle transport and segregation of chromosomes during mitosis. All of these events are highly controlled by the interplay between extracellular factors, the surface receptors that they activate and the resulting cascade of intracellular signaling molecules. The cytoskeleton is composed of three major functionally coordinated and connected filamentous systems: microtubules, the intermediate filaments and the actin filaments. While all three structural components and their interactions are critical to cell behavior, this work will focus on proteins regulating actin filaments. Illustrating its importance in cells, actin is highly conserved throughout eukaryotic evolution and present at near millimolar concentrations, constituting ~5-20% of total cellular protein content.

Actin is a 42 kDa monomer (G-actin) that self-assembles into semi-flexible polymers (F-actin) under physiological conditions [1]. The rate-limiting step in this polymerization is the spontaneous formation of a nucleus consisting of 3 actin monomers, to which additional actin monomers then assemble onto the free end at a fast rate. G-actin molecules carry tightly bound ATP molecules that are hydrolyzed to ADP shortly after assembly to a polymer. The nucleotide hydrolysis changes the critical concentration (C_c) for polymerization at the two ends of the filament. In purified systems under physiological conditions, i.e. containing potassium, divalent ions, ATP and actin concentrations above the C_c for the slowest end, actin will be nearly completely polymerized. At equilibrium in the presence of ATP, because the C_c of the two ends are different, monomers will disassemble from the pointed end (-) and reassemble at the fastest growing end, called the barbed end (+). This dynamic process, called treadmilling, allows F-actin to remain at constant length while continuously exchanging monomers. It also allows proteins that interact with the filament ends to either promote disassembly (e.g. when binding the barbed end) or assembly (e.g. when binding the pointed end). ADP-containing subunits that dissociate off from the pointed end are recharged by ATP in the solution. In a cell, the ionic and salt conditions are optimal for actin assembly and the concentration of actin is well above the C_c needed for actin polymerization from both ends. But resting cells only contain ~50 % of their total actin as F-actin, which is arranged into higher order structures, while the rest is G-actin that is sequestered by monomeric actin binding proteins [2, 3]. When cell motility is required upon cell activation, actin can shift from the monomeric pool into the filamentous pool. The reversible assembly and the organization of filaments into more complex three-dimensional structures are regulated by hundreds of actin binding proteins (ABPs). These proteins control actin

assembly, disassembly, turnover and filament lengths, sequester monomers, and organize fibers into complex architectures in response to signaling cascades initiated by various stimuli. Most of the G-actin is in complex with cytosolic G-actin-binding proteins (thymosin β4 and profilin), which prevent incorporation onto the pointed end of F-actin. Since actin assembly occurs ~ 10 times faster from the barbed ends than the pointed ends, actin assembly and filament length are controlled by barbed end capping/uncapping, filament severing/annealing, and *de novo* formation of nuclei. Gelsolin is the founding member of a family of barbed end capping proteins and is activated by a rise in the intracellular calcium concentration to sever filaments. Phosphoinositide metabolism in the cell membrane attracts and sequesters gelsolin and other capping proteins from the newly formed barbed filament ends to ensure fast polymerization. Although it is not clear to what extent it occurs in the cell, gelsolin can also bring together two G-actin molecules, forming a barbed-end capped nucleus from which actin can assemble in pointed end direction. Other ABPs (e.g. Arp 2/3 complex) form a nucleus with the barbed end exposed, leading to fast actin assembly.

The actin filaments are ordered into complex structures by bundling (e.g. α-actinin), branching (e.g. Arp 2/3 complex) and crosslinking (e.g. filamin A) proteins. The shape and surface topology of a cell is dependent on the architecture of the underlying actin filaments in the vicinity of, and anchored to, the plasma membrane. For example, actin bundles in platelets give rise to long thin filopods that bind to fibrin strands to form a three-dimensional blood clot. A more two-dimensional actin network consisting of orthogonally arrayed short actin filaments makes up the cellular lamellipodium, which directs the motility of the cell, pulling it across a surface and plugging injured vasculature [3, 4].

Upon tissue injury, large amounts of actin can be released from damaged cells into the extracellular space. Since the ionic conditions in the extracellular fluid favor actin polymerization, high amounts of F-actin could be released to potentially increase the viscosity of blood and perturb blood flow through the microvasculature. The actin severing protein gelsolin has a secreted plasma isoform called plasma gelsolin, which is constitutively active in the high extracellular calcium concentrations of plasma. Plasma gelsolin severs extracellular F-actin to short filaments, and by capping barbed ends, prevents polymerization and favors monomer release. Another plasma protein, Gc-globulin, which binds G-actin, rapidly clears monomerized actin in the liver.

This thesis investigates the two actin binding proteins gelsolin and filamin A. Whereas gelsolin solates actin filament gels (thereof its name), filamin A efficiently forms orthogonal three-dimensional F-actin gels (Figure 1). The two first papers in this thesis are work directed towards understanding the role of plasma gelsolin in the extracellular environment and examine if there are other functions for gelsolin in the circulation besides actin severing and scavenging. They especially focus on its role in inflammation. The third paper describes a structural basis for orthogonal filament crosslinking by the protein filamin A. It provides a novel explanation for how it binds to actin filaments at a high angle and simultaneously can interact with other binding partners.

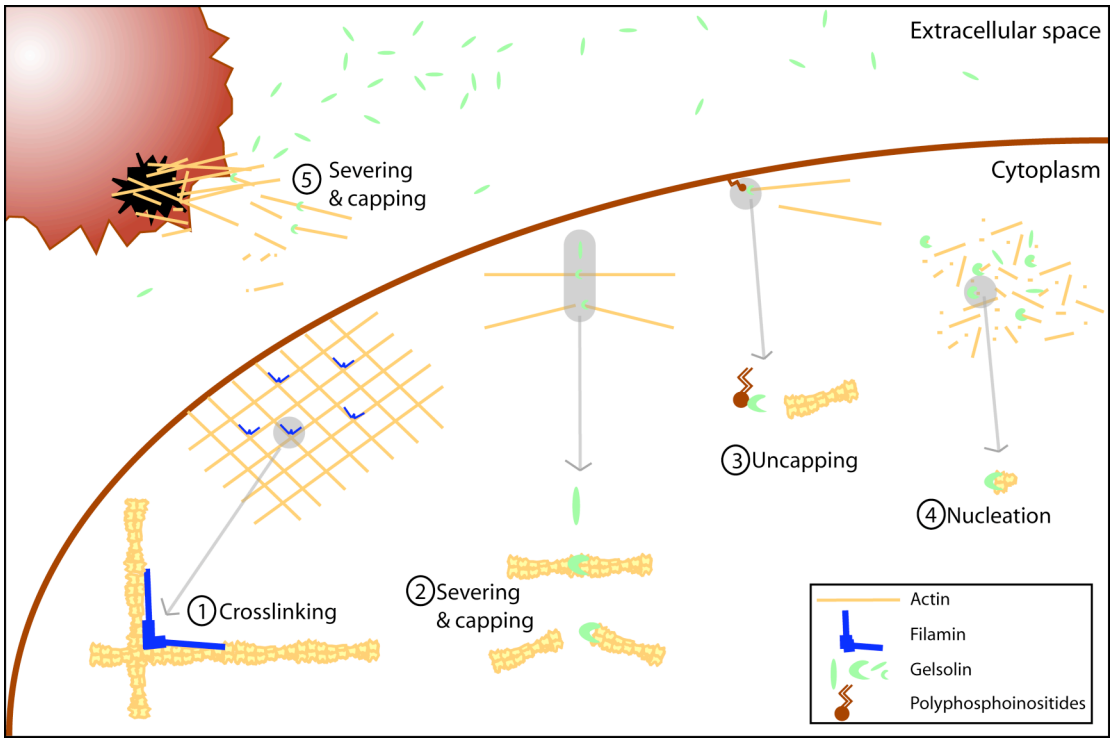

Figure 1. Functions of gelsolin and filamin A. Gelsolin and filamin A participate in actin organization in cells. 1) Filamin A crosslinks F-actin into orthogonal networks; 2) gelsolin can sever actin filaments to shorter pieces, and cap the barbed ends; 3) the barbed ends serve as templates for fast polymerization when gelsolin is uncapped by membrane phosphoinositides; 4) gelsolin binds actin monomers to form a nucleus from which F-actin can elongate in the slow-growing direction; 5) there is a plasma isoform of gelsolin that severs F-actin that leaks out into the extracellular space during cell lysis and tissue injury.

2. BACKGROUND AND LITERATURE REVIEW

2.1 Filamin A

Actin ultrastructures range from parallel bundles to three-dimensional gel networks, determined by ABPs. The diversity of actin networks provides flexibility for cell shape changes, prevents large organelles from being displaced while permitting passage for small structures, and ensures rigidity to the cell upon intra- and extracellular forces. FLNa is a F-actin crosslinking protein and an important component of three dimensional actin networks. By crosslinking F-actin, it accommodates cell motion over a surface or shape change, and upon mechanical stress, formation of these crosslinks is essential for mechanoprotection (cytoskeletal adaptations to mechanical stresses). It also mediates actin-membrane connections and serves as a scaffold for numerous different (over 50) cellular binding partners [5].

The discovery of filamin A

Filamin was purified in 1975 as the first non-muscle actin-binding protein. It precipitated and sedimented with F-actin at low centrifugal forces and exhibited some characteristics similar to erythrocyte spectrin. It was named actin binding protein (ABP) [6], and later ABP280 due to the molecular weight of its polypeptide chain (280.5 kDa), but since many other homologous actin binding proteins now are identified, the name was changed to filamin A (FLNa) [5]. The ability of FLNa to form a dense array of tangled filaments was soon identified [7], and it was shown that FLNa is a potent actin gelation factor [8, 9].

Filamin A structure

The name filamin (A) fits the "filamentous" appearance of this dimeric protein. Monomer subunits are ~80 nm long assemblies built from 24 immunoglobulin-like (Ig-like) repeats of ~96 aa, numbered 1-24 from N-T to C-T [10]. The Ig-like repeats are, just like immunoglobulins, composed of anti-parallel β-pleated sheets made of 7 β strands [10, 11]. Repeats align linearly, perhaps slightly overlapping each other, and are divided into two rod-like structures by a 27 aa strand called hinge 1 (H1), proposed to give the molecule flexibility. Ig-like repeats 1-15 form rod 1, and 16-23, rod 2. Between repeat 23 and the self-association domain is a second 35 aa (~3.5 nm) sequence insertion called hinge 2 (H2) [10]. The hinges contain calpain-cleavage sites [12]. The N-T Ig-like repeat is preceded by a stretch of 275 aa containing the actin-binding domain (ABD). The ABD consists of two calponin homology subdomains that form an α-helical globular domain [13]. This sequence motif has also been recognized in β-spectrin, dystrophin, α-actinin, calponin, nesprin, plectin, fimbrin and utrophin [14]. FLNa monomers connect at the C-T by self-association of repeat 24 [15] (Figure 2).

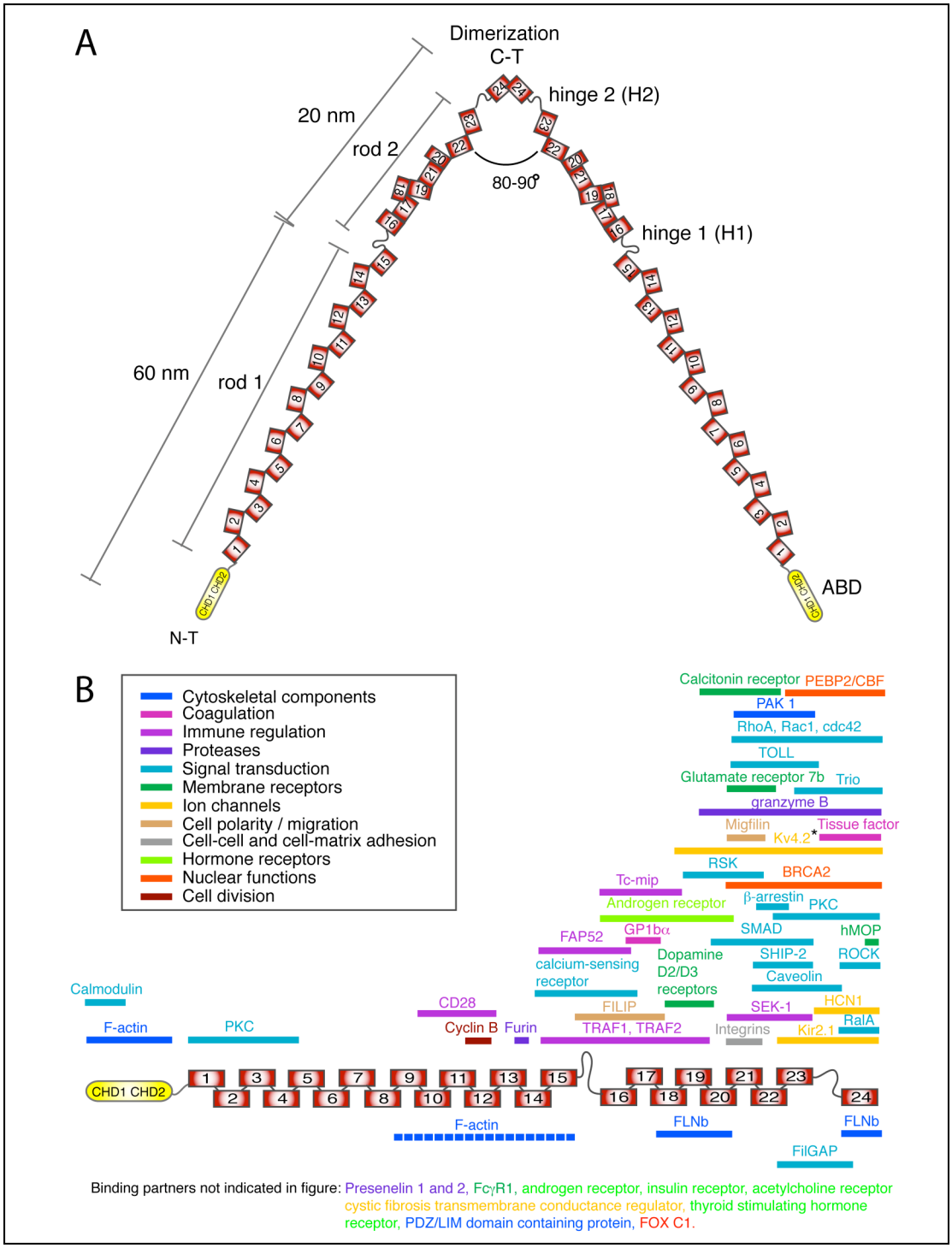

Figure 2. Filamin A structure and binding partners. A) shows the general structure of the FLNa dimer. Each FLNa molecule is a dimer of ~80 nm in length and built from a N-T ABD similar to that of other spectrin superfamily members, followed by 24 immunoglobulin-like repeats. The repeats are interrupted by hinges and dimerization is mediated by repeat 24. Repeats 1-15 are called rod 1 and repeats 16-23 are rod 2. Rod 2 does not interact with actin, and is where most FLNa partners bind (paper III). B) delineates the binding sites for certain binding partners [16-48]. The dashed blue line adds our newly identified binding site for F-actin, described in detail in paper III.

Atomic force microscopy has been used to learn more about the mechanical properties of the FLNa subdomains. Using a *Dictyostelium discoideum* relative of

human FLN, ddFLN, containing only 6 Ig-like repeats, it was shown that individual repeats unfold before the dimer is broken. A force of ~200 pN is necessary to break a dimer and it was shown that one segment (ddFLN4) unfolded and refolded more easily than the others [49]. Such unfolding might modulate interactions with this domain, and regulate protein binding and signal transduction during mechanical stress. Vertebrate filamin domains unfold under different forces [49, 50]. Paper III describes the FLNa rod 2 domain as a compact region, whose appearance might derive from additional inter-domain interactions [46], and provide another source of "elasticity" in the FLNa molecule.

Filamin isoforms and expression

There are three filamin genes in humans, *FLNA*, *FLNB* and *FLNC*, that encode the unique proteins filamin A, B and C which have 70% sequence homology in the repeat segments and 45% homology over the hinges [51]. Hinge 2 is present in all isoforms, but hinge 1 is lacking in some splice-variants of FLNb and FLNc [52, 53]. Alternative splicing of sequences encoding a region of 8 aa in repeat 15 of FLNa has been reported [10]. Furthermore, there are FLNa and FLNb splice variants (filamin A_{var-1} and filamin B_{var-1}) that are widely expressed at low levels and have an internal deletion of 41 aa between repeats 19 and 20 [54]. The gene for FLNa is located on the X-chromosome at Xq28 [55], making FLNa the only variant that is X-linked [56].

Studies have shown overlapping cellular and tissue expression patterns for FLNa, b, and c. Of the three, FLNa is the most abundant and widely expressed variant in human tissue. Most cells express 1-3 μM FLNa [57]. FLNb also has a broad distribution, but is less abundant than FLNa. FLNc, though widely expressed during development, is mainly found in skeletal and cardiac muscle cells in adults [5].

Actin binding

The FLNa ABD has two calponin homology domains (CH1 and CH2) separated by a linker sequence [58, 59]. CH1 contains two putative actin-binding sites (ABS1 and ABS2) and CH2 has one (ABS3). ABS2 has a hydrophobic stretch that is important for binding to actin. The nature of binding to the two other sites is less clear [60, 61]. The FLNa ABD is dissociated from F-actin by Ca^{2+}-calmodulin (holocalmodulin) [30]. Despite having a similar ABD to other crosslinking proteins, FLNa binds to F-actin with higher affinity. In paper III, evidence for a model of how FLNa interacts with F-actin is presented.

Actin gels crosslinked by filamin A

The properties of cytoplasm are complex. Like polymer gels, cytoplasm exhibits viscoelastic behavior, i.e. behaves like a solid in response to certain forces, with minimal deformation, but when stressed over a longer period of time, entanglements can resolve as filaments slide past each other in a fluid-like manner [62]. The forces imposed on a cell can be external mechanical forces, such as shear stress, that can regulate cell shape, migration [63], gene expression and apoptosis, or internally generated forces such as those during protrusion, contraction, phagocytosis and cytokinesis [64]. Crosslinked FLNa and F-actin form gels that behave similarly to covalently crosslinked networks (avidin-biotin), in that they are quite resistant to

deformation induced by constant shear stress [65], implying that these crosslinks are stable and important for protection of cell shape in response to shear forces experienced *in vivo*.

Cortical cytoplasm close to the plasma membrane consists of F-actin bundles and orthogonal crosslinks that determine the cell's mechanical properties [66]. F-actin networks, reconstituted using gelsolin-shortened F-actin to obtain physiologically relevant lengths (~1 μm), behave mechanically as a living cell when they are crosslinked by FLNa and subjected to a large pre-stress (the pre-stress creates a physical environment for the actin network that mimics the intracellular setting where the filaments are always under stress due to connections to other cellular structures). The hinge 1 is essential for this function [67].

FLNa is the most potent F-actin crosslinking protein identified and creates a F-actin gel at concentrations lower than any other known protein [68, 69]. A stoichiometry of one FLNa dimer per actin filament is sufficient to induce gelation [70]. Since most F-actin-crosslinking proteins have two F-actin binding sites of similar affinity as does FLNa, the affinity and number of ABDs alone cannot explain the efficiency of FLNa in creating an actin gel. Instead, the answer lies in the strikingly orthogonal geometry with which FLNa arranges actin filaments (Figure 3) [5]. The precise properties required for FLNa to promote this high angle-branching are not clear, but dimerization [71], and N-terminal ABDs are necessary [60, 72]. An extended end-end length, and the proposed flexible hinges, in combination with more rigid staggered subunit structures, has been suggested to provide FLNa with a "leaf-spring like" composition, i.e. a mix of flexibility and stiffness [10]. In paper III, the characteristics of FLNa important for its actin crosslinking function are demonstrated in greater detail.

Localization of filamin A in the cell

FLNa is distributed diffusely and uniformly in un-polarized neutrophils and macrophages, with a slightly enhanced distribution to the cortex [73, 74]. Upon cell activation, FLNa accumulates at the leading edge, in the ~1 μm margin of the lamellipodium localized closest to the plasma membrane, where the cytoskeleton is composed of a three-dimensional orthogonal network of short filaments. FLNa is present in these structures at X-, T- and Y-shaped junctions in rabbit macrophages, human platelets, and tumor cells. The inter-branch distances are shorter in the platelet cytoskeletons, consistent with their higher FLNa content, in agreement with an inverse proportional relationship between inter-branch distance and FLNa concentration [57, 75, 76]. Although demonstrated to be at the actin junctions by immunogold labeling of cell cytoskeletons, FLNa has never been observed at crosslinks by electron microscopy in the absence of antibodies. Thus, it has not been known how individual FLNa molecules "sit" on actin at junctions. An explanation for this and a demonstration of how they interact is presented in paper III.

In mice, FLNa expression is abundant in cell soma and at the leading processes of migrating neurons, and reaches very high levels in the ventricular zone during neurogenesis. FLNa is involved in the neuroblast migration during vertebrate cortical development, and a condition where this process is disrupted is described below [77, 78].

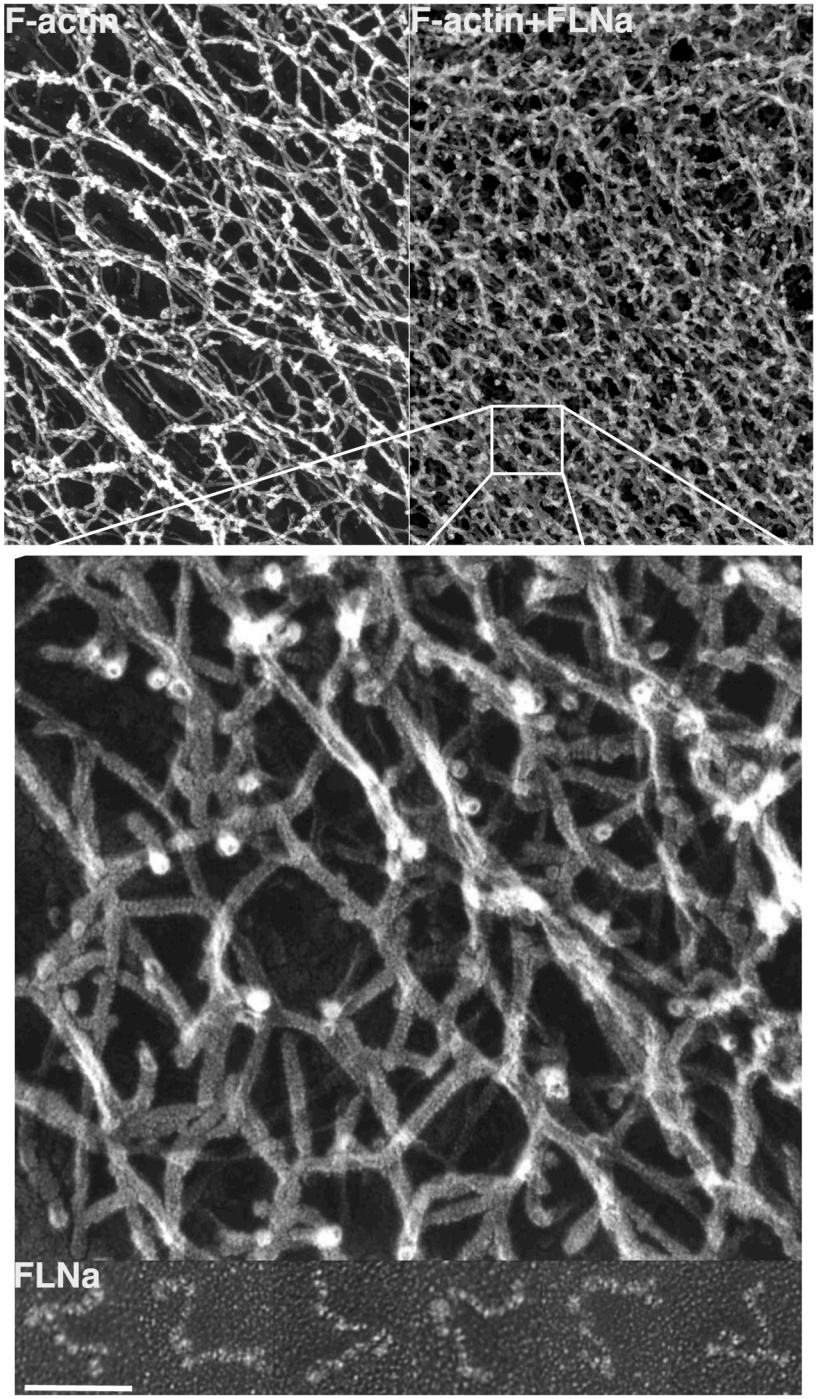

Figure 3. Effect of filamin A on actin networks. FLNa at a 1:50 G-actin ratio (upper right corner) creates a dense and orthogonal F-actin network, compared to actin polymerized in the absence of FLNa (upper left corner). At this ratio of FLNa to actin, the inter-branch distances approximate the arm length of FLNa (lower images). Scale bar is 100 nm. Electron micrographs of networks are a courtesy of Dr. John Hartwig.

Binding partners

Filamins have, in addition to actin, over 50 binding partners of great functional diversity. Most of the interactions are in rod 2 (see Figure 2), although often the exact domains of binding have not been identified. Partners include intracellular proteins, cofactors and membrane receptors. The interactions of FLNa with membrane structures link the actin scaffold to the membrane and provide mechanical stability as well as maintain cell-cell and cell-matrix connections. By binding to small G-proteins (Rho family GTPases) that are involved in controlling actin polymerization, and some of their regulatory cofactors, FLNa can organize polymerizing actin filaments into 3D structures. Some of the FLNa binding partners that are involved in regulating actin

assembly are: RhoA, Rac, cdc42, RalA (GTPases) [79], Trio (a guanine nucleotide-exchange factor [GEF] for Rac and RhoG) [48], Pak1 (a downstream effector of Rac that promotes actin assembly) [19], ROCK (an effector of RhoA) [80], and Lbc (a RhoGEF) [81].

In paper III, FLNa partner-binding in the presence of F-actin is studied by use of FilGAP, an ~ 84 kDa RhoGTPase-activating protein (GAP). FilGAP is specific for Rac GTPase and complements Trio in controlling the activity of FLNa-associated Rac to affect actin polarization. FLNa-binding targets FilGAP to sites of membrane protrusion, where it antagonizes Rac *in vivo*. When FilGAP is removed by knockdown with small interference RNA, spontaneous lamellae formation occurs through elimination of ROCK-dependent suppression. Forced expression, on the other hand, induces numerous blebs around the cell periphery that can be suppressed by a ROCK-specific inhibitor. Kidneys have the highest FilGAP mRNA levels. The expression level of FilGAP varies between cell types, but the relative molar ratio of FilGAP to FLNa is probably around 1:100. FilGAP has a pleckstrin homology (PH) domain, a RhoGAP domain and a coiled-coil (CC) domain. The FLNa binding domain, including the essential CC domain (residues 552-748), is ~16 kDa. The FilGAP binding site on FLNa is on repeat 23 [47] within the rod 2 domain of FLNa, where most partners interact.

FLNa also binds to transmembrane proteins that are involved in cell adhesion, cell shape, activation and locomotion. The first identified FLNa binding partner was the glycoprotein (GP)1bα [82, 83], a component of the platelet von Willebrand factor receptor, whose cytoplasmic tail binds to Ig repeat 17 of FLNa (and with less affinity to repeat 19). The crystal structure shows that a groove is formed by β-strands C and D of repeat 17 into which a short region of the cytoplasmic tail of GP1bα fits in a lock-and-key fashion. Tight binding results from the interaction of each subunit molecule with both alpha chains of a single VWF receptor. Bonds are of both hydrophobic and hydrogen-bonding nature [31]. This interaction is similar to the integrin β7 cytoplasmic tail binding to a site in Ig repeat 21 [45]. The integrin family of adhesion receptors provides an essential connection between the extracellular matrix and the actin cytoskeleton. This link is necessary for many integrin-mediated processes, including cell migration, fibronectin matrix assembly and focal adhesion formation [84, 85]. FLNa binds to several β-integrins in addition to integrin β7 [45, 86]. Although the primary sequence of FLNa-binding sites in the cytoplasmic tails of GP1bα and integrin β7 are not conserved, the binding interactions in Ig repeat 17 and 21 are similar. In both cases the receptor tail binding site is a β-strand flanked by prolines that fits in a groove formed by the C and D β-strands of the FLNa repeat. Since FLNc has also been shown to self-associate using the C and D strands of Ig repeat 24 [87], the C and D strands appear to be a common interaction surface for binding partners [31].

Since cells are constantly exposed to mechanical stress, such as fluid flow, they must be able to adapt to tension-changes in the cell membrane in order to maintain membrane integrity, cell shape, and adhesion to the extracellular matrix. In cells subjected to shear stress, β1 integrin directly associates with FLNa to induce signals resulting in cell stiffening. FLNa is both recruited locally to cortical areas of increased tension, and its production is up-regulated. Cells lacking FLNa do not exhibit this stiffening. Stiffening in response to external stress is called mechanoprotection [88, 89].

Just recently, the resolution of the structure of FLN Ig repeats 19-21 revealed an unexpected arrangement affecting the integrin interaction. Instead of the repeats being linearly arranged, repeat 20 is partially unfolded, which brings repeat 21 close to repeat 19. Importantly, the N-T of repeat 20 forms a β-strand that interacts with the C and D face of repeat 21 and occupies the binding site for integrin cytoplasmic tails. Disruption of this interaction is required to enhance integrin binding [46]. This is the first example of autoinhibition of partner binding in FLNa, which might be important for interactions with other binding partners as well. In paper III, electron microscopy images of the rod 2 domain and measurements of its length support the nonlinear C-T repeat structure.

FLNa is phosphorylated by several serine/threonine protein kinases such as protein kinase A, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II and p90 ribosomal S6 kinase [90-94]. The reason for these phosphorylations remains to be determined, but it might alter intra- and inter-repeat structures and thereby modulate partner interactions.

Human and cellular consequences of lacking functional filamin A

Cell-lines

Several human malignant melanoma cell lines do not express FLNa and are poorly motile. Cells from one of these lines, called M2, have unstable surfaces [95, 96], are unable to extend a flat ruffling lamellae upon stimulation, and are thus unable to achieve the polarization that is necessary for motility. Instead, they protrude and retract blebs from their surfaces (spherical aneurysms), because the FLNa lacking cell cortex cannot withstand the internal hydrostatic pressures generated by myosin II-based contraction. When FLNa cDNA is introduced to these cells, they crawl with velocities proportional to the amount of FLNa they express. If levels are increased above WT values, the cells slow down [95].

Human conditions involving FLNa

Mutations in the *FLNA* gene that completely blocks its expression are associated with an X-linked condition called (bilateral) periventricular nodular heterotopia (PNH) [97-99]. PNH is characterized by nodules of neurons in an inapt location adjacent to the walls of the lateral ventricles, a condition resulting from failed neuronal migration into the cortex [100, 101]. Despite this lack of neurons in the cortex, the intelligence of affected individuals is normal or only mildly compromised. These accumulations cause epileptic seizures in the patients, usually starting in the second decade of life. PNH patients have an unusually high incidence of vascular complications due to congenital cardiovascular abnormalities, small joint hyperextensibility and gut dysmotility. Mutations in this X-linked FLNa gene causes most males to die *in utero*, suggesting that FLNa is essential for embryonic cell migration [5].

Mutations of the FLNa gene are also associated with the otopalatodigital (OPD) syndrome spectrum, which includes: OPD 1, OPD 2, frontometaphyseal dysplasia and Melnick-Needles syndrome. Altogether, 45 mutations of FLNa have been reported in patients with PNH or ODP spectrum [99, 102-104].

2.2 Gelsolin

Gelsolin (GSN) is an ubiquitous [105, 106] actin filament severing, capping and actin nucleation protein of eukaryotes. It was identified and isolated from rabbit lung macrophages in 1979 as a protein that in the presence of micromolar calcium concentrations, solated cytoplasmic actin filament gels crosslinked by filamin A [107]. It is widely studied, its three dimensional structure is determined, and it exists as both an intracellular (cytoplasmic gelsolin, cGSN) and a secreted protein (plasma gelsolin, pGSN).

GSN is the founding member of a larger superfamily of conserved proteins present in eukaryotes. Proteins of this family include gelsolin, villin, advillin, adseverin, CapG, supervillin and flightless I. Family members share related structures, being composed of three or six homologous segments or domains (Figure 4) named gelsolin-like domains, G1-G6 (also called S1-S6). The three repeat domain structure likely evolved by gene triplication of a prototypical single domain containing precursor protein followed by gene duplication to yield the 6 domain protein [108, 109]. Individual domains are related to cofilin in structure. All family members share the capacity to bind (cap) the barbed ends of actin filaments.

Gelsolin isoforms and expression

The GSN gene locates to chromosome 9 in humans, and it is alternative mRNA splicing that determines production of the different isoforms [109]. There is still much to be learned about the regulation of expression of the GSN isoforms in different settings. GSN expression has been observed to be both up-regulated and down-regulated during cell differentiation [110, 111], and an increased production was observed as a response to corticoid hormones [112]. Additionally, it is not known if there are any specific regulatory factors determining cGSN or pGSN production. The GSN protein sequence is highly conserved among different species [108] and GSN has been found also in invertebrates [113]. Cytoplasmic (~0.2-5 μM) and plasma concentrations (~1.5-3 μM) are approximately equal.

Gelsolin structure

cGSN is a globular protein of 80.3 kDa composed of 730 aa assembled from 6 repeat domains, G1-G6 [107]. pGSN is slightly larger (~ 85.7 kDa), and identical to cGSN with the exception of a N-T 25 aa plasma extension of unknown function [109] and a 27 aa peptide that signals for secretion and is cleaved off in the ER lumen during translocation to the extracellular space. cGSN has five cysteine residues, all in the free thiol state. In pGSN, the cysteines at position 188 and 201 are oxidized in the rough ER, resulting in a disulfide bond [114] (Figure 4). Recently, a second cytoplasmic isoform was discovered, gelsolin-3, 11 aa longer than cGSN, and present in oligodendrocytes mainly in the brain, lungs and testis [115].

Determination of the crystal structure of horse pGSN shows that the 6 subdomains are composed of a five- or six-stranded mixed β-sheet and 2 α-helices, and the domains fold into a compact protein in the absence of calcium (Ca^{2+}) [116] incapable of binding to

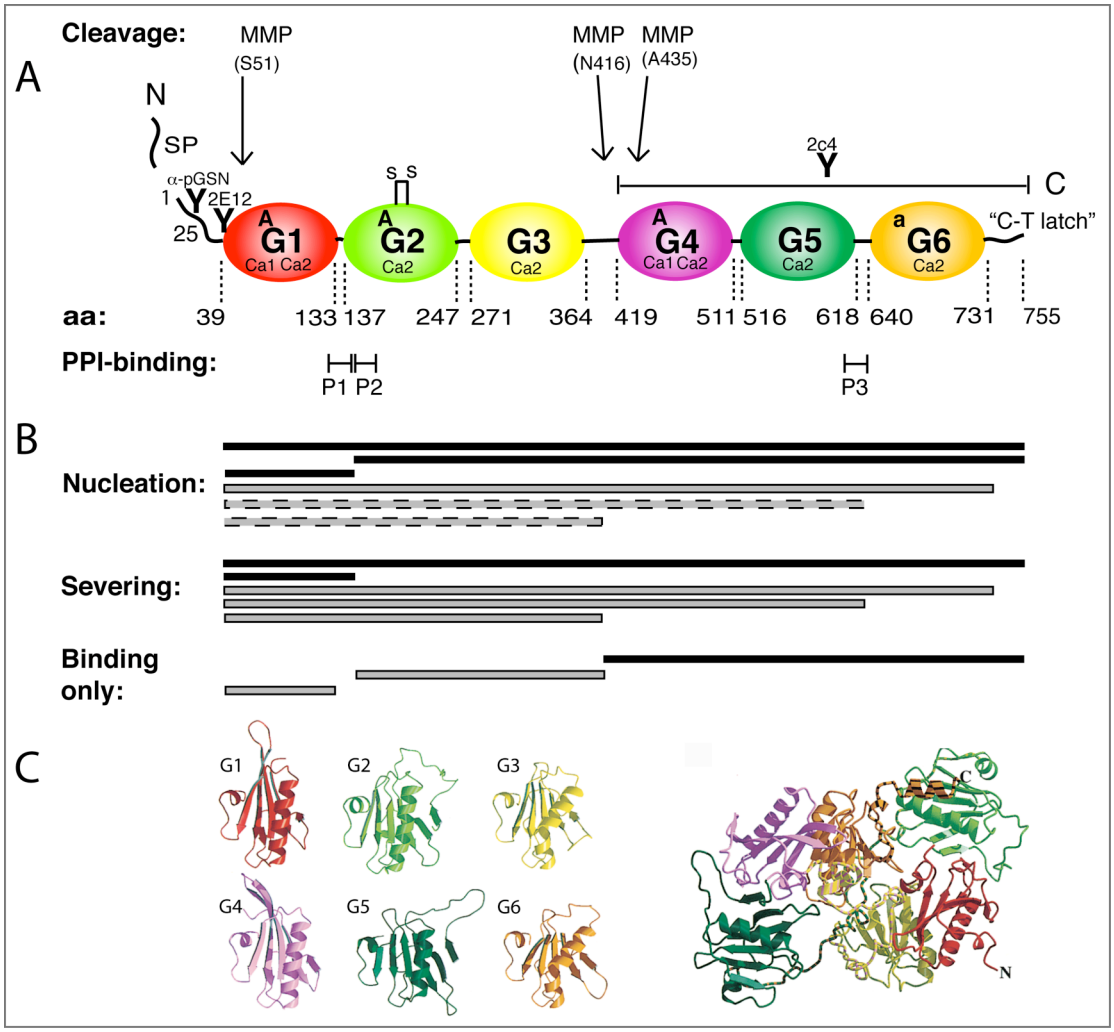

Figure 4. Structure/function of plasma gelsolin. Emphasis is on features important for actin, Ca^{2+} and phospholipid interactions. A) Domain structure of pGSN. GSN is built from 6 globular domains (G1-G6). The main identified differences between the cGSN and pGSN molecule is the addition of a cleavable signaling peptide and the 25 aa plasma extension of pGSN. pGSN also has a disulfide bridge in G2. GSN has eight Ca^{2+} binding sites, of them the primary regulatory Ca^{2+} binding site, locates in the C-T of the protein. G1 contains a high affinity G-actin and F-actin binding domain. G2 has a F-actin binding domain. G4 has a G-actin binding and F-actin binding domain of lower affinity than G1. The binding sites for phosphoinositides are aa 135-149 (P1), 160-169 (P2, also defined as 150-169) in the N-T and 621-634 (P3) in the C-T. The binding sites of three antibodies used to detect pGSN, cGSN, or both isoforms are indicated. 2c4 monoclonal antibody recognizes the C-T half of GSN, hence both pGSN and cGSN are detected by it. pGSN is recognized by a specific antibody that binds epitopes in the plasma extension. 2E12, specific for cGSN detects a domain in GSN hidden by the plasma extension. MMPs cleave pGSN *in vitro* and their cleavage sites are indicated (last aa in the N-T fragment). Residues are numbered as in human pGSN [114, 116-134]. B) Effects of truncation on GSN activities are indicated. Removing the very C-T of GSN results in loss of Ca^{2+}-regulation of nucleation and severing. When cut in half, the N-T half has weak nucleation, whereas the C-T has no nucleating effect [117, 121, 123-125, 127, 128]. C) As revealed by the crystal structure, G1 and G4, G5 and G2 and G3 and G6 are similar in structure. In the absence of Ca^{2+} the C-T latch (black and orange) interacts with G2 and the molecule has a closed conformation [116]. Key: aa = amino acids, SP = 27 aa signaling peptide, Ca2 = type II Ca^{2+}-binding site, Ca1 = type I Ca^{2+}-binding site, **A** = actin binding domain, **a** = possible actin binding domain, s — s = disulfide bond, dashed line = low nucleation (~5-10% of the activity of the full-length protein), black bars = activity requires Ca^{2+}, gray bars = activity does not require Ca^{2+}. The 3D structure in Figure 4C is reprinted from Cell, Vol. 90, L. Burtnick, E. Koepf, J. Grimes, Y. Jones, D. Stuart, P. McLaughlin, R. Robinson; The crystal structure of plasma gelsolin: Implications for actin severing, capping and nucleation, p. 661-670, with permission from Elsevier Ltd.

actin. Ca^{2+} binding to GSN induces a conformational change that opens up the molecule [135, 136], exposing its actin binding sites, which allows it to either nucleate actin assembly and/or bind along actin filaments, sever them, and then cap their barbed ends [135-137].

Gelsolin's effect on actin and practical use in different assays

GSN can bind to G-actin, F-actin sides or ends, nucleate actin polymerization, and sever actin filaments. When mixed with actin monomers in ~0.1 M KCl, 1.5 mM $CaCl_2$, 2 mM $MgCl_2$ 0.5 mM ATP, GSN binds to two actin monomers, and forms a complex from which actin can polymerize in the pointed end direction [138, 139]. The first actin monomer adds to GSN at a slow rate [140, 141]. However, once one monomer is bound, the second adds at a 1000 fold higher rate [142]. This Ca^{2+}-dependent [143] effect is called nucleation and can be utilized to generate F-actin of defined lengths (Figure 5) since the length of F-actin will be determined by the GSN:actin ratio. Further, since the rate limiting step in actin assembly is nucleation, GSN accelerates the rate of assembly in a dose-dependent fashion, allowing the amount of GSN in a sample to be determined. *In vitro* assays for fast determination of GSN concentration in unknown samples uses fluorescently labeled actin such as N-(1-pyrenyl)iodoacetamide-labeled actin (pyrene actin), which fluoresces with higher intensity as a polymer.

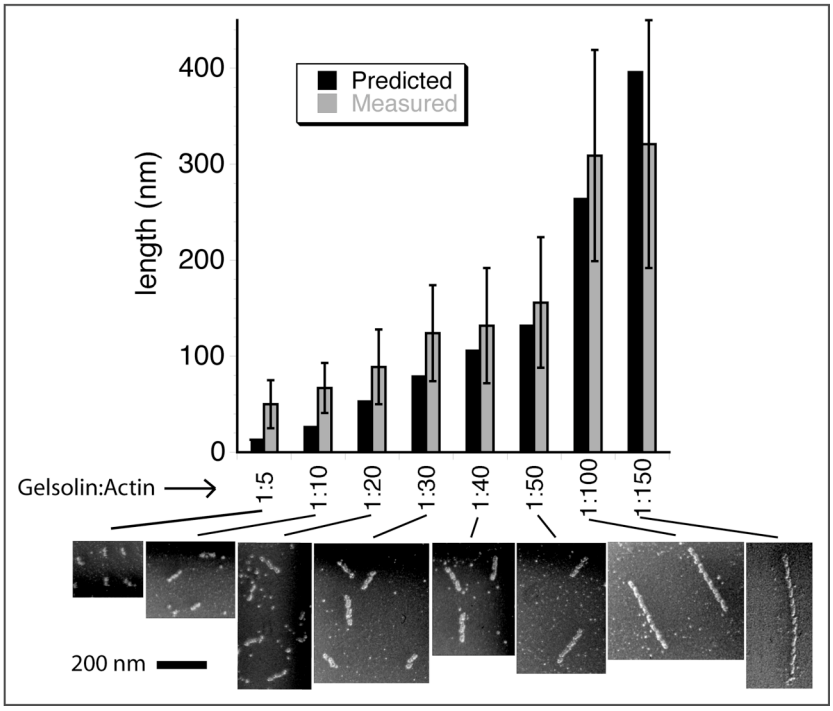

Figure 5. Gelsolin's effect on F-actin length. Filaments become shorter as the GSN to actin ratios increase. Black bars indicate the predicted values based on the ratio of actin monomers to GSN mixed in polymerization studies (14 monomers = 37 nm). Grey bars indicate the measured lengths. Data are means (SD), N = 40 - 400. Electron micrographs show representative gelsolin nucleated F-actin at the indicated pGSN:actin ratio.

The mechanism of gelsolin binding to actin

The G1-3 and G4-6 halves of GSN are held together by a linker sequence that makes up 8% of the GSN total molecular mass and is sufficiently long (~66 Å) to stretch across the actin filament for optimal positioning of both N-T and C-T actin binding sites on the actin filament [116, 128]. In its closed structure, the N-T and C-T halves of GSN are held together by interactions between G6 (mediated by the C-T tail and called the "C-T latch") and the F-actin binding helix in G2, rendering the actin

binding domains inaccessible to actin. The addition of Ca^{2+} induces a dose-dependent conformational change that opens up the GSN molecule [144-146] increasing both maximum linear dimension as well as radius of gyration [147]. First, the C-T latch releases from G6, displaying the actin-binding site in G2 [116, 146]. G2 then initiates actin severing by binding along the side of the filament, followed by release of the first domain from the third domain in each triplet in order to expose actin binding sites in G1 and G4 (Figure 6) [128]. Once tight binding is established, GSN shares two Ca^{2+} molecules with actin [128, 148]. In the severing process, GSN changes the actin conformation, twisting the filament and ultimately disrupting the noncovalent bonds between actin subunits in the filament [116].

The affinities of the various GSN domains for actin differ. The actin-binding site in G1 binds actin monomers and filaments with high affinity (K_d = 5 pM) without requiring Ca^{2+}. G4 has a calcium dependent G- and F- actin-binding site (K_d = 1.8 μM alone, 25 nM if part of G4-6) and G2 binds F-actin with micromolar affinity (K_d = 5-7 μM) [121, 149, 150]. When the last 20 amino acids in the C-T tail of GSN are removed, Ca^{2+} regulation of actin binding is lost [117] (Figure 4).

GSN has been found to be tyrosine-phosphorylated by $pp60^{c\text{-}src}$ [151] at predominantly tyrosine 438 in subdomain G4 [152]. It has been suggested that tyrosine phosphorylation induces a change in conformation of gelsolin that promotes actin severing [153].

The interaction of calcium with gelsolin is complex

There are two different types of Ca^{2+}-binding sites in GSN. Type I sites occupy positions shared between GSN and actin while the type II sites are localized to residues within the GSN molecule. Among the at least eight Ca^{2+} binding sites [118], varying in affinities from ~0.1 μM to ~1 mM [154-157], two sites are of type I and six are of type II [118] (Figure 4). A high affinity (0.1 μM) type II site in G2 is important for opening up the closed conformation [145, 146, 155]. However, in order to bind actin, higher Ca^{2+} concentrations and Ca^{2+} occupancy of sites in both N-T and C-T are needed [119, 146]. During physiological ionic conditions with GSN and actin concentrations resembling those in cells, GSN binding to F-actin is half-maximum at 0.14 μM. However, in order for half-maximal effect of severing, 0.4 μM Ca^{2+} is required [154]. Requirements of higher Ca^{2+} concentrations for binding, severing and nucleation have also been reported [107, 116, 137, 146, 158, 159]. Decreasing the pH reduces the Ca^{2+} dependency for actin nucleation and severing, and if the pH is low enough, actin modulation can occur without Ca^{2+} [137].

Although different molar calcium requirements have been reported, a uniform conclusion is that in the cytosol of resting cells, where the Ca^{2+} concentrations (< 100 nM) are below the values of any reported dissociation constants, GSN binds actin very slowly. Cell activation increases the intracellular Ca^{2+} concentration by >100 fold, resulting in an increase in GSN-actin binding and severing rate [154, 158]. In contrast, pGSN, present in the circulation, is constantly exposed to mM levels of Ca^{2+}, and thus always in an active conformation, ready to sever filaments and sequester any monomeric actin that may have leaked out from damaged cells (Figure 6).

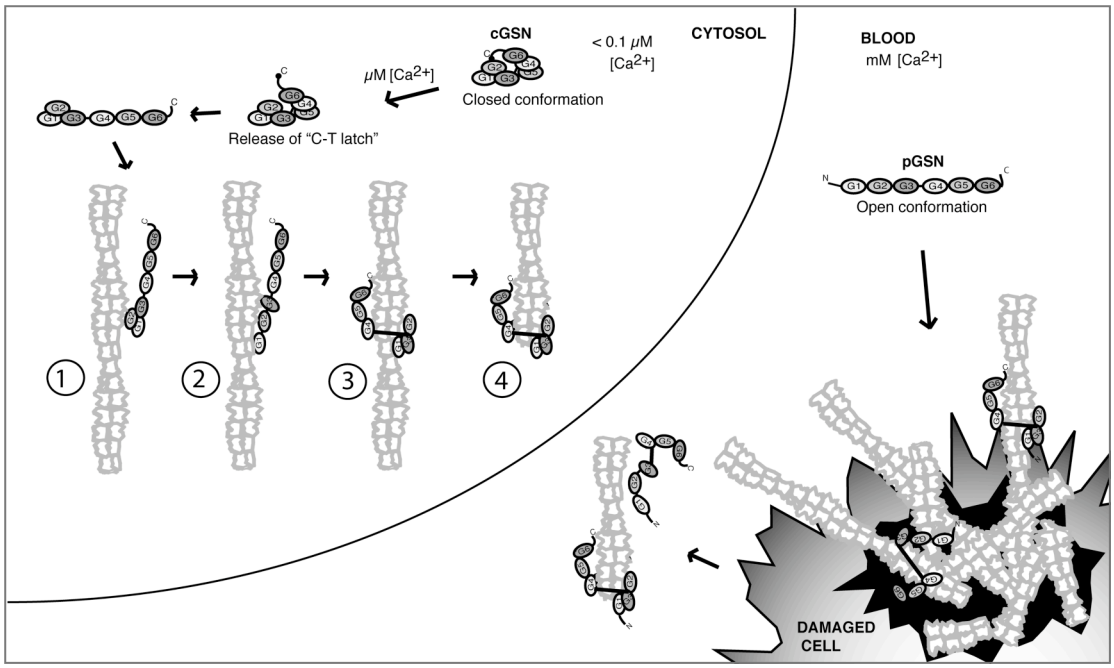

Figure 6. Actin severing by gelsolin in cytosol and plasma. cGSN activity is tightly regulated in cells and requires the cell to increase its intracellular Ca^{2+} concentration to convert it to its active conformation. Because the actin binding sites are hidden in the closed conformation, severing, in response to Ca^{2+}, requires a series of conformation changes. 1) The actin-binding site in G2 is exposed when Ca^{2+} binds to the C-T and the C-T latch releases from G6. G2 can then bind along the side of the filament while G1-G3 and G4-G6 undergo structural rearrangements. 2) G1 and G3 attaches to actin and G1 disrupts the actin-actin contact below G2. 3) G4-G6 stretch across the actin filament, bind the adjacent actin subunit and disrupt the second actin-actin contact. 4) GSN stays on the severed filament, capping the barbed end [116, 118, 128]. pGSN is active and ready to sever filaments and sequester any monomeric actin leaked out from damaged cells because of the presence of high concentrations of free Ca^{2+} in blood.

Phosphoinositides (PPIs), phosphorylated derivatives of phosphatidylinositol, are acidic phospholipids composed of a phosphatidic acid backbone that connects through a phosphate group to the inositol sugar headgroup. The inositol sugar can be phosphorylated at different locations, which generates functionally different species. PIP_2 and phosphatidylinositol 4-monophosphate are the two most abundant PPIs in the plasma membrane. Binding of phosphatidylinositol lipids to GSN prevents binding to actin and under certain conditions dissociates gelsolin from filament ends [3, 132, 160, 161]. The K_d for gelsolin binding to PIP_2 as determined by gel filtration is 40.2 μM and 305.4 μM in the presence and absence of Ca^{2+}, respectively. The N-T half has much higher affinity for PIP_2 (3-7 μM) than the C-T or the full-length protein. Decreased pH increases PIP_2-binding [162]. GSN binds to PIP_2 both as a (>5%) constituent of a bilayer membrane and in pure micelle forms [163].

Three phosphoinositide binding sites have been mapped in gelsolin. Using deletional mutagenesis and synthetic peptides, two sites within the N-T half of gelsolin have been identified [131, 132, 161]. Sequences at 150-169 (P2) [131] and 135-149 (P1)[132] that are enriched in basic residues have been found to interact with the negatively charged phosphates on the inositol headgroup followed by formation of hydrophobic bonds between non-polar GSN side chains and the fatty acid tail of the lipid [116, 130]. In

addition, a C-T PPI-binding site (P3) has been identified that requires the diacylglyceryl moiety as well as the inositol headgroup [133]. Binding of phosphoinositides to the P2 region in GSN domain G2 changes its conformation from a β-sheet into α-helix [164]. This change in conformation destabilizes the G2 F-actin-binding sites in domain 2 [164], and hence, F-actin binding is disrupted when GSN is bound to PIP_2 [164]. Molecular dynamics and circular dichroism studies support the idea that PPI lipid binding is driven both by electrostatic and hydrophobic forces [130, 164].

LPA (mono-acylglycerol-3-phosphate) is the smallest and simplest of the glycerophospholipids, consisting of a glycerol backbone, an acyl chain (alternatively alkyl or alkenyl chain) in either the *sn*-1 or *sn*-2 position and a phosphate headgroup. It has multiple biological actions as a lipid mediator in addition to its role as a precursor in phospholipid biosynthesis, spanning from inflammation to neurogenesis and tumor progression. pGSN binds LPA with high affinity (K_d = 6 nM) [165], and LPA inhibits the F-actin severing activity of GSN and can uncap GSN from barbed ends [166]. LPA has been reported to bind to P2 and binds presumably also to P1 (and possibly P3) [165-167]. In contrast to PIP_2, LPA interacts with GSN at a 1:1 ratio in solution [166]. PIP_2 and LPA increase the GSN tyrosine phosphorylation rate by 25-30 fold [152].

Gelsolin binding to nucleotides

Adenosine triphosphate (ATP) binding to GSN [168] involves both halves of GSN. The phosphate groups of ATP interface with basic residues on G5, sharing C-T binding sites with PIP_2. The binding is stronger to ATP than ADP [169] and is decreased in the presence of Ca^{2+} [170]. ATP and other nucleotides (ADP, GDP, GTP, CTP, UTP, and UDP) bind and can elute GSN from affinity columns [171, 172]. Gelsolin also binds to a diadenosine, *diadenosine 5',5"'-P1,P3-triphosphate (Ap3A)*, with a K_d of 0.3 μM. The binding is non-covalent, and stronger than for ATP or other nucleotides. [173].

Main identified functions of cytoplasmic gelsolin involving the cytoskeleton

GSN is important for cell motility and shape changes, since GSN has the ability to rapidly change actin filament lengths and expose free filament ends for polymerization or depolymerization in response to calcium, and thereby reorganizing the cytoskeleton. Crawling cells show GSN dependent motility [174-177] that is increased by overexpression of GSN [178]. GSN expressing fibroblasts have higher F-actin turn-over rates and move faster than cells from GSN KO mice, thus actin severing by gelsolin is important for fast motility [179]. GSN null cells also have decreased ruffling activity, increased amount of F-actin in stress fibers, and crawl with a pseudopod-like extension process [180].

Details about GSN's intracellular role are plentiful and beyond the scope of this thesis. In addition to its role in cell motility and shape changes, GSN is involved in e.g. apoptosis [181-184], phagocytosis [180, 185, 186], cancer [187-193], and nuclear receptor translocation [194], and the list of functions is constantly growing.

2.2.1 Plasma gelsolin

pGSN is a 782 aa long GSN isoform, also called brevin [195] and actin-depolymerizing factor [196] in the literature. Most cells secrete pGSN, but smooth, skeletal and cardiac muscle cells transcribe large amounts of pGSN mRNA and devote 0.5-3% of their protein biosynthetic activity to the production of pGSN [105, 197, 198]. Since skeletal muscle accounts for the bulk of tissue mass, it is believed to be the major source of pGSN [105].

The plasma level of pGSN in humans is 200 ± 50 mg/l, spread in a Gaussian distribution (T. Osborn, unpublished data). Isolated human and rabbit pGSN has a long half-life of 2.3 days when injected intravenously in rabbits [199], indicating that pGSN circulates for at least a few days in plasma. Because it derives from muscle tissue, pGSN must pass through interstitial fluid of the extracellular matrix to localize in blood. pGSN is also present in human cerebrospinal fluid (CSF) [200], and high levels of GSN mRNA are present in the mouse choroid plexus [201]. Bronchial epithelia secrete GSN into the airway surface liquid [202], and pGSN is presumably present in synovial fluid (SF) since it can be produced by chondrocytes [203] and GSN mRNA is present in synovial fibroblasts [204].

While certain functions for the cytoplasmic isoform have been established, the function(s) of the abundant plasma isoform remains a mystery. Most of the information regarding pGSN involves its decrease during acute inflammation, and its role in severing and scavenging extracellular actin, but pGSN might also bind to bioactive phospholipids or other mediators in the circulation. The known literature regarding pGSN and potential binding partners will be discussed.

Gelsolin amyloidosis

In familial amyloidosis of Finnish type (FAF, Finnish hereditary amyloidosis), an autosomal dominant disease, a 654G-A or 654G-T mutation in the GSN gene results is an amyloid protein [205-207]. These changes lead to a loss of Ca^{2+} binding, creating conformational alterations within domain 2 and rendering GSN more likely to be cleaved to a 68-kDa fragment (C68) by furin (α-gelsolinase) in the *trans*-Golgi compartment [208, 209]. Membrane associated type I matrix metalloproteinases (β-gelsolinase) cleave C68 further [210]. Therefore, plasma from FAF patients contain, in addition to full-sized pGSN, a number of lower molecular mass C-T fragments of the protein [211-213]. The 8 kDa and 5 kDa cleavage products make up the amyloid deposits [207, 214] in the extracellular matrix of tissues. Patients with the mutation are at risk for peripheral neuropathy, corneal lattice dystrophy, skin changes, as well as renal and cardiac manifestations. Homozygotes usually do not survive past their third decade, whereas heterozygotes typically have a normal lifespan with symptom onset between ages 30 and 50 [213].

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurological disease that leads to irreversible loss of neurons particularly in the hippocampus and cortex. Extracellular plaques contain β-amyloid peptides (Aβ peptides), which are cleavage products of the

β-amyloid precursor protein, and neurofibrillar tangles of hyperphosphorylated tau protein. Aβ-protein is present both in human plasma and CSF and the $A\beta_{42}$ peptide is especially important for the pathogenesis [215]. pGSN and Aβ bind and Aβ peptides co-immunoprecipitate with pGSN from plasma [216]. The pGSN-Aβ complex is stable in the presence of sodium dodecyl sulphate, but disrupted by reducing agents, suggesting that disulfide formation stabilizes the binding. There are two saturable Aβ binding sites in pGSN. Their K_ds are 1.38 μM and 2.55 μM [216] in the absence of calcium; these values might be different in physiological plasma calcium concentrations where pGSN has a different conformation. The binding of pGSN to Aβ inhibits Aβ fibril formation [216, 217]. In a recent study plasmid DNA coding for pGSN was introduced to two different amyloid-depositing transgenic mouse models for AD, and led to reduced brain deposits of Aβ [218]. This implies that pGSN might be protective in AD by preventing Aβ aggregate formation. Additional research is needed to learn more about the levels and distribution of pGSN in patients with AD. Interestingly, in a pilot study, the pGSN levels increased with disease duration in two different animal models for AD (T. Osborn, unpublished data).

Plasma gelsolin in acute inflammation

Although the information about pGSN function is sparse, it is well established that pGSN levels decrease in blood in acute inflammatory conditions that involve tissue damage. A decrease was first observed in leukemia patients receiving cytostatika [219]. Since then, pGSN depletion in many additional conditions have been reported.

pGSN levels decrease by ~60-65% in patients with acute respiratory distress syndrome (ARDS) and actin is detected in the patients' plasma. Levels in patients with bacterial pneumonias also decrease, but to a lesser extent (~50%) [220]. Importantly, pGSN levels are predictive of the clinical outcome of critically ill surgical patients [221, 222], as patients having pGSN levels lower than 61 mg/l remain longer at the intensive care unit, have prolonged ventilator dependence, and increased mortality [221].

During hematopoietic stem cell transplantation (HSCT), pGSN measurements might be valuable in predicting development of the, often fatal, complication idiopathic pneumonia syndrome (IPS), since levels fall shortly after allogenic HSCT in patients where IPS is developed. Values lower than 100 mg/l correlate strongly with development of fatal IPS [223].

pGSN levels are decreased progressively also during acute oxidant injury in mice and are an early indicator of injury. The mildness of the injuries was confirmed by the fact that the actin concentrations in plasma never were higher than 20 μg/ml [224] (compared to animal models for sepsis where actin levels are >10 times as high [225]). Hyperoxia-induced lung injury was less severe in animals that received pGSN compared to control animals receiving BSA, and pGSN decreased neutrophil infiltration in the pulmonary interstitium and alveolar space [226]. pGSN levels dropped to 10% of normal values in a rat model for inflammation-induced lung injury within 12 h of insult and remained low for almost a week. Gc-globulin values also fell, but to a lesser extent, and only transiently. Administering pGSN to the animals prior to damage, including one follow-up boost after 8 hours, prevented the increase in pulmonary microvascular permeability [227].

The decrease of pGSN in sepsis (the systemic inflammatory response system that occurs during infection [228]) was confirmed in a recent study. Following LPS- or cecal ligation and puncture (CLP)-induced sepsis, the pGSN values were reduced to 50% and 25% of the normal values, respectively. To determine whether levels of pGSN not only predict disease, but are also important in the defense against acute systemic inflammation, pGSN levels were maintained constant in a cohort of the mice by subcutaneous administration of 8 mg recombinant human pGSN (rhpGSN) immediately upon septic insult. The animals that were given rhpGSN survived significantly longer than control animals given subcutaneously injected saline solution [225].

Other conditions where pGSN levels are low and correlate with disease are during acute liver failure, acute myocardial infarction, myonecrosis, acute hepatitis, falciparum malaria and septic shock [229-232]. Although only a few studies examined pGSN levels after recovery, they have suggested that pGSN normalizes [230, 231, 233]. All reported studies where pGSN has been re-administered to injured animals have demonstrated a beneficial effect. While as of yet, there is no established mechanism to explain this behavior, there are, however, a few proposed functions for pGSN.

Proposed functional roles of plasma gelsolin

Extracellular actin scavenger system

Upon tissue damage and cell lysis, a large amount of actin is potentially released into the extracellular space. The physiological salt concentrations in blood strongly favor actin polymerization, so released F-actin remains as filaments. Long actin filaments that develop would increase the viscosity of blood. However, while actin is predicted to polymerize in the buffer conditions of plasma, no polymerization is seen when added to actual plasma, suggesting that factors in blood prevent polymerization [234]. pGSN, together with Gc-Globulin (Vitamin D-binding protein) have been proposed to be the main players in a system that removes actin from the circulation, namely the extracellular actin scavenging system (EASS; Figure 9). Plasma gelsolin severs F-actin and caps the barbed ends, whereas Gc-globulin sequesters monomeric actin for removal from the circulation in the liver. The $T_{1/2}$ of F-actin injected in rabbits is 30 minutes in healthy animals, thus the clearance of actin-Gc-globulin complexes is much faster than the 12-24 hour $T_{1/2}$ for Gc-globulin alone [199, 235, 236]. The EASS has also been detected during burn injury in burn wound fluid [237].

Injection of large amounts of G-actin into rats (>10 mg) results in polymerization, suggesting that the EASS is saturable. This high dose of actin leads to sudden cardiopulmonary arrest and right heart dilatation. Microthrombi, enmeshed in a dense network of F-actin, were visible in pulmonary arteries and capillaries [234]. These effects may result from F-actin itself or from nucleotides bound to actin, since they are very potent cell activators. Each actin molecule in a filament has an ADP or ATP bound, and ADP bound to actin activates platelets [238-240]. Platelet aggregation induced by F-actin was decreased in the presence of pGSN and Gc-globulin [238]. Actin might also interact directly with fibrin during clot formation [241, 242]. G-actin (or the ADP that it holds) is directly toxic to cultured sheep pulmonary artery endothelial cells, and the toxicity is reduced in the presence of pGSN [243].

While actin and actin-GSN complexes have been detected in serum during tissue damage [219, 244] actin filaments have been harder to find. However, recently Lee et al. observed actin in sedimentable form in plasma from LPS and CLP treated mice [225] and preliminary data suggest that F-actin is present on the outside of certain cell-derived microparticles (MP) isolated from GSN KO mice subjected to sepsis but not on MP from WT mice (T. Osborn, unpublished data). In these animal models for sepsis, actin concentrations in plasma reach ~250-500 mg/l, a portion of which sediments upon centrifugation, consistent with F-actin. Exogenously administered rhpGSN decreases sedimentable actin, presumably due to severing. Interestingly, the total plasma actin content was higher in rhpGSN treated mice compared to controls [225]. This may represent mobilization of trapped F-actin from the injury site by rhpGSN as a mechanism to "clean up" cellular debris.

Extracellular bioactive lipids that may be modulated by plasma gelsolin

Lysophosphatidic acid

LPA is a multifaceted phospholipid that is present in blood and in cells. A summary of the diverse LPA functions is listed in table 1. The variety of LPA effects derives from the broad tissue distribution of its receptors and their coupling to several different G-protein subfamilies [245]. There are at least five G-protein coupled receptors, named LPA_{1-5} [246-248], that are activated by LPA. The receptors are different in structure and distribution, and have a distinct preference for albumin-bound vs. non-albumin-bound LPA as well as for different LPA species [249, 250]. In addition, there is an intracellular receptor, PPARγ (peroxisome proliferator-activated receptor γ), important for atherogenesis that is activated by LPA traversed from the extracellular environment [251].

Autotaxin (lysophospholipase D), a constitutively active enzyme, produces most of the LPA present in blood by cleaving the abundant (>100 μM) phospholipid lysophosphatidyl choline (LPC) to LPA [252-254]. Importantly for atherosclerosis, LPA is also formed by mild oxidation of low-density lipoproteins [255, 256]. Due to its rapid enzymatic degradation, and possibly also auto-inhibition of autotaxin by LPA [257], circulating LPA concentrations are kept between 0.1-1 μM [258, 259]. Locally, in atherosclerotic plaques, the concentrations can be higher [256]. LPA can be degraded by plasma membrane lipid phosphate phosphatases or by acylation by LPA acyl transferases [245]. Termination of LPA signaling occurs primarily at the receptor level, by receptor desensitization and internalization [248, 260].

Although the plasma concentrations of LPA [259, 261] are far in excess of that required to saturate the nanomolar K_d of the known LPA receptors [262], plasma has no LPA-related activity on cells, suggesting the presence of "factors" in plasma that can prevent LPA from activating their membrane bound receptors [261]. pGSN is one possible LPA-buffer, binding LPA with higher affinity than albumin [165, 166], and the hypothesis of pGSN as a lipid-buffering agent in blood has been proposed [165, 263]. In contrast to plasma, serum exerts LPA-like biological activity [264] and contains ~5μM LPA [259], an amount in excess to pGSN (~3μM). Although albumin binds to LPA [264], its concentrations are so high (~ 600 μM), that if albumin served as a main buffer for LPA, serum would not have LPA-like activity. That albumin is not a main buffer is further supported by the fact that it facilitates the delivery of LPA to cells in blood [250, 264]. pGSN also facilitates the delivery of LPA to cells at low concentrations, but at

physiological concentrations of pGSN this activity is suppressed [165].

Table 1. Main biological and pathological events induced by LPA

Effect or event	Tissue and/or cell type	Comments	Refs.
Increases cell proliferation and survival	Many cell types (incl. EC, SMC, FB, TC, MQ)	Both normal and tumor transformed cells are affected.	245, 265
Increases cell migration, invasion and chemotaxis	Many cell types (incl. EC, SMC, TC)	Normal and tumor transformed cells are equally affected.	245, 265
Pro-inflammatory, increases cytokine production	FB, AC, LC, SMC, EPC, DC, EC, MC, carcinoma	Cytokines produced are: MCP-1, IL-1β, IL-3, IL-6, IL-8, Gro-α, GM-CSF.	245, 266
Promotes wound healing	Skin, intestinal epithelium	Supports proliferation and migration of mesenchymal- and EPC, stimulates production of cytokines, promotes fibronectin assembly, and triggers MFB contraction. Topical application on skin.	245, 267-269
Promotes blood clotting and thrombus formation	PT, VSMC	Induces VSMC contraction, PT shape change and aggregation.	256, 261, 265, 270, 271
Promotes atherosclerosis and cardiovascular disease	PT, VSMC, MQ, EC	Induces dedifferentiation of VSMC, and PT-MO aggregation. Affects contraction and permeability of VSMC and EC. LPA i) accumulates in atherosclerotic lesions (produced from PT), ii) is a constituent of mox-LDL iii) triggers PT activation, iiii) sustains inflammation.	272-275 256, 265, 271, 276
Angiogenesis	Vasculature, EC	Promotes proliferation and migration of ECs in vitro, enhances or reduces monolayer permeability in different EC systems, and induces cell contraction of SMC and MFB.	276-280
Promotes cancer progression	Ovarian, colorectal, prostate, gastric	Activated PTs are thought to be the source of LPA in the tumor microenvironment.	245, 281
Fertility (female)	uterus	Promotes BC implantation.	282
Nervous system development/biology	Neurons, NB, AC	Induces growth cone collapse and neurite retraction, reversal of AC stellation, and stimulates neuronal differentiation.	264, 283-287

AC = astrocytes, BC = blastocyst, DC = dendritic cells, EC = endothelial cells, EPC = epithelial cells, FB = fibroblasts, GM-CSF = granulocyte-macrophage colony-stimulating factor, Gro-α = Growth-regulated oncogene-α, IL = interleukin, LC = leukocytes, MC = mast cells, MCP = monocyte chemoattractant protein, MFB = myofibroblasts, MO = monocyte, mox-LDL = mildly oxidized low density lipoprotein, MQ = macrophages, NB = neuroblasts, PT = platelets, Refs. = references, SMC = smooth muscle cells, TC = T-lymphocytes, VSMC = vascular smooth muscle cells.

Platelet-activating factor

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent pro-inflammatory phospholipid with diverse physiological and pathological effects (listed in table 2) that mediates many different types of signaling (juxtacrine, paracrine, endocrine, autocrine and intracrine) [288-292]. Plasma PAF levels in healthy humans are very low to non-detectable but elevate in the plasma of septic patients where they can reach ~40 nM [293]. PAF is not maintained in cells as a pre-formed mediator, but is rapidly synthesized in a highly regulated manner [289, 292, 294, 295]. The

main sources of PAF are neutrophils, monocytes, platelets and endothelial cells. Neutrophils synthesize PAF during inflammation [296] and release ~30-40% as soluble forms [292], the rest being exposed on the cell surface [289] or associated with released microparticles [297]. PAF synthesized from activated endothelial cells is retained on the cell surface for juxtacrine signaling and contributes to the recruitment of neutrophils and monocytes to inflamed tissues by promoting adherence to the endothelium. Platelets can also retain some of the PAF they produce, an event possibly important for thrombotic events. In contrast, monocytes release most of the PAF they synthesize [289]. The major pathway for production during pathological inflammation and hypersensitivity responses [298] is from choline-containing membrane phospholipids, especially phosphatidylcholines (PC) by the enzyme cytosolic phospholipase A_2, that hydrolyzes long chain fatty acids esterified at the *sn*-2 position to form lyso-PAF [292]. Lyso-PAF is acetylated by an acetyltransferase to produce bioactive PAF [299]. Since PAF is produced from PC membrane lipids with different hydrocarbon chains, PAF is a heterogeneous family of molecules.

Table 2. Some important physiological and pathophysiological effects of PAF [292, 299-303]

Body system	Physiological effect	Pathophysiological effect
Whole body	Hemostasis, platelet aggregation and secretion, chemotaxis and activation of neutrophils and eosinophils, activation of monocytes/macrophages, stimulation of B lymphocytes, cell-cell-interactions, intra- and inter-cellular signal transduction, cell differentiation, apoptosis	Acute inflammation, allergic disorders, endotoxic shock, anaphylactic shock, disseminated intravascular coagulation, cancer, organ transplant rejection
Blood	Platelet aggregation and secretion, chemotaxis and activation of neutrophils and eosinophils, activation of monocytes, stimulation of B lymphocytes	Thrombocytopenic purpura
Central nervous system	Synaptic plasticity	Ischemic brain damage, convulsion
Cardiovascular system	Hypotension, negative inotropic effect, angiogenesis.	Myocardial ischemia.
Respiratory system	Bronchoconstriction, bronchial hyperreactivity	Bronchial asthma, acute lung injury, ARDS
Gastrointestinal system	Smooth muscle contraction, portal vein hypertension, glycogenolysis (liver)	Peptic ulcer, liver cirrhosis, ischemic bowel necrosis, pancreatitis
Urinary system	Proliferation of mesangial cells, inhibition of renin release	Glomerulonephritis
Reproductive system	Ovulation, ovoimplantation, stimulation of embryo, pregnancy	

PAF carries out its actions by binding to a G-protein-coupled seven-transmembrane spanning receptor, the PAF receptor (PAFR). The PAFR is expressed on diverse cells (including basophils, eosinophils, hepatocytes, keratinocytes, lymphocytes, monocytes/macrophages, osteoclasts, platelets, neutrophils, renal mesangial cells, vascular endothelial cells and vascular smooth muscle cells) [292, 300, 304]. PAF binds to its high-affinity receptor at concentrations in the picomolar range [305]. The PAFR couples to both pertussis sensitive and insensitive G-proteins, and activation involves many different signal transduction pathways [304] leading to cell polarization, cell motility and

cell spreading as well as up-regulation of pro-inflammatory molecules including cytokines, chemokines, lipid mediators, cell-surface molecules and oxygen radicals [289].

PAF is highly regulated, and a dysfunctional regulatory system is associated with disease [306]. One mechanism that controls for excessive PAF activation is receptor desensitization [306]. In addition, PAF is inactivated to lyso-PAF by a constitutively expressed, lipoprotein-associated enzyme PAF-acetylhydrolase (PAF-AH), present at 0.5-1 μg/ml in plasma. The $T_{1/2}$ of PAF in plasma, unless it is bound to plasma proteins, is estimated to be ~5 minutes [307-309]. Importantly, there is a class of PAF-like lipids that mimic the bioactivity of PAF and that might contribute to pathology by activating the PAFR. Although PAF synthesis is highly regulated, the synthesis of these lipids by oxidative fragmentation is not [292, 310].

Platelet-activating factor in acute inflammation
PAF activity and circulatory $T_{1/2}$ is increased in sepsis [307, 311-314] and PAF contributes to inflammation [290, 315]. Mortality from CLP- or LPS-induced sepsis is reduced by administration of PAF-AH [316]. But while administration of recombinant PAF-AH to septic patients in a phase II clinical study reduced the mortality of patients by 23% [317], a larger phase III study did not confirm the effect [318], and clinical trials conducted on PAFR antagonism have not been successful [319-322].

ARDS develops secondary to clinical conditions such as sepsis, severe burns, acute pancreatitis, hemorrhagic shock and trauma. Development of this complication significantly increases mortality. The pathogenesis derives from activated leukocytes that migrate to the pulmonary interstitium and increase endothelial permeability, leading to pulmonary edema and proteinaceous exudates. One important pro-inflammatory factor produced is PAF, whose responses are increased in ARDS [323-325]. In an animal model for ARDS, PAFR deficient mice survive better than WT mice, suggesting that decreasing PAF-signaling is beneficial [326].

In summary, PAF is involved in severe states of inflammation like sepsis and ARDS. In these conditions PAF levels increase and pGSN levels diminish. Repeatedly, studies have shown that re-administration of exogenous pGSN is beneficial. Paper I investigates whether pGSN may modulate PAF-induced cell-activation.

Other interactions potentially important in plasma
Other factors that might interact with pGSN in the extracellular environment are: ATP and Ap3A that were mentioned as binding partners previously, and are present in plasma in addition to the cytosol, amyloid β-peptides as described in the section about AD, and LPS, fibrin, fibronectin, and matrix metalloproteinases (MMPs) which will be discussed below. Table 3 summarizes factors potentially important for understanding the role of plasma gelsolin in the circulation.

Plasma gelsolin binds to LPS from various gram-negative bacteria with high affinity (K_d = 1 nM). LPS inhibits GSN's actin severing activity at lower concentrations than either LPA or PIP_2 [327] and a GSN peptide from P2 binds to LPS with higher affinity than to LPA [167]. LPS binds to toll-like receptor-4 and binding induces translocation of NF-κB to the nucleus, resulting in transcription of various cytokines. GSN can

Table 3. Potential binding partners for gelsolin in blood plasma

Binding partner	Binding site, K_d (stoichiometry)	Effect of pGSN, comments	Method for binding documentation
Actin [121, 128, 149, 150, 219, 220, 225, 233, 234, 332]	K_d: G1: 5pM, G2: 5-7 μM, G4: 1.8 μM, G4-G6: 25 nM (GSN 1: 2 G-actin, GSN 1: 1 F-actin)	Severs F-actin in the circulation and in tissues upon damage, and might scavenge G-actin. Preliminary data suggest that actin can be present on the outside of cell-derived MPs, and is cleaved off by pGSN (T. Osborn, unpublished data).	*In vitro* and *in vivo*: e.g. co-immunoprecipitated with pGSN from plasma
Aβ-Peptide [216-218, 333]	K_d: 1.38 μM and 2.55 μM. Disulfide bridge might be important for binding.	Binds to Aβ, disassembles preformed Aβ fibers, prevents fibrillization of soluble Aβ.	*In vitro* and *in vivo*. Co-immunoprecipitated with pGSN from plasma
ATP [168, 169, 334]	K_d: 0.28 μM G5 is important. Binding spans over N-T and C-T halves, and mainly occurs in the closed GSN conformation.	ATP is released upon cell-activation from e.g. neutrophils and is important for fMLF-induced superoxide formation and chemotaxis. pGSN in blood is in the open conformation, thus it is uncertain how much ATP it would bind.	Equilibrium dialysis
Ap3A [173]	K_d: 0.3 μM	Primes neutrophil respiratory burst and induces Ca^{2+} release. Binding to pGSN might prevent this cellular activation.	Affinity chromatography through Ap3A-agarose
Fibrin [241, 335]	unknown	Binds to fibrin oligomers and fibrin clots (results in lower pGSN levels in serum, than in plasma).	Binding of radiolabeled GSN to fibrin clots
Fibro-nectin (FN) [330]	K_d: ~ 1 μM (GSN 1:1 FN)	Binds to both native and polymerized FN. Uncertain function. pGSN might bind FN in blood and localize it to exposed actin for participation in actin clearance.	Solid-phase binding assays, turbidity of FN/GSN solution, quasi-elastic light scattering, gel-filtration chromatography, sucrose gradient centrifugation
LPS [167, 225, 327]	K_d: ~ 1 nM* for whole pGSN protein and 180 nM for P2 peptide. Might also bind P1 and P3.	Inhibits TLR-4-induced events in astrocytes and HAEC, but not fibroblasts or monocytes. Might have both inhibitory and stimulatory functions. Binding inhibits GSN-induced actin severing	Solid-phase binding assays, fluorescence measurements and actin depolymerization assays, isothermal titration calorimetry
LPA [165-167, 263]	K_d: 6 nM for pGSN, 920 nM for P2 peptide. Binds P1, P2, and presumably P3	Binds LPA and delivers it to RCMs at low concentrations, inhibits LPA-induced platelet P-selectin up-regulation in the presence of albumin, binding inhibits pGSN's F-actin severing function.	Equilibrium dialysis, actin depolymerization, isothermal titration calorimetry
PAF [263]	Unknown interaction site(s), but is inhibited by P2 .	Inhibits PAF-induced P-selectin up-regulation on platelets and O_2^- production from neutrophils.	Increases pGSN's nucleating function, competes with Alexa488-labeled LPS for binding to pGSN (T. Osborn, P. Lee unpublished observations)

*approximation [327], A = actin, Ap3A = diadenosine 5',5''-P1,P3-triphosphate, ATP = adenosine triphosphate, fMLF = formyl-methionyl-leucyl-phenylalanine, HAEC = human aortic endothelial cells, LPA = lysophosphatidic acid, LPS = lipopolysaccharide, MP = microparticle, O_2^- = superoxide ion, PAF = platelet-activating factor, RCM = rat cardiomyocytes, TLR-4 = toll-like receptor-4.

influence the physiological effect of LPS. pGSN inhibits LPS-induced NF-κB translocation in astrocytes, but not in fibroblasts [327]. Furthermore, in astrocytes and human aortic endothelial cells, LPS-induced disassembly of the actin cytoskeleton is inhibited by pGSN [327]. GSN did not significantly inhibit LPS-induced monocyte activation as determined by tumor necrosis factor-α production [225].

Ap3A is stored in dense granules of platelets, chromaffin cells and neuronal cells. After release into the extracellular space, this dinucleotide exhibits divergent biological effects on a variety of target cells and organs (e.g. primes respiratory burst and induces calcium transients in neutrophils). In contrast to ATP, Ap3A has a long $T_{1/2}$ in the blood [328, 329].

Fibronectin (FN) binds to several plasma proteins and is present in fibrin clots. GSN binds to both fibrin and FN. The K_d for FN is 1 μM at a 1:1 ratio *in vitro*. FN binding does not compete with actin binding [330].

pGSN is a substrate for MMPs and is cleaved *in vitro* by MMP-3 (most potent), MMP-2, MMP-1, MMP-14 and MMP-9 [120, 331]. MMPs are zinc-containing endopeptidases that participate in both normal and pathological processes. They are particularly increased during cancer and inflammatory conditions e.g. rheumatoid arthritis (RA) and atherosclerosis. They normally degrade extracellular matrix components, however, conditions that increase MMP production result in excessive degradation. One study of burn wound fluids found an inverse relationship between pGSN and metalloproteinase levels; gelsolin proteolytic fragments of 49 kDa were also detected [237].

Plasma gelsolin in rheumatoid arthritis

RA is a chronic inflammatory disease of unknown etiology that destroys synovial (diarthrodial) joints. Although the initiating event is unknown, persistent and progressive synovitis develops in peripheral joints. The progression, extent and pattern of the inflammatory response are highly variable and depend on both genetic and environmental factors. The earliest changes occur in the synovial microvasculature characterized by occlusion of the vessel lumen, endothelial cell swelling and capillary leakage that results in proliferation of cells lining the joint cavity, congestion, edema and fibrin exudates. Nodular aggregates of lymphocytes accumulate in the synovium early in the disease followed by other inflammatory cells. The release of inflammatory cytokines results in hyperproliferation of blood vessels, synovial fibroblasts and the synovial lining layers leading to a hypertrophic synovium. Granulation tissue (called pannus) extends to the cartilage and invades and destroys the cartilage, bone and other surrounding soft tissue. MMP production from synovial fibroblasts and monocytic phagocytes results in collagen and proteoglycan degradation. In erosive RA, the destruction reaches the bone [336].

One important aspect of the healthy joint altered in RA is the SF, where the volume increases and the composition changes. In healthy joints, there exists only a thin film of SF (less than 3 ml of fluid). Normally, SF functions as a lubricant to reduce friction upon motion, acts as a shock absorbing cushion during activity and serves as a dynamic metabolic reservoir for the proteins trafficking from the plasma, synovial tissue and cartilage. Synovial microvessels control solute flow via fenestrations that

allow diffusion-based exchange of small molecules between plasma and the interstitium in a size selective fashion also limited by the plasma flow rate and the narrow diffusion path between synovial lining cells. In healthy joints, small solutes (e.g. O_2 and CO_2), are in equilibrium with plasma, but plasma proteins enter SF passively by an unknown mechanism (possibly involving the fenestrae, intercellular junctions or cytoplasmic vesicles) and do not exist at equilibrium with plasma (~20% of the total plasma protein). Due to size-selectiveness, small proteins are overrepresented in SF, whereas large proteins are underrepresented. Transport of protein out of the synovial space, on the other hand, is not size dependent and occurs by diffusion through lymphatic vessels [336].

In RA, due to the underlying microvascular insufficiency, the plasma supply is limited and gradients arise resulting in abnormal joint spaces with low oxygen pressure, low glucose, low pH, high lactate and high carbon dioxide pressure. These conditions adversely affect surrounding cells and SF protein function. In addition to the biochemical changes, the endothelium becomes leaky, allowing more protein to enter SF. Protein production from surrounding cells may also be increased. Protein affluent is counteracted by an increase in lymphatic drainage, which occurs faster than in a healthy synovium, as well as local consumption in the synovium, as has been described for complement components [336]. pGSN may enter the joint space by multiple mechanisms. In addition to transport from blood, it may be locally produced by the joint space. Chondrocytes derived from osteoarthritic cartilage secrete gelsolin, and interestingly, Gc-globulin *in vitro* [203] and synovial fibroblasts produce gelsolin. pGSN might also be released from invading inflammatory cells. While cGSN production is known to be down-regulated by at least 30% in animal models of rheumatoid arthritis [204], as of yet, pGSN levels have not been measured in RA or in a healthy joint.

Inflammatory mediators produced by invading leukocytes play a devastating role in the progression of RA. Neutrophils migrate to the SF due to the presence of many chemotactic factors, especially cytokines such as IL-8, formulated peptides (e.g. fMLF) and activated compliment components (e.g. C5a). Once in the joint space, neutrophils are activated by various inflammatory mediators, phagocytose cellular debris, and produce superoxide anions, cytokines, prostaglandins and leukotrienes. This sequence leads to PAF production in the SF [337]. Cartilage and bone tissue damage ensues from the prolonged inflammation and is likely to result in extracellular actin exposure. In addition, fibronectin [338-341] and fibrin [342, 343] are present at greater than normal concentrations in the inflamed joint. Since plasma gelsolin has affinity for both actin, fibrin [335] and fibronectin [330], it might bind these proteins in SF. Furthermore, it may interact with other inflammatory mediators present, e.g. PAF [263], and possibly be a substrate for MMPs *in vivo* [120].

Since the potent inflammatory mediator PAF is present in SF of arthritic joints and in blood of RA patients at high concentrations [344], attempts have been made to use PAF antagonists as therapy against RA. Although animal studies were successful in decreasing synovial inflammation and cartilage depletion [345, 346] and preliminary studies of humans with RA showed an improvement of disease activity [347], a larger clinical study failed to prove effectiveness of PAF antagonism for RA [348]. Since pGSN levels are decreased during acute systemic inflammation, and pGSN has a protective effect against more severe injury and death, presumably by anti-

inflammatory mechanisms, paper II aims to begin to understand pGSN's potential role in RA.

3. THE GOALS OF THIS THESIS

The essential goal of this thesis is to further explore the physiological functions of two important actin binding proteins, plasma gelsolin and filamin A.

Plasma gelsolin is the extracellular isoform of the actin severing and capping protein gelsolin. Although much is known about cytosolic gelsolin, very little information has been generated on its secreted counterpart. Most of the research of plasma gelsolin has been directed towards measuring levels in plasma during different acute inflammatory states of disease, leading to the general conclusion that its levels drop during severe tissue injury. Furthermore, it has been shown that re-administration of recombinant plasma gelsolin to injured animals improves their outcome and survival. However, the mechanism behind the survival augmenting function(s) of gelsolin in plasma has not been delineated.

Filamin A is a potent actin filament crosslinking protein that also interacts with many other binding partners, including cell-surface receptors and signaling molecules. It is essential for cell motility and membrane stability and is believed to be important in mechanoprotection. It is an ~160 nm long, V-shaped homo-dimer that contains amino-terminal actin binding domains. However, as of yet there have not been enough details concerning filamin A's structure to fully explain its function as a multi-potent protein scaffold that can also organize actin filaments into orthogonal gel-like networks.

This thesis is based on research, organized into three manuscripts, investigating the following questions:

1) What is the mechanism behind plasma gelsolin's protective role in inflammation?

2) Are plasma gelsolin levels decreased in chronic inflammatory conditions as have been documented in acute inflammatory conditions?

3) How does filamin A form high affinity actin crosslinks?

4) Are filamin A crosslinks rigid or flexible?

5) Do filamin A-F-actin interactions affect binding to partner molecules?

6) What sub-domains of the filamin A molecule define orthogonal F-actin branches?

4. RESULTS AND DISCUSSION

Identification of a new function for plasma gelsolin – modulation of phospholipid-induced cell activation (paper I)

A new function for pGSN has been identified, as an inhibitor of cellular activation induced by two bioactive phospholipids, LPA and PAF *in vitro*, suggesting a novel physiological role for GSN in blood. The nature of their respective inhibition varies slightly, and is discussed below. A hypothesis for an *in vivo* anti-inflammatory mechanism is proposed.

Plasma gelsolin-mediated inhibition of platelet responses to LPA

It has previously been shown that low concentrations of pGSN can deliver LPA to cellular receptors, and that this effect is absent at physiological pGSN concentrations [165]. Here, pGSN causes a small enhancement of platelet P-selectin exposure in the absence of BSA, but in the presence of BSA, it inhibits LPA-induced P-selectin up-regulation dose-dependently. This inhibition might be more pronounced *in vivo* where albumin levels (~ 600 μM) are higher and the molar ratio of pGSN to LPA is larger.

The overall responsiveness to LPA is donor-dependent, as has previously been reported [249, 272]. The reason for this difference has not been explored further, but could come from differences in donor receptor subtypes and quantities.

Although diversion from the LPA receptors is a likely explanation for the observed inhibition by pGSN since it is known to bind to LPA, other mechanisms for the inhibition might exist. It has been shown that LPA-induced platelet aggregation can be completely inhibited by apyrase (which catalyzes hydrolysis of nucleotides such as ADP and ATP) and antagonists of the platelet ADP receptors P2Y1 and P2Y12. Hence, ADP-mediated receptor activation may play a central role in LPA-induced platelet aggregation [272]. As pGSN binds ADP and other nucleotides [171] pGSN might blunt the ADP-induced cellular activation.

Plasma gelsolin inhibits PAF-induced P-selectin up-regulation on platelets and superoxide anion production from neutrophils

In contrast to LPA, PAF-induced platelet P-selectin expression is inhibited by pre-incubation with pGSN both in the presence and absence of BSA (Figure 7). The inhibition is dose-dependent, and more pronounced than for LPA. PAF-induced superoxide anion production is also decreased by pGSN. An inhibition, although less pronounced, is observed in both the platelet and the neutrophil cell systems without pre-incubating pGSN with PAF. This suggests fast binding to pGSN, inhibition of secondary effects from subsequently released mediators (e.g. PAF, ADP, ATP) or a quenching of the signal. Quenching of the signal is unlikely, since there is no gelsolin-dependent decrease with control peptides. Release of ADP upon platelet stimulation by PAF has been inferred indirectly from a decreased P-selectin expression in the presence of apyrase (T. Osborn, unpublished data). Since GSN binds ADP [171], as discussed above, this might explain at least part of the inhibitory effect pGSN has on cell-activation by bioactive phospholipids.

At a 10 times higher ratio of pGSN to phorbol 12-myristate 13-acetate (PMA) than what was used for PAF, superoxide production is inhibited by 50%. This could reflect the effect of a direct interaction between pGSN and PMA, or an indirect effect where pGSN sequesters other mediators released from cells exposed to PMA [349].

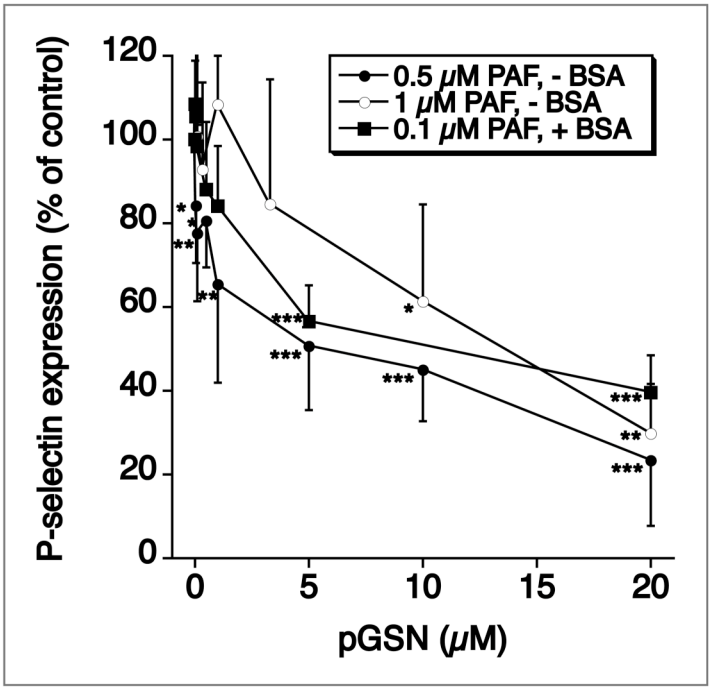

Figure 7. PAF-induced platelet P-selectin expression is inhibited by plasma gelsolin. Inhibition is observed both in the presence and absence of BSA (1:4 BSA:PAF). Control is PAF in the absence of pGSN. Data are mean (SD); n = 5-9, ($p < 0.0001$ ***, $p < 0.001$ **, $p < 0.05$ *).

PAF increases plasma gelsolin-induced actin nucleation

Since lipid binding to GSN (PIP_2, LPA and LPS) decreases its severing and capping functions [160, 166, 327], but not its nucleation activity, similar experiments were performed with PAF. Unexpectedly, GSN's severing function was not inhibited by PAF, and its actin nucleation activity was increased. PAF did not influence the actin polymerization rate in the absence of GSN, suggesting that the increased nucleation is a direct effect of PAF on pGSN. LPA on the other hand, did not affect pGSN's actin nucleation rate.

The plasma gelsolin-PAF interaction

"Classical" phospholipid interactions within GSN involve electrostatic attractions between the P1, P2 and P3 sites and the negatively charged phosphate group(s), as well as some association between hydrophobic residues in gelsolin and the acyl chains of the lipids [130-133]. Electrostatic forces are thought to initially drive the phospholipid binding. Hydrophobic forces then strengthen the interaction. PAF is composed of a phosphocholine head-group lacking a net negative charge (see Figure 8 for differences in structure among pGSN binding partners); hence, its interaction with GSN suggests other unique binding sites [350]. However, data that a peptide (QRLFQVKGRR) mimic of the P2 phospholipid-binding site in GSN domain G2 inhibits superoxide anion production induced by PAF, supports that the P2 site is involved.

pGSN can bind to phospholipids as single molecules, membrane components or micelles. Both types of interactions might be physiologically relevant, since PAF *in vivo* is membrane bound in cells and MPs [351] and can be both free or albumin bound in plasma [352, 353]. The nature of the interaction in my experimental setting is not known [354] and the binding stoichiometries remain to be investigated.

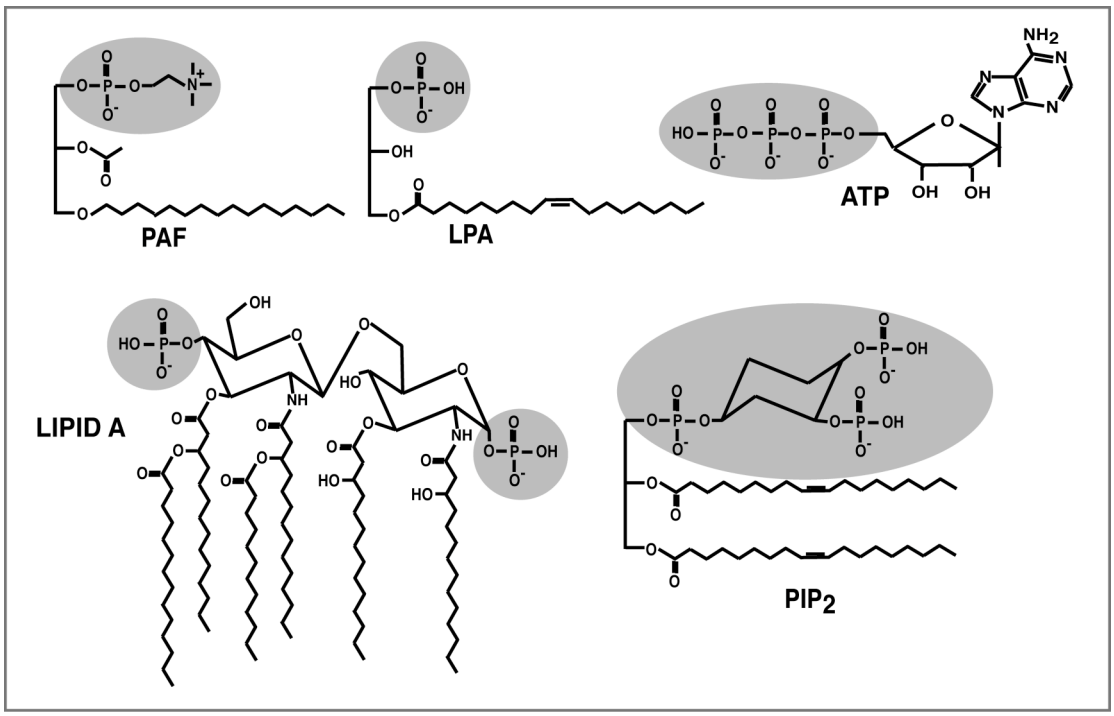

Figure 8. Molecules that interact with gelsolin. LPA (shown: 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), Lipid A (from e-coli LPS), PIP_2 (shown: 1,2-dioleoyl-*sn*-glycero-3-[phosphatidyl-4,5-bisphosphate]) and ATP are structurally different, but have in common that they contain net negative charges due to phosphate groups. PIP_2, LPA and LPS are likely to share the same binding sites in P1, P2 and P3. PAF (shown: 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) activity is inhibited by pGSN and interacts with the P2 site (a P2 derived peptide inhibited PAF) and possibly also the P1 and P3 sites and/or other unidentified sites. Shadowed areas contain the different phosphate "head-groups" which are thought to be important for interactions with GSN.

Protein binding to PAF

As reflected by the lack of protein-PAF-binding data reported in the literature, binding studies with PAF have proven troublesome to perform. Solubility difficulties of PAF, susceptibility to hydrolysis, difficulty in labeling by fluorescent compounds, a low critical micelle concentration, and high affinity binding to surfaces such as glass, Teflon and polystyrene have been blamed [352]. Binding studies of PAF to pGSN by a modified equilibrium dialysis system using ^{3}H-PAF or protein-coated beads did not show saturable binding for either pGSN or BSA, and binding was complicated by non-specific adherence of PAF to surfaces (T. Osborn, unpublished data). Additionally, previous studies have reported significant decomposition of PAF during long dialysis experiments [352]. A competition ELISA binding assay, pairing Alexa-568-labeled LPS versus LPA or PAF suggested that an excess of PAF and LPA can compete with LPS for binding to GSN (T. Osborn, P. Lee, unpublished data).

The new hypothesis of plasma gelsolin function in blood

The ability of pGSN to modulate biological responses to LPA, PAF [263], and possibly other inflammatory mediators, might be important in homeostasis and inflammation. Binding of inflammatory agents to pGSN prevents undesired cell activation, while maintaining and protecting a pool of bioactive lipids or other mediators from degradation [355]. Delivery to cells following vascular damage might be initiated by release of actin from disrupted cells, causing pGSN to release bioactive lipids to participate in defense and repair. Alternatively, diminished local concentrations of pGSN may lead to phospholipid delivery and release at receptor sites [165]. However, when tissue damage is severe, and a large amount of actin is released to the extracellular compartment, the bulk of pGSN shifts to binding actin. This releases bound inflammatory mediators that now initiate more severe secondary events. Figure 9 illustrates this theory.

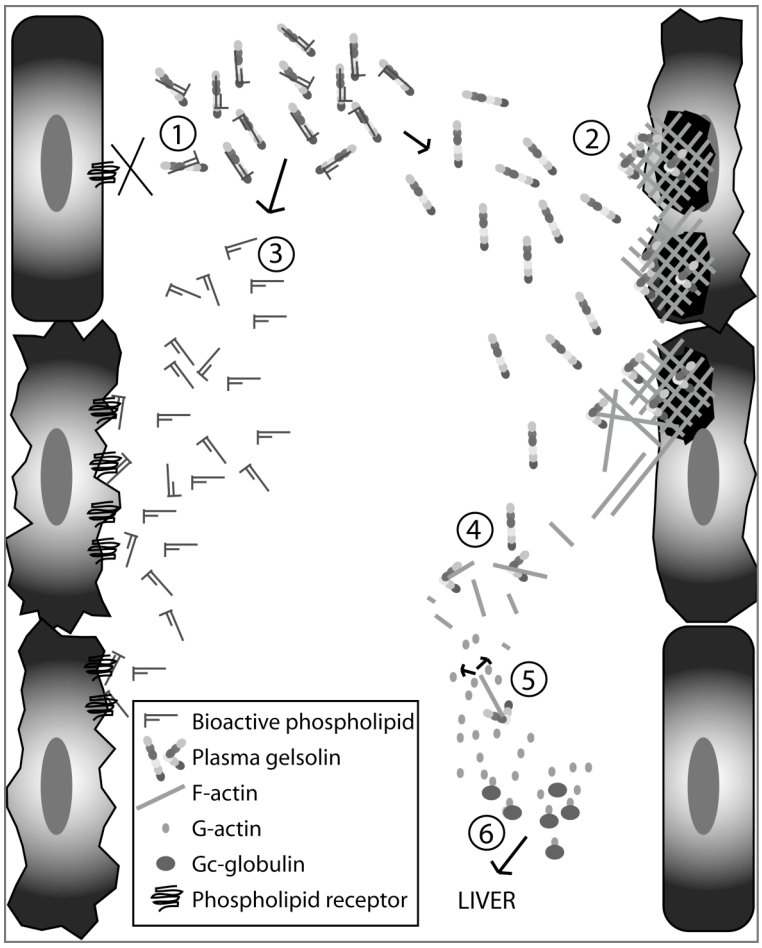

Figure 9. New hypothesis of plasma gelsolin function in blood. 1) Binding of inflammatory agents to pGSN prevents undesired cell activation, while maintaining and protecting a pool of bioactive lipids or other mediators from degradation. 2) Tissue injury exposes actin to the extracellular environment, attracting pGSN, resulting in a shift of binding from phospholipids to F-actin. 3) Unbound phospholipids are free to activate cells and mediate secondary injuries by potentiation of the inflammatory response. 4) Released F-actin is sequentially severed to shorter strands by the high pGSN amounts in plasma. 5) Actin monomers dissociate from the pointed end as pGSN caps the barbed ends. 6) Monomers are captured by Gc-globulin and transported to the liver for clearance.

Importance of plasma gelsolin's effect on bioactive phospholipids in inflammation

The biochemical readouts chosen for monitoring plasma gelsolin effects were platelet P-selectin expression and neutrophil superoxide anion generation, both important for the inflammatory response. P-selectin mediates the adhesion of neutrophils to the surface of activated platelets, and neutrophil superoxide anions are precursors for reactive oxidants intended to kill pathogens, but responsible also for damaging host cells at the site of inflammation. The ability of plasma gelsolin to inhibit the

bioactivity of LPA and PAF suggests a mechanism for its anti-inflammatory and pro-survival function during acute inflammation.

Potential importance for atherosclerosis

Atherosclerosis is a chronic, progressive disease affecting the vasculature that increases the risk of cardiovascular events such as myocardial infarction and stroke. Bioactive lipids such as PAF and LPA are increased in the atherosclerotic plaque and are produced from oxidative modification of LDL. Because of this, and demonstrated decreases in pGSN levels in plasma during inflammatory conditions, a study of 512 plasma samples obtained from the Women's Health study [356] was performed to investigate whether the plasma levels of pGSN, like CRP [356, 357], would be predictive of future cardiovascular events. Unfortunately, this study failed to detect such a relationship (T. Osborn, unpublished data), but it is the largest population of "healthy' individuals that have been studied for pGSN levels and thus provides information about the normal range and variability of pGSN levels in women, where it follows a normal bell-shaped distribution curve (T. Osborn, unpublished data). The distribution in men has not been investigated, but would be expected to be similar.

While plasma gelsolin might not be useful as a diagnostic tool for cardiovascular disease, it may provide important information regarding disease progression and could potentially be therapeutic. Key events in atherosclerosis include oxidative modification of lipoproteins, monocyte migration into the vessel wall, macrophage activation and foam cell formation. PAF is believed to play a part in this disease by inducing expression of adhesion molecules and release of chemotactic stimulants. In addition, other oxidized phospholipids structurally related to PAF produced in lesions can activate the PAFR. LPA is also produced in atherosclerotic lesions and, in a similar manner, is intimately involved in atherosclerosis [271]. Therefore, pGSN therapy to inhibit these phospholipids could represent an important intervention for atherogenesis. Interestingly, LPA enhances the production of MMPs [358], which might be a pathway that removes pGSN, since it is cleaved by various MMPs *in vitro* [120].

Potential importance for sepsis

pGSN is protective against various models of sepsis [225]. While binding to LPS has been demonstrated, there are opposing data regarding neutralizing effects of pGSN on LPS-induced cell activation [225, 327], and the fact that pGSN protects against sepsis induced by insults other than LPS injection, such as CLP, suggests different or additional mechanisms of protection. Since PAF is a potent inflammatory mediator, much work has been undertaken to inhibit PAF signaling in sepsis. Septic patients have high PAF receptor occupancy and diminished receptor numbers on their platelets due to receptor internalization, suggesting that PAF is an active component of their inflammatory response [312]. However, both PAF-receptor antagonists and PAF-AH (a PAF degrading enzyme) therapy have failed in clinical studies [319-322]. The reasons for this may be many, but inhibition focusing on one factor in such a complex condition might simply be insufficient. The effect of pGSN on human sepsis is still to be determined, but the findings that pGSN has protective effects against both PAF and LPA, and improves outcomes in animal models of acute inflammation, suggests that it might be a broad spectrum anti-inflammatory molecule with potential for future therapy targeting severe inflammation.

Future studies on plasma gelsolin

The inflammatory response is complex and includes the production and release of many different mediators of cell activation. Additional research is needed to fully understand the role of GSN in inflammation, focusing on not only phospholipids, but also other potential binding partners. Preliminary studies have been carried out to identify binding partners in human serum, using a GSN-column, solid phase extractions and gas-chromatography mass-spectroscopy. These data, although preliminary, suggest that pGSN might bind to cholesterol, or alternatively lipoproteins (T. Osborn, unpublished data). More binding partners are sure to be identified.

Preliminary data also suggests that F-actin is exposed on a fraction of MPs produced *in vitro* from platelets following stimulation with various types of agonists. pGSN can remove this F-actin at physiological concentrations, and when LPS is injected into WT and GSN KO mice, MPs with surface-exposed actin can be isolated from the plasma of GSN KO mice, but not from WT mice. This suggests yet another function of pGSN in stripping F-actin from potentially thrombogenic MPs. This interesting finding requires further investigation.

Plasma gelsolin levels are decreased in patients with RA compared to controls and even lower in the synovial fluid (paper II)

Circulating pGSN levels were lower in patients with RA compared to healthy controls and even lower in SF. Since there is considerable cell lysis and tissue damage in the synovium during RA, I confirmed that pGSN is present in the joint. Plasma origin was also supported by a correlation between pGSN levels in plasma and SF in the matched pair of samples. The levels of pGSN correlated weakly, and inversely, with levels of CRP, a sensitive marker of inflammation, but were not related to other inflammation markers investigated. This is the first evidence that pGSN levels are modulated not only in acute inflammatory conditions but also during chronic inflammation. Mechanistically, pGSN might be sequestered, consumed and/or rapidly cleaved in the joint during chronic arthritis. This knowledge could potentially lead to new understandings of both pGSN function and the pathogenesis of RA.

It is intriguing to speculate that pGSN might be involved in the pathogenesis of RA, since it interacts with many of the factors present in the inflamed joint space, such as fibrinogen, fibronectin, MMPs and actin; PAF is also presumably released. Preliminary results suggest that at least some of the plasma from RA patients contain actin (data not shown). Actin in plasma can be in a monomeric state or complexed to GSN, but could also be in a particulate form, which has been shown for sepsis [225] and in preliminary data on cell derived MPs. Plasma MP counts correlate with disease activity in RA [359] and MPs are also present in SF [360].

It is important to analyze SF from control samples in order to better understand the pGSN distribution within the healthy joint space. While albumin is generally considered a protein tracer for diffusion from plasma during inflammation, and is indeed lower in RA patient plasma, with levels being even lower intra-articularly, the distribution for pGSN is different from the one reported for albumin [361]. This distinction cannot be attributed to a size difference between the two proteins. In addition to size, the distribution of proteins also depends on local production and/or consumption among other factors. pGSN might be produced and secreted locally by

chondrocytes [203] and fibroblasts [204], suggesting that the actual consumption at the inflamed joint might be larger than what is detected by decreased blood levels.

pGSN levels were equally low in patients with non-erosive and erosive RA. This is not surprising as multiple factors influence levels by modifying transport, production and degradation. In both cases there is tissue damage that can lead to actin exposure as well as other molecules known to bind pGSN such as fibrin, which presents in the synovium early. Both actin and fibrin might act as sponges for pGSN, soaking it up from blood and SF at sites of tissue injury. pGSN levels might also be affected by local production in the inflamed joint as fibroblasts proliferate forming the pannus (granulation tissue). In late stage RA, the decreased vascularity (and presumably decreased accessibility for proteins) observed due to granulation tissue, fibrosis and joint immobility, might be compensated by enhanced local production of pGSN into the SF by the proliferating cells. Lastly, pGSN might be cleaved in the inflamed synovium by MMPs [120], although we did not detect any cleavage fragments in plasma or SF of RA patients.

Future experiments to better understand the role of pGSN in RA
Future experiments on pGSN's role in RA could include measurements of binding to inflammatory mediators by determining "free" and 'bound" pGSN. This can be done by comparing values obtained from the nucleation assay to the severing assay, since actin-severing is inhibited by many factors known to interact with pGSN (e.g. LPA, LPS and actin), while nucleation is not. By identifying binding partners in SF (e.g. actin, fibronectin, LPA, PAF, and other potential undefined molecules), possibly in complex with pGSN, we will understand more about the role of pGSN in SF and in RA.

Since pGSN is protective against death due to sepsis of various etiologies in animal models [225], it would be interesting to see what effect pGSN treatment has on animal models for RA. The idea of pGSN being a broad spectrum anti-inflammatory agent makes it a perhaps possible candidate for the treatment of inflammatory arthritis.

New insights to the FLNa structure and interaction with F-actin (paper III)
Important domains of FLNa, involved in F-actin binding and crosslinking, have been identified and studied in the electron microscope. A novel explanation is provided for how FLNa binds to F-actin and creates orthogonal crosslinks with sufficient rigidity to withstand thermal forces without compromising its ability to accommodate binding partner interactions. Figure 10 summarizes these new findings.

The structure of the filamin A molecule – topological differences of rod 1 and rod 2
The FLNa molecule has been sequentially truncated from its N-T to C-T to define structure-function relationships. Because the structure of each of FLNa's 24 subunits resembles those in immunoglobulins, the domains are named IgFLNa domains. Removal of the C-T dimerization domain (IgFLNa24), results in monomeric molecules. The contour length of FLNa is ~160 nm as previously reported [362] and molecules appear flexible but have V- or U-shapes at their midpoints. Truncated molecules of the rod 1 domain (ABDIgFLNa1-15) have the predicted length. IgFLNa16-23, called the rod 2 domain, is shorter than expected and has a compact globular appearance in agreement with the crystal structure of IgFLNa19-21 (see

Figure 2 for the new model of FLNa structure)[46]. Stereo paired images taken at a ± 10° tilt reveal that the molecules attach flat to the mica surface, except for the most N-T ~10-20 nm that tend to protrude in a "foot like" fashion. Since the C-T adheres tightly to the surface, despite having a globular configuration, measurements of the C-T angle can be made in "two-dimensions".

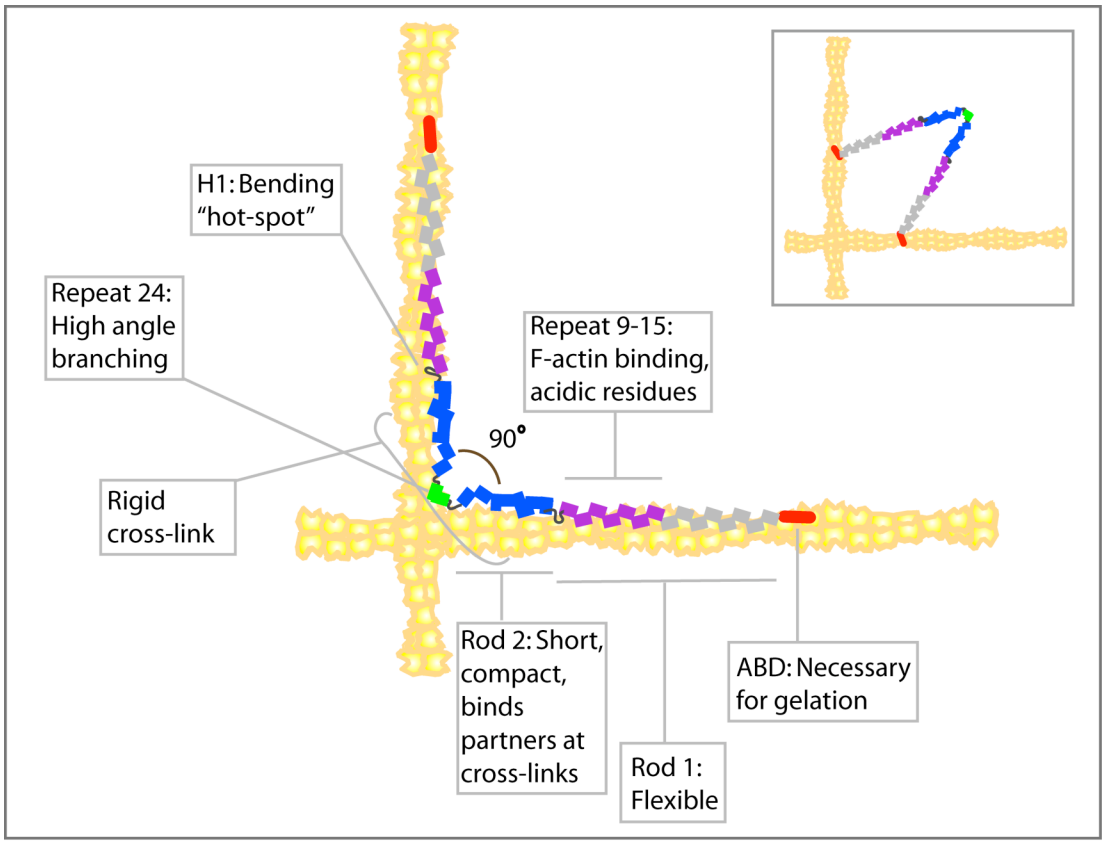

Figure 10. New characteristics of filamin A crosslinking of F-actin. FLNa's ABD is required for gelation, but its affinity for F-actin is 1/1000 less than for FLNa. Additional domains, IgFLNa9-15, in rod 1 bind to F-actin and increase the affinity (avidity). Dimerization results in high avidity. The insert depicts FLNa on F-actin as it has been illustrated prior to our findings of the rod 1 binding sites. Our results show that the orthogonality of the F-actin branches derives from the angle created in the FLNa molecule by self-association. Rod 2 is shorter and more compact than expected and can still bind partners when FLNa attaches to F-actin. Rod 1 is flexible. A bending 'hot-spot' is present between rod 1 and rod 2 (hinge 1, H1), which increases the overall flexibility. Flexible rod domains may increase the likelihood of locating filaments to a crosslink. The C-T accounts for rigid crosslinks.

The basis of high angle branching comes from the angular organization of the self-association domains

Measurements of the C-T V-shaped angle in the FLNa dimer show that it is approximately orthogonal, consistent with data from X-ray crystallography of the FLNc dimerization domain [363]. Keeping the dimerization domains, removing rod 2, and reattaching parts of rod 1 to hinge 2, does not change the angle formed by the self-association domain. To determine whether hinge 2 is also involved in creating the high angle, similar measurements were performed on FLNa molecules constructed to lack hinge 2. Measurements of the angle formed at the C-T on these hinge-2-less

molecules were identical, thus, supporting that the dimerization domain alone accounts for the high angle branching.

Filamin A rod domains are freely flexible with the exception of hinge 1, a bending "hot-spot"

Analysis of the contour of the FLNa molecule reveals that the rod 1 domain is equally flexible throughout. The compact rod 2 domain is also somewhat flexible. The rod domain flexibility is further confirmed by the finding that despite a N-T to C-T contour length of ~80 nm, the end-to-end distance (shortest path between the two termini) is only ~50 nm. A hot-spot for bending is observed at ~60 nm from the N-T at the position of hinge 1, where the angle of divergence is highly variable. Deletion of hinge 1 results in molecules lacking this bending point. Although the name implies that the hinge 1 structure is more flexible than the rest of the molecule, this has not previously been shown by measurements.

Novel actin binding sites in the rod 1 domain contribute to high avidity actin filament binding that enhances filamin A's potency as a crosslinker

Although the ABD is essential for crosslinking and gelation, constructs lacking the domain bind and bundle F-actin at high concentrations, provided that the dimerization domain is present. The ABD was previously identified as the main actin-binding site in the FLNa molecule, but the affinity of the ABD alone for F-actin, which is similar to that of other spectrin superfamily members, did not match FLNa's potency as a crosslinker [65], its ability to withstand high applied stress [67], or the full-length protein's 1000-fold higher affinity for F-actin. Identification of additional binding sites in the flexible rod 1 domain together with high avidity binding explains these discrepancies. The flexibility of rod 1 might aid in attaching a second actin filament, thus speeding up the "on-rate" for crosslinking. IgFLNa9-15 was identified as the binding region in rod 1 that attaches FLNa along the F-actin filament. Interestingly, this region is more acidic than the other Ig repeats. The exact mechanism that attaches these domains to F-actin remains to be investigated but the flexibility of the rod 1 domain might aid in its positioning in the groove of the twisting F-actin helix. Given its location aligned parallel to the actin filament long axis, it has previously been difficult to observe FLNa molecules at actin filament junctions in the EM. Visualization of FLNa at F-actin crosslinks *in situ* by immunoelectron microscopy using gold-labeled antibodies that recognize different epitopes throughout the protein, supports the notion that a large portion of FLNa binds along F-actin.

Filamin A-F-actin crosslinks create rigid structures

The FLNa-F-actin junctions are not deflected by the forces induced by thermal motion despite the finding that these forces are strong enough to oscillate regions of the actin filaments distal to the crosslink. This result is consistent with observations that high external strains are needed to deform FLNa-F-actin networks [67]. Based on compact appearance noted in the EM images, and recent crystal structure [46] the globular rod 2 domain may be able to unfold under stress. If this is true, the interdomain-structure of rod 2 is likely to contribute to this increased elasticity upon large strain in addition to the unfolding of individual domains [49]. Additional experiments using fluid flow (shear stress) over FLNa-F-actin branches may define the force-resistance relationships of individual crosslinks. FLNa constructs that lack specific repeats of rod 1 and 2 but contain the actin binding regions will have utility in

further understanding how domains generate the mechanical properties of the crosslink.

Importance of a globular rod 2 domain for binding partners – FilGAP
As illustrated in Figure 2, the rod 2 domain contains binding sites for many different binding partners although it does not appear to contribute to the binding to F-actin. In agreement with this, FilGAP, which binds at IgFLNa23, can interact with both free and F-actin-attached FLNa. Moreover, the compact or globular structure of rod 2 can reversibly unfold to influence partner interactions (see below for example). This opens up a new area of research regarding FLNa regulation and, because FLNa is the connecting link between many molecules and the cytoskeleton, cytoskeletal regulation in general.

The globular rod 2 domain – a theory of integrin-binding and mechanotransduction
The crystal structure of IgFLNa19-21 reveals that IgFLNa20 is partially unfolded, and that the first strand (A) of IgFLNa20 interacts with IgFLNa21 stands C and D [46]. By doing so, it forms a folded structure instead of an elongated rod as described for ddFLN where the domains attach sequentially [364]. A short and compact structure of rod 2 is consistent with my EM images. The strands that IgFLNa20 interacts with on IgFLNa21 are the same as for integrin binding [46]. Thus, in this folded state, the integrin binding site is hidden. It is intriguing to speculate that upon mechanical stress, which results in the application of strain on the cytoskeleton, the overlapping domains might disengage to open up the integrin binding site. Since the entire rod 2 domain appears compact, it is likely that other domains in rod 2 may behave similarly, especially since the sequence of IgFLNa20 is the most similar to IgFLNa16, 18 and 22. This might be a means of auto-regulating the attachment of several binding partners, where some may bind preferentially in the "closed" state as compared to the "open" configuration. This may explain in part how FLNa can have such diversity in partners, a situation that certainly needs intricate regulation. During mechanoprotection, when FLNa is accumulated at the area of applied force [88], this regulation might be especially important.

Overall, a better comprehension of the function of individual domains within the FLNa molecule will bring us closer to understanding the various phenotypes associated with the growing list of mutations in the human *FLNA* gene, and enhance the likelihood of finding treatment targets, whether it involves binding partners, cortical/cytoskeletal stability or signal/mechanotransduction.

5. CONCLUDING REMARKS

This thesis reveals novel functions of two ubiquitous actin binding proteins, plasma gelsolin and filamin A.

Plasma gelsolin is shown to inhibit cellular activation induced by two bioactive phospholipids important for inflammation, lysophosphatidic acid and platelet-activating factor. It has not previously been recognized that interactions of this protein with endogenous inflammatory mediators compete for receptor activation. Furthermore, these results provide a mechanism by which gelsolin acts as an anti-inflammatory agent in plasma. Plasma gelsolin levels are reduced in patients with rheumatoid arthritis, showing that plasma levels of this protein decrease not only during acute inflammatory conditions but also during chronic inflammation.

The main conclusions concerning plasma gelsolin are:

1) **Plasma gelsolin inhibits cellular activation *in vitro* induced by two potent lipid mediators, lysophosphatidic acid and platelet-activating factor.** Readouts for these effects were P-selectin expression on platelets and superoxide anion production from neutrophils, two events important for inflammation. These results suggest a new model for anti-inflammatory actions of plasma gelsolin in addition to its participation in extracellular actin scavenging. We propose that plasma gelsolin might act as a phospholipid-buffering agent in the circulation, protecting from excessive cell activation. During tissue injury, release of large amounts of actin clears gelsolin from the circulation, resulting in loss of binding to inflammatory mediators, which can then activate cells and inflict secondary injury.

2) **Plasma gelsolin levels are reduced not only in acute, but also in chronic inflammation, such as rheumatoid arthritis.** Plasma levels are diminished in patients with rheumatoid arthritis compared to age and gender matched healthy controls, and even lower in the patients' synovial fluid. Plasma gelsolin may be sequestered and consumed locally in the inflamed joint.

The mechanism with which filamin A interacts with actin is dissected from electron microscopy studies and binding assays using the whole protein, as well as truncated molecules. These investigations explain the diverse functions of filamin A as an actin-filament crosslinker and protein scaffold.

The main conclusions regarding filamin A are:

1) **Filamin A achieves high avidity F-actin-binding by attaching along its side to F-actin.** This requires binding sites within repeats 9-15 of the rod 1 domain, in addition to the previously known actin binding sites at the amino-termini, as well as dimerization.

2) **Filamin A crosslinks are rigid structures that can withstand forces imposed by Brownian motion.** Despite appearing as a rather flexible molecule in the electron microscope, filamin A forms rigid F-actin crosslinks. The self-association domain at the carboxyl-terminus of the filamin A molecule confers crosslink rigidity, since both rod 1 and hinge 1 are flexible structures.

3) **The rod 2 domain confers a breathing space in the crosslinks, explaining how filamin A can interact with partners while firmly attached to actin.** Electron microscopy of the filamin A molecule reveals a compact organization in the rod 2 domain, which is where most known partners interact. Rod 2 is globular and shorter than constructs built from the same number of repeats derived from rod 1. A binding partner that interacts with repeat 23 in the rod 2 domain can bind to filamin A while filamin A is still attached to F-actin, proving for the first time that filamin A links binding partners to the actin cytoskeleton. The globular structure of this region also evokes a new hypothesis of FLNa binding autoregulation.

4) **The filamin A carboxyl-terminal dimerization domain defines a V-shape that gives rise to perpendicular branching.** Perpendicular F-actin branching requires filamin A side binding, but the large crosslinking angle reflects the structure defined in the dimerization domain located in the carboxyl-terminal of FLNa.

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