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PDZ-LIM DOMAIN PROTEINS AND α-ACTININ AT THE MUSCLE Z-DISK

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Abstract

The Z-disk is a sophisticated structure that connects adjacent sarcomeres in striated muscle myofibrils. α-Actinin provides strength to the Z-disks by crosslinking the actin filaments of adjacent sarcomeres. α-Actinin is an antiparallel homodimer, composed of an N-terminal actin binding domain (ABD), the central rod domain, and two pairs of C-terminal EF-hands. The PDZ-LIM domain proteins interact with α-actinin at the Z-disk. Of these proteins, only the actinin-associated LIM protein (ALP), Z-band alternatively spliced PDZ-containing protein (ZASP/Cypher) and C-terminal LIM protein (CLP36) have a ZASP/Cypher-like (ZM) motif consisting of 26-27 conserved residues in the internal region between the PDZ and LIM domains. The aim of this work was to understand the molecular interplay between the ZM-motif containing members of the PDZ-LIM proteins and α-actinin. To unveil the biological relevance of the interaction between the PDZ-LIM proteins and α-actinin, naturally occurring human ZASP/Cypher mutations were analyzed.

Two interaction sites were found between ALP, CLP36 and α-actinin using recombinant purified proteins in surface plasmon resonance (SPR) analysis. The PDZ domain of ALP and CLP36 recognized the C-terminus of α-actinin, whereas the internal regions bound to the rod domain. Further characterization showed that the ALP internal region adopts and extended conformation when interacting with α-actinin and that the ZM-motif partly mediated the interaction, but did not define the entire interaction area. ZASP/Cypher also interacted and competed with ALP in binding to the rod domain. The internal fragments containing the ZM-motif were important for co-localization of ALP and ZASP/Cypher with α-actinin at the Z-disks and on stress fibers. The absence of ALP and ZASP/Cypher in focal contacts indicates that other interacting molecules, for instance vinculin and integrin, may compete in binding to the rod in these areas or additional proteins are required in targeting to these locations. The co-localization of the ZASP/Cypher with α-actinin could be released by disrupting the stress fibers leading to an accumulation of α-actinin in the cell periphery, whereas ZASP/Cypher was not in these areas. This suggests that an intact cytoskeleton is important for ZASP/Cypher interaction with α-actinin. Earlier studies have shown that mutations in the ZASP/Cypher internal region are associated with muscular diseases. These mutations, however, did not affect ZASP/Cypher co-localization with α-actinin or the stability of ZASP/Cypher proteins.

The Z-disk possesses a stretch sensor, which is involved in triggering hypertrophic growth as a compensatory mechanism to increased workloads. α-Actinin is a docking site of molecules that are involved in hypertrophic signaling cascades mediated by calsarcin-calcineurin and protein kinase C (PKC) isoforms. The internal interaction site may be involved in targeting PKCs, which bind to the LIM domains of ZASP/Cypher, to the Z-disks. The similar location of the internal interaction site with calsarcin on the rod suggests that ZASP/Cypher, ALP and CLP36 may regulate calsarcin-mediated hypertrophic signaling.

Keywords: α-actinin, PDZ-LIM proteins, sarcomere, stress fiber, ZM-motif

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Abbreviations

ABD	actin binding domain
ALP	actinin-associated LIM protein
CD	circular dichroism
CH	calponin homology
CHO	chinese hamster ovarian
GFP	green fluorescent protein
LIM	a protein domain named after the cell-lineage protein (LIN-11), insulin gene-enhancer binding protein (Isl1), and a protein required in specification of mechanosensory neurons (MEC-3)
CLP36	C-terminal LIM protein (with a molecular weight of 36 kDa)
kDa	kilodalton
MAGUK	membrane associated guanylate kinase
MLP	muscle LIM protein
NMR	nuclear magnetic resonance
PDB	protein data bank
PDZ	a protein domain named after the postsynaptic-density protein (PSD-95), discs large protein (Dlg), and zona occludens protein (ZO-1)
PIP2	phosphatidylinositol (4,5) -bisphosphate
PIP3	phosphaditylinositol (3,4,5) -trisphosphate
PKC	protein kinase C
RIL	reversion-induced LIM protein
SPR	surface plasmon resonance
ZASP/Cypher	Z-band alternatively spliced PDZ-containing protein
ZM-motif	ZASP/Cypher-like motif

List of original articles

This thesis is based on two original articles and one manuscript, which are referred to in the text by their Roman numerals.

I Klaavuniemi T, Kelloniemi A & Ylänne J (2004) The ZASP-like motif in actinin-associated LIM protein is required for interaction with the α-actinin rod and for targeting to the muscle Z-line. J Biol Chem 279: 26402-10.

II Klaavuniemi T & Ylänne J (2006) ZASP/Cypher internal ZM-motif containing fragments are sufficient to co-localize with α-actinin – analysis of patient point mutations. Exp Cell Res 312: 1299-311.

III Klaavuniemi T, Alho N, Hotulainen P, Kelloniemi A, Permi P, Mattila S & Ylänne J (2006), Characterization of the interaction between actinin-associated LIM protein (ALP) and the rod domain of α-actinin. Manuscript.

Contents

1 Introduction

The contractile machinery of muscle cells is composed of myofibrils, which contain repeating contractile units called sarcomeres. The integrity of the sarcomeres is essential for muscle function. Connecting adjacent sarcomeres are complex structures known as Z-disks that function both as structural entities and as mechanochemical signaling centers. α-Actinin is a major component of the Z-disk where it crosslinks actin filaments from adjacent sarcomeres and thus provides strength to the Z-disks.

In addition to α-actinin, a large number of structural and signaling proteins localize at the Z-disks. PDZ-LIM proteins form a family of α-actinin interacting Z-disk proteins containing seven members: actinin-associated LIM protein (ALP), C-terminal LIM protein (CLP36), reversion-induced LIM protein (RIL), Mystique, Z-band alternatively spliced PDZ-containing protein (ZASP/Cypher), Enigma and Enigma homology protein (ENH). These proteins have a conserved PDZ-domain at the N-terminus, and one to three conserved C-terminal LIM domains. The internal region between the PDZ and LIM domains is less-conserved, although ALP, CLP36 and ZASP/Cypher have a conserved ZASP/Cypher-like motif (ZM) in the internal region. Mice knock-out studies of ALP and ZASP/Cypher have confirmed that these proteins are important for proper sarcomeric organization.

The aim of this work was to understand the molecular interplay between the ZM-motif containing members of the PDZ-LIM proteins and α-actinin. To unveil the biological relevance of this interplay, an analysis of naturally occurring human ZASP/Cypher mutations was performed.

2 Review of the literature

2.1 Muscle structure and function

The primary function of muscle is to contract. There are three muscle types which all have the ability to contract: skeletal muscle, cardiac muscle and smooth muscle. Skeletal muscle consists of myofibers that are large, cylindrical multinuclear cells. In myofibers there are many myofibrils, which are the contractile assemblies containing actin (thin) and myosin (thick) filaments. The basic contractile unit of a myofibril is a sarcomere. In skeletal and cardiac muscle the sarcomeres of adjacent myofibrils are aligned, which generates the striated appearance of these tissues in microscopic preparations. The smooth muscle, instead, does not have the striated pattern. (Burkitt *et al.* 1993.)

Based on the striation of skeletal and cardiac muscle the different zones of the sarcomere were named as bands and lines (Fig. 1). Now we understand the basic molecular organization of these areas. The A-band is composed mainly of myosin, whereas the major component of the I-band is actin. The C-zone defines the area in the A-band, where actin and myosin filaments overlap. The M-line dissects the A-band in the middle, and the Z-disk (also called Z-band or Z-line) divides the I-band. One sarcomere is the area between two Z-disks, and thus the Z-disk connects the actin filaments from neighboring sarcomeres. (for a review, see Clark *et al.* 2002.)

In addition to myofibrils, there are also other specialized structures in striated muscle cells. Other cytoskeletal filament systems (for a review, see Stromer 1998) connect myofibrils to the sarcolemma (plasma membrane) and its invaginations (T-tubules) as well as to the sarcoplasmic reticulum (endoplastic reticulum) (Burkitt *et al.* 1993). Muscle nuclei are organized beneath the sarcolemma and muscle mitochondria (also called sarcosomes) are characteristically located within the I-bands (Burkitt *et al.* 1993).

Cardiac and skeletal muscles differ at the tissue level, although they have a similar sarcomeric organization. In cardiac muscle the cells are not fused together to form continuous multinucleated myofibers. Instead, the adjacent myocytes are connected to each other by intercalated discs, which have three main junctional complexes (reviewed in Clark *et al.* 2002). The gap junctions enable the chemical connection between adjacent cardiomyocyte, adherens-junctions and desmosomes connect the filamentous systems,

such as actin, to the membrane. Cadherin-cadherin interactions at adherens-junctions link the adjacent cardiomyocytes.

The cytoskeleton of smooth muscle cell is not organized into myofibrils, although some similarities exist (reviewed in Small & Gimona 1998, Stephens 2001). In smooth muscle, the Z-bodies, also called dense bodies, correspond to the Z-disks (Bond & Somlyo 1982). More precisely, dense bodies are located in the cytosol, whereas dense bands/dense plaques are electron-dense areas close to the cell membrane (Geiger *et al.* 1981, Stephens 2001). Both dense bodies and dense bands are anchoring sites for actin filaments of opposite polarities and together these dense bodies with actin and myosin filaments form the contractile unit of smooth muscle (Bond & Somlyo 1982).

Different cytoskeletal filament systems provide the basis of striated muscle organization and function. In the following chapters these filaments, muscle contraction and muscle differentiation are described in detail.

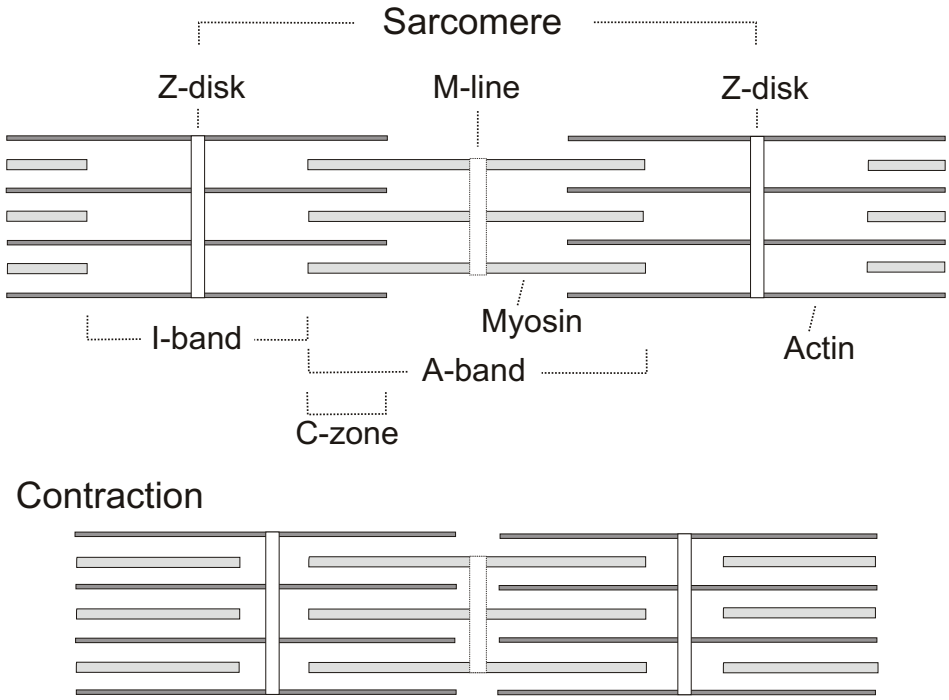

Fig. 1. The sarcomeres connected by the Z-disks are the basic contractile units of myofibrils. The sliding movement of myosin and actin filaments generates contraction.

2.1.1 Filamentous systems in striated muscle

Traditionally there are four major filamentous systems in myofibrils (Clark *et al.* 2002). Filamentous actin and myosin are responsible for contraction. The giant proteins titin and nebulin are closely connected to myofibrils constituting the third and the fourth filamentous systems. The novel giant protein obscurin can be added as a fifth filamentous system. The intermediate filaments and microtubules can be seen as the sixth and seventh filamentous systems in muscle, although they are not perceived as part of the contractile apparatus *per se*. Finally, the cortical cytoskeleton forms the eighth filamentous network.

2.1.1.1 The contractile cytoskeleton: actin and myosin

Actin proteins are found in most eukaryotic cells. Actin has a versatile role in mediating various cellular processes, for instance the role of actin polymerization in cell motility has been extensively studied (reviewed in Carlier *et al.* 2003). Globular actin monomers (Kabsch *et al.* 1990) polymerize to form actin filaments, which have the appearance of two twisted helices (Hanson & Lowy 1963).

Actin isoforms can be classified in terms of their isoelectric mobility into classes α, β and γ and further based on their expression patterns into 6 isoactins in mammals. α-skeletal and α-cardiac actins are striated muscle isoforms, whereas α-vascular and γ-enteric actins are expressed in smooth muscle (also termed α- and γ- smooth muscle isoforms). Nonmuscle cells express β- and γ- actins, which are also called cytoplasmic actins. The identity of the actin isoforms at the protein sequence level is 93-99.5 %, being higher between muscle actins than between cytoplasmic isoforms. (for references, see Vandekerckhove *et al.* 1986, Mounier *et al.* 1997.) Despite the high sequence identity, actins are functionally diverse. Actin expression is developmentally regulated and tissue-specific. Although several isoforms can be expressed simultaneously in a single cell, they are specifically compartmentalized. During muscle development, the muscle isoforms begin gradually to predominate. Striated muscle actins, which are found in sarcomeres, form more stable polymers than nonmuscle actins. Nonmuscle β- and γ-actin are also present in striated muscle, where they localize at submembraneous regions and at costameres (for reviews, see Herman 1993, Khaitlina 2001.) The nonmuscle actins are major components of the cortical cytoskeleton (chapter 2.1.1.4).

In sarcomeres the fast-growing barbed ends of actin polymers point toward the Z-disks (Ishikawa *et al.* 1969). The barbed ends are capped by CapZ (Casella & Torres 1994 and references therein) and the pointed ends by tropomodulin (Almenar-Queralt *et al.* 1999). In muscle, actin interacts with tropomyosin and troponins, which are important in the regulation of muscle contraction (reviewed in Squire & Morris 1998). It is essential that the actin length remains the same in sarcomeres. Several models of how this can be achieved have been proposed (Littlefield & Fowler 1998). Length regulation extends from the Z-disk portion along the filament up to the M-line, and involves, for instance, the actin capping proteins CapZ and tropomodulin, and the giant titin and nebulin proteins.

Myosins form the second filamentous system of myofibrils. There are 15 classes in the myosin superfamily. As a general feature, myosins can bind actin and act as motor proteins. Filament forming muscle- and nonmuscle myosins constitute class II. Class II myosins are hexameric enzymes composed of two heavy chains and two pairs of light chains. Head and tail regions constitute the myosin molecule. The head region contains the N-terminal parts of the heavy chains and the regulatory and essential light chains. The head region is the motor domain binding to actin, hydrolyzing ATP and generating movement. The myosin tail region contains a coiled-coil forming region, which enables the dimerization of two myosin heavy chains in a bipolar order. In striated muscle, these hexameres are bundled together by parallel interactions along the filaments to form thick myosin filaments. A single thick filament in a sarcomere can contain up to 600 myosin molecules. (for reviews, see Reggiani *et al.* 2000, Sellers 2000.)

Based on the motor domain sequence, class II myosins can be divided into two subclasses. Sarcomeric myosins form one group, and smooth-muscle and nonmuscle myosins the second (Sellers 2000). There are a number of isoforms of both myosin heavy and light chains, which can combine with each other to produce various isomyosins. The expression of isomyosins is regulated to fulfill the different contraction requirements depending on muscle type. (reviewed in Reggiani *et al.* 2000.)

Myosins are the major constituents of the A-band. The head domain is seen as "cross-bridges" constituting the C-zone, where thick filaments interact with thin filaments (Clark *et al.* 2002). The tails of bipolar myosins are anchored at the M-line, where myosin filaments are crosslinked by M-bridges. Myomesin and M-protein are potential candidates for crosslinking thick filaments at the M-line. Myomesin is expressed in all muscle types, in contrast to M–protein. (for references, see Agarkova & Perriard 2005.) Myomesin forms antiparallel dimers. This suggests that myosin filaments are crosslinked by myomesin in the M-line in a similar way that actin filaments by α-actinin in the Z-disk. (Lange *et al.* 2005.) Myosin together with titin may position thin filaments in sarcomeres (Littlefield & Fowler 1998). As in the case of thin filaments, the constant length and arrangement of myosin in sarcomeres is crucial for efficient muscle function (reviewed in Barral & Epstein 1999).

2.1.1.2 The giant proteins: titin, nebulin and obscurin

Titin, also known as connectin, is a giant (3-3.7 megadaltons) multifunctional protein spanning from the Z-disk to the M-line. The N-terminal parts overlap in the Z-disk, and the C-terminal parts at the M-lines. Thus, two titin molecules form a continuous system through the entire sarcomere. The majority of the titin molecule is composed of immunoglobulin-like and fibronectin type 3 domains. Titin can be separated into different regions based on the sarcomeric location and function. These are the Z-disk, M-line, A-band and I-band regions. The A-band region may act as a template in thick filament assembly. The M-line region contains a kinase domain, which links titin to signaling cascades. The I-band region acts as a molecular spring, which is responsible for the elasticity of muscle. Titin regulates the assembly of actin and myosin filaments and maintains sarcomeric organization. Titin maintains the thick filaments in register during

contraction cycles, (for reviews Clark *et al.* 2002, Guglieri *et al.* 2005.) and interacts with both thick (myomesin) (Obermann *et al.* 1997) and thin filament (α-actinin) (Sorimachi *et al.* 1997, Ohtsuka *et al.* 1997a, Ohtsuka *et al.* 1997b) crosslinkers. The Z-disk region of titin contains Z-repeats, which have been implicated in the regulation of the Z-disk width (Young *et al.* 1998, Luther *et al.* 2002, Luther & Squire 2002). Alternative splicing generates diversity for titin (Clark *et al.* 2002).

Nebulin is another giant sarcomeric protein (500-900 kilodaltons, kDa) composed mainly of small modular motifs. Alternatively splicing accounts for its size variation. (Labeit & Kolmerer 1995 and references therein.) Nebulin spans the entire length of thin filaments acting most likely as a ruler for thin filament assembly during myofibrillogenesis. It has been proposed that the organization of nebulin into modular motifs enables its interaction with actin, monomer by monomer. Nebulin may also regulate contraction by preventing actin-myosin interaction. It was thought that nebulin was not expressed in heart. Instead, a smaller nebulin-like protein, nebulette (107 kDa) was found in cardiac muscle. Nebulette does not seem to extend the entire length of the thin filaments, and thus may not act as a ruler. However, now nebulin expression has also been detected in heart. (for a review, see McElhinny *et al.* 2003.) Nebulin may also be involved in defining the Z-disk width (Millevoi *et al.* 1998).

A third giant sarcomeric protein, named obscurin, was originally found as an interaction partner to titin (Sanger & Sanger 2001). Obscurin, 800 kDa in size, is composed of immunoglobulin-like and fibronectin type 3 -domains, like titin. Obscurin has also signaling domains, such as the Rho-guanine nucleotide exchange factor (Rho-GEF). In addition to titin, obscurin most likely interacts also with myosin. Obscurin surrounds both the Z-disks and M-lines. Recent findings suggest an important role for obscurin in myofibrillogenesis, in particular in incorporation of myosin into A-bands and in formation of the M-lines. Obscurin may connect myofibrils to the sarcoplasmic reticulum via its interaction with ankyrin. (Borisov *et al.* 2006, Kontrogianni-Konstantopoulos *et al.* 2006, and references therein.)

2.1.1.3 Intermediate filaments and microtubules

The contractile machinery is not isolated from the rest of the cytoskeleton; intermediate filaments are especially important. Intermediate filament polypeptides are composed of an N-terminal head, a central rod and a C-terminal tail. The rod region mediates dimerization of two intermediate filament molecules, thus initiating the assembly of intermediate filaments. Intermediate filament proteins can be categorized into classes I-V. Some intermediate filament proteins can form heteropolymers. Homopolymeric class III members desmin and vimentin are found in muscle. (for references, see Bellin *et al.* 1999, reviewed in Paulin & Li 2004.) The intermediate filaments surround the Z-disks and M-lines connecting them at the sarcomeric level as well as in a transverse orientation to adjacent myofibrils (Wang & Ramirez-Mitchell 1983). Desmin filaments have been extensively studied in muscle, where they associate with the Z-disks (Lazarides & Granger 1978) and interact with nebulin (Bang *et al.* 2002). Desmin connects costameres to the Z-disks (for references, see Frank *et al.* 2006) and probably meditates the

connections to microtubules as well (Clark *et al.* 2002). Although desmin is expressed at early stages in myofibrillogenesis, desmin deficiency in mice does not affect muscle development, but it is required to maintain the sarcomeric organization in mature muscle (Milner *et al.* 1996, Li *et al.* 1997, and references therein). On the other hand, plectin deficiency in mice is lethal. Plectin links intermediate filaments to actin and microtubules. (reviewed in Clark *et al.* 2002.) The dispensability of desmin indicates that compensatory mechanisms, probably mediated by other muscle intermediate filaments, do exist. Synemin, a novel muscle intermediate filament protein, is connected to Z-disks and costameres via interactions with α-actinin and vinculin (Bellin *et al.* 1999, Bellin *et al.* 2001). The results of Bellin and colleagues (Bellin *et al.* 1999, Bellin *et al.* 2001) indicate that synemin can only form heteropolymers with desmin and vimentin. Recent studies have also revealed cytokeratins as intermediate filament components of muscle (Ursitti *et al.* 2004, Stone *et al.* 2005).

Microtubules composed of α- and β-tubulins are also present in muscle (Clark *et al.* 2002). In muscle cells, microtubules are concentrated at the subsarcolemmal area. They are also associated with myofibrils and nuclei. (Boudriau *et al.* 1993.) Interference with microtubule dynamics by taxol, which promotes tubulin polymerization, prevents cell fusion and differentiation (Antin *et al.* 1981). The disruption of microtubules by colcemid or nocodazole leads to fragmentation of myotubes (Saitoh *et al.* 1988). Colchicine (an agent that inhibits microtubule polymerization) treatment in patients may cause muscle myopathy in some cases (Fernandez *et al.* 2002 and references therein). Microtubules are important for contraction in heart, where they probably affect Ca^{2+}-signaling (for references, see Clark *et al.* 2002).

2.1.1.4 Cortical cytoskeleton

Nonmuscle actins form a filamentous network beneath the sarcolemma in muscle cells. γ-Actin is especially enriched at costameres and with mitochondria, whereas β-actin associates with neuromuscular junctions. (reviewed in Herman 1993, Khaitlina 2001.) This cortical actin network is connected to the Z-disk components and to the sarcolemma (reviewed in Ervasti 2003) thus bridging myofibrils to the sarcolemma and extracellular matrix. Costameric actin (γ-actin) is connected to the sarcolemma largely by spectrin family proteins (reviewed in Broderick & Winder 2002, Broderick & Winder 2005) dystrophin, α-actinin (reviewed in chapter 2.2) and spectrin. In short, α-actinin connects γ-actin to integrins, dystrophin to glycoprotein complexes and spectrin via ankyrin to NaK-ATPase. γ-Actin interacts also with other components in the costameric area. (reviewed in Ervasti 2003.) Dystrophin provides a strong connection between costameric actin and the sarcolemma. In dystrophin deficient mice this connection is fragile. (Rybakova *et al.* 2000.) An overexpression of utrophin, which is highly homologous to dystrophin, can rescue the linkage of costameric actin to the sarcolemma in dystrophin deficient mice (Tinsley *et al.* 1998, Rybakova *et al.* 2002). Mutations in dystrophin and dystrophin associated proteins are the most common causes of human muscular disorders (reviewed in Dalkilic & Kunkel 2003). The total absence of dystrophin leads to Duchenne muscular dystrophy (DMD) (Hoffman *et al.* 1987).

A spectrin cytoskeleton was originally found in erythrocytes, where it maintains the characteristic biconcave shape of the cells and provides the elasticity that is required when entering small capillaries. The spectrin cytoskeleton of erythrocytes is composed of spectrin tetramers that are connected to short actin filaments at their ends to form a continuous lattice. This lattice is attached to transmembrane proteins via ankyrin and band 4.1 proteins. Deficiencies in spectrin lead to fragility of erythrocytes. (Alberts *et al.* 1994.) Spectrin is also found in other cell types including muscle (reviewed in Baines & Pinder 2005). In *Caenorhabditis elegans*, spectrin is important in connecting muscle to the hypodermis in the developing worm (Norman & Moerman 2002). In addition to the sarcolemmal localization in muscle, spectrins also associate with internal structures, such as the sarcoplasmic reticulum and Z-disks and M-lines of sarcomeres. They are also detected in intercalated discs of cardiac muscle. Different spectrin isoforms and the spectrin associated proteins ankyrin and 4.1 proteins are compartmentalized specifically in cardiac muscle cells indicating isoform-specific functions. Although spectrin is shown to be important in heart development in mice, the function of spectrin in muscle is not clear. Spectrin may be important in localizing ion channels and pumps and thus contribute to Ca^{2+} regulation in muscle. (Baines & Pinder 2005.)

2.1.2 Contraction

The contraction of skeletal muscle is under somatic nervous system control. In skeletal muscle, motor neurons branch within a muscle to form motor units, which supply a group of muscle fibers. (Burkitt *et al.* 1993.) Acetylcholine is released from the nerve terminal at the neuromuscular junction, where the neuronal end is attached to the muscle membrane. The binding of acetylcholine to its receptors on the sarcolemma causes the receptor channels to open, which enables Na^{2+}-influx leading to the depolarization of sarcolemma. (Alberts *et al.* 1994.) The depolarization extends to the myofibrils via the T-tubule system, which leads to Ca^{2+}-release from the sarcoplasmic reticulum. The cytosolic Ca^{2+}-concentration is increased about 100-fold. Ca^{2+} binds to troponin C on the troponin-tropomyosin complex located on thin filaments. In relaxed muscle, tropomyosin blocks the myosin binding site on actin. Ca^{2+} binding to troponin C causes a shift in the tropomyosin position and exposes the myosin binding site. Actin filament binding, in turn, activates the myosin power cycle. The myosin head domain mediates the connection to the actin filaments and the power stroke, where the energy of ATP-hydrolysis is converted to mechanical work. This results in a sliding movement of actin and myosin filaments at the sarcomeric level and as muscle contraction at the organ level. (reviewed in Berchtold *et al.* 2000, Clark *et al.* 2002.) Slight differences to this conventional steric-blocking mechanism have been suggested (Squire & Morris 1998). The motile force of muscle contraction is directed by special attachment sites, such as myotendious junctions (reviewed in Clark *et al.* 2002) to bones. The contraction is essential for muscle tissue vitality, dysfunction of neuronal supply leads to degeneration of muscle fibers (Burkitt *et al.* 1993).

In contrast to skeletal muscle, cardiac muscle has an intrinsic ability to contract without stimulation from the central nervous system. The stimulation of cardiac

contraction is mediated by modified cardiac cells known as pacemakers that initiate action potentials. The normal heartbeat begins with an action potential in the pacemaker cells located in the sinoatrial node that spreads rapidly through the electrically coupled cells of the atria. After a slight delay, the action potential stimulates the depolarization of the atrioventricular node and the impulses are transmitted to the ventricles via the bundle of His and Purkinje fibers. However, the intrinsic contraction rate is regulated by the autonomous nervous system and hormones. (Gould & Keeton 1996.) The contractile machinery and the trigger of the sliding motion of thin and thick filaments are similar to that of skeletal muscle, but in cardiac muscle the cells are not fused. Instead, they are a connected by intercalated discs, which permit spreading of the depolarization from one cell to another (Burkitt *et al.* 1993).

Smooth muscle contraction is under the control of the autonomous nervous system and hormones (Gould & Keeton 1996). The Ca^{2+}-flux from the sarcoplasmic reticulum is also the first initiator after a nerve signal in smooth muscle (Somlyo & Somlyo 1994). Instead of a troponin-tropomyosin system, the contraction is regulated by a calmodulin/myosin-light chain kinase/phosphatase system in smooth muscle (Stephens 2001). Ca^{2+} binds to calmodulin, which activates the myosin light-chain kinase (MLCK). MLC-kinase phosphorylates Ser19 on the myosin regulatory light chain. This allows myosin ATPase activation by the thin filament leading to the contraction. (reviewed in Somlyo & Somlyo 1994.) The differences in cytoskeletal organization and function between smooth and striated muscle are largely caused the tissue specific expression of the contractile proteins. For instance, the smooth muscle myosin isoform differs from the striated muscle isoform - instead of a helical bipolar arrangement, smooth muscle myosin forms nonhelical side-polar filaments, where the crossbridges project in opposite directions on the opposite sides of a thick filament. The side-polarity may explain the ability of smooth muscle to shorten more during contraction when compared to skeletal muscle. (Xu *et al.* 1996.) Herrera and co-workers (Herrera *et al.* 2005) suggest that in contrast to striated muscle, the contraction machinery of smooth muscle is flexible. The changes in the number of contractile units and the changes in thick filament length account for the length changes of smooth muscle cells (Herrera *et al.* 2005).

2.1.3 Myofibrillogenesis

How is the fine-tuned sarcomeric organization formed? There are two models for myofibrillogenesis. In the premyofibril theory, the organization of thin and thick filaments is a synchronous process, where three stages can be distinguished (Dabiri *et al.* 1997, Sanger *et al.* 2002, Du *et al.* 2003). The premyofibrils are characterized by Z-bodies, which are precursors of the Z-disks, rich in α-actinin. Actin filaments are connected to the Z-bodies and to nonmuscle myosin. In nascent myofibrils, Z-bodies start to fuse laterally. At this stage muscle myosin and titin are recruited. During the differentiation process nonmuscle myosin gradually disappears. In mature myofibrils, nonmuscle myosin is absent. In the second model, thin and thick filaments are assembled independently (Dlugosz *et al.* 1984, Schultheiss *et al.* 1990, reviewed shortly in Epstein & Fischman 1991, Holtzer *et al.* 1997, Kontrogianni-Konstantopoulos *et al.* 2006). The I-

Z-I complexes composed of Z-disk components and actin filaments are combined with myosin thick filaments by the action of titin to form mature sarcomeres. The stress fiber-like structures serve as a template to assist the assembly. In this model, nonmuscle myosin is not required for the formation of myofibrils, although Du and colleagues (Du *et al.* 2003) argued that in this model the apparent absence of nonmuscle myosin was due to inadequate antibodies. In conclusion, the final outcome in both models is the same, although there are some disagreements in the pathways.

2.1.4 Z-disk

In mature myofibrils, the adjacent sarcomeres are connected by Z-disks, which are essential structures for muscle integrity and function. The Z-disks are composed of a large number of proteins and other molecules. The major proteins are actin filaments crosslinked by α-actinin. Also titin, nebulin and intermediate filaments are partially located in the Z-disks (Fig. 2). In addition to acting as a mechanical stabilizer, the Z-disk functions also as a platform for signaling cascades. Moreover, the Z-disks have been implicated in stretch sensoring. The Z-disk is a dynamic structure. For instance, some of the Z-disk components can translocate to nuclei. (for recent reviews, see Frank *et al.* 2006, Hoshijima 2006.) Myofibrils are connected at the Z-disk level to costameres, which resemble focal adhesion complexes in nonmuscle cells having a similar protein composition (for reviews, Ervasti 2003, Samarel 2005).

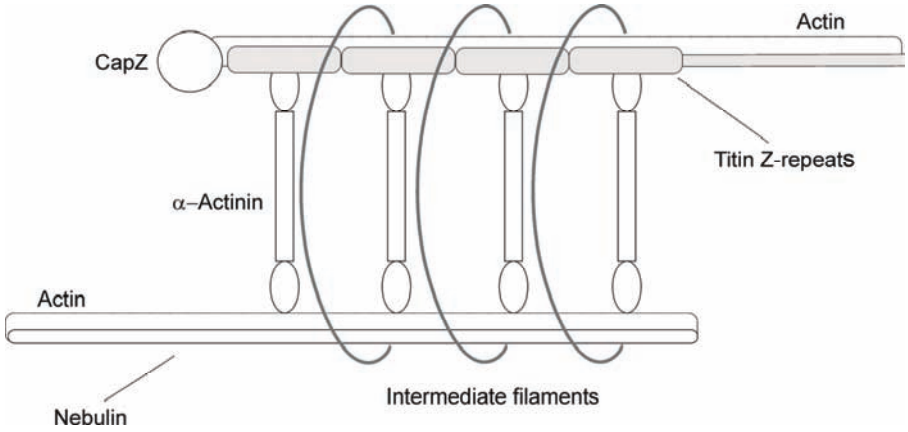

Fig. 2. Major components of the Z-disk. α-Actinin crosslinks antiparallel actin filaments. Titin Z-repeats may regulate the number of α-actinin crosslinks.

Muscle fibers can be classified into slow- and fast-twitch fibers, which differ in the width of the Z-disks. Cardiac and slow skeletal muscle fibers have wide Z-disks (100-140 nm), and fast muscle fibers have narrow Z-disks (30-70nm). In longitudinal electron micrograph sections of Z-disks, a characteristic zigzag pattern is observed. The number of zigzag-layers is higher in wide than in narrow Z-disks. Each zigzag-layer is composed of Z-links. An important component of these Z-links is α-actinin, which crosslinks the

actin filaments. The Z-disk width reflects the number of crosslinks mediated by α-actinin. One zigzag-layer may contain more than one Z-link. (Luther *et al.* 2002 and references therein.) The Z-disk width can also vary within a single sarcomere (Luther *et al.* 2003), which may be a transitional stage of myofiber type from slow to fast or *vice versa*. Ca^{2+}-handling and the expression pattern of various myosin isoforms determine to a great extent which muscle fiber is fast and which is slow (for reviews, Berchtold *et al.* 2000, Reggiani *et al.* 2000). The number of titin Z-disk Z-repeats correlate with the width of the Z-disks and with the amount of Z-links (Luther *et al.* 2002). It has been proposed that the number of titin Z-repeats regulates the amount of Z-links mediated by α-actinin in a one to one manner (Young *et al.* 1998, Figure 2). However, the size of a titin Z-repeat in the α-actinin bound state does not match with the distance between the Z-links. It is possible that two Z-repeats, instead of one, may account for the periodicity of Z-links. (Luther & Squire 2002.) One theory suggests that nebulin may also be involved in regulating the Z-disk width (Millevoi *et al.* 1998). To briefly summarize, the Z-disk is a sophisticated 3D-lattice of α-actinin mediated crosslinks of actin repeated at defined intervals.

2.1.5 Stress fibers as a model for myofibrils

Stress fibers of nonmuscle cells have been widely used to study the functional properties of sarcomeric proteins. Originally, it was thought that stress fibers were found only in cultured cells, but similar structures have been found in cells under mechanical pressure, such as in endothelial cells of heart ventricles (Wong *et al.* 1983, Franke *et al.* 1984). Although stress fibers cannot be perceived as analogues of myofibrils, there are similarities in the protein compositions. For instance, they both contain actin, myosin and α-actinin (Sanger *et al.* 1983, Langanger *et al.* 1986, Katoh *et al.* 1998, and references therein). More than 20 years ago, a sarcomeric model for stress fiber structure was proposed (Sanger *et al.* 1983) resembling the organization of a sarcomere known today. The actin filaments of stress fibers have an alternating polarity (Cramer *et al.* 1997, and references therein) similar to sarcomeres. Furthermore, the formation of the transverse arc –type of stress fibers, which do not terminate at focal contacts, resembles the formation of premyofibrils (Hotulainen & Lappalainen 2006), where myosin is incorporated into preformed α-actinin -crosslinked actin bundles (reviewed in chapter 2.1.3). α-Actinin bundles the thin filaments forming a periodic pattern of dense areas (also called dense bodies) on stress fibers (Sanger *et al.* 1983.) It has been reported that the myosin of stress fibers would have a bipolar arrangement enabling contraction (Langanger *et al.* 1986).

The contraction of stress fibers is regulated by Ca^{2+} and phosphorylation of the myosin regulatory light chain (Katoh *et al.* 1998), which is similar to smooth muscle contraction (chapter 2.1.2). There are also differences between smooth muscle and stress fibers. For instance, myosin in stress fibers is apparently organized into bipolar minifilaments (Verkhovsky *et al.* 1995) instead of the side-polar arrangement in smooth muscle (Xu *et al.* 1996). Focal adhesions terminating both ends of stress fibers resemble myotendius junctions at the ends of muscle and intercalated discs with respect to their protein composition (for references, see Katoh *et al.* 1998). Stress fiber-like structures may serve

as templates in myofibrillogenesis, where one dense band corresponds to one Z-disk in the mature sarcomere (Dlugosz *et al.* 1984), in contrast the fusion of Z-bodies to Z-disks as in the premyofibril theory (Dabiri *et al.* 1997, Sanger *et al.* 2002, Du *et al.* 2003).

2.2 α-Actinins

A protein that precipitated actomyosin was discovered over 40 years ago (Ebashi & Ebashi 1964). This protein was named as α-actinin (Ebashi & Ebashi 1965). α-Actinin is a member of the spectrin protein superfamily that also includes spectrins, dystrophin and utrophin. The general features of these proteins are the specific number of spectrin repeats and their ability to bind filamentous actin via the actin binding domain (ABD). These proteins also have two or four EF-hand motifs that may bind to Ca^{2+}. α-Actinin has only four spectrin repeats indicating that it is the ancestor molecule for the whole family. (reviewed in Broderick & Winder 2002, Broderick & Winder 2005.) The shorter the distance between the ABDs, the tighter the bundle formed (for references, see Puius *et al.* 1998). α-Actinins, as well as the whole spectrin family, can also be classified as a member of the filamentous actin binding proteins. As a common feature, all the members of this superfamily have an ABD, but otherwise the domain organization is varied. In addition to the spectrin family of proteins, this group includes, for example, filamin and fimbrin. (for a review, see Puius *et al.* 1998.)

In humans, there are four α-actinin genes encoding proteins at about 80 % sequence identity (Millake *et al.* 1989, Youssoufian *et al.* 1990, Beggs *et al.* 1992, Honda *et al.* 1998, Nikolopoulos *et al.* 2000). α-Actinin 2 was most likely the ancestor of the α-actinin gene family (Dixson *et al.* 2003). α-Actinins 2 and 3 are restricted to striated muscle, where they are enriched especially at the Z-disks (Lazarides & Granger 1978), but can also be found in the sarcolemma (Hance *et al.* 1999). α-Actinin 3 is absent in cardiac muscle (Beggs *et al.* 1992). α-Actinin 3 is also absent in approximately 16% of humans, but there is no obvious phenotype (North *et al.* 1999). Instead, α-actinin 3 deficiency in congenital muscular dystrophy patients (North & Beggs 1996) is a secondary phenomenon (North *et al.* 1999). α-Actinin 2 is expressed in all fiber types, but α-actinin 3 only in type 2 (fast) fibers (North & Beggs 1996). Nonmuscle α-actinins 1 and 4 are located in focal contacts, stress fibers and cortical networks (Lazarides & Burridge 1975, Sanger *et al.* 1983). The localization pattern of isoforms 1 and 4 differ. α-Actinin 1 is concentrated at the ends of stress fibers in focal contacts and adherens junctions, whereas α-actinin 4 localizes on stress fibers and can also translocate to nuclei (Honda *et al.* 1998). The isoform expressed in smooth muscle dense plaques and dense bodies (Geiger *et al.* 1981, Small 1995) is a product of alternative splicing of the α-actinin 1 gene (Waites *et al.* 1992).

2.2.1 Domain composition

α-Actinin is an antiparallel homodimer, composed of an N-terminal ABD, the central rod domain composed of four spectrin repeats (R1-R4), and two pairs of C-terminal EF-

hands (reviewed in Blanchard *et al.* 1989, Fig. 3). α-Actinin is a rod shaped molecule, with a size around 30-40 nm and a molecular weight of a dimer in nondenaturing solvents around 200 kDa (Podlubnaya *et al.* 1975, Suzuki *et al.* 1976, Condeelis & Vahey 1982). Advanced technologies have enabled 3D-reconstructions of the whole α-actinin molecule (Tang *et al.* 2001, Liu *et al.* 2004).

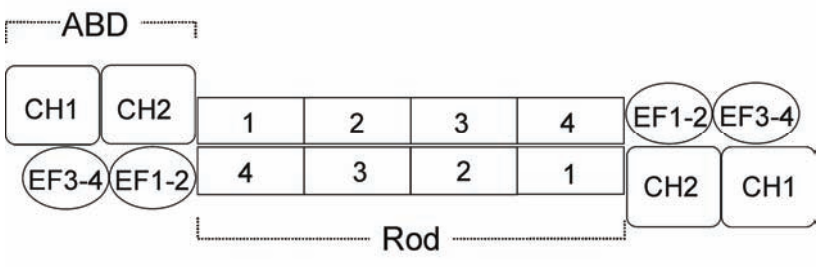

Fig. 3. Domain composition of α-actinin. The ABD is composed of two CH domains, the central rod of four spectrin repeats, and the C-terminal part of two pairs of EF-hands.

The ABD is composed of two calponin homology (CH) domains, CH1 and CH2. CH-domains are globular modules composed of four 11-18 residue α-helices, which are connected by loops. The N-terminal CH1 is responsible for the binding to actin, whereas the adjacent CH2 acts as a facilitator increasing the affinity for actin in spectrin family members. (reviewed in Gimona *et al.* 2002.) The crystal structure shows that α-actinin 3 ABD is in a "closed" conformation where the CH domains are in close proximity (Franzot *et al.* 2005) as in spectrin (Carugo *et al.* 1997). In contrast, an "open" conformation, where the CH domains are separated by an extended conformation of the linker region, is found in the spectrin family members dystrophin and utrophin (Keep *et al.* 1999, Norwood *et al.* 2000). Using cryoelectron microscopy, both "open" and "closed" conformations of chicken smooth muscle α-actinin have been observed (Liu *et al.* 2004).

The spectrin repeats, which are the most variable parts of the α-actinin molecule (Virel & Backman 2004), mediate α-actinin dimerization (Wallraff *et al.* 1986, Imamura *et al.* 1988). A spectrin repeat is a triple-helical coiled-coil bundle, varying in size between 106 in spectrin to 122 residues in α-actinin (for reviews, see Broderick & Winder 2002, Broderick & Winder 2005). Different views of the dimerization of α-actinin exist. According to Flood and co-workers (Flood *et al.* 1995), all four spectrin repeats are required for dimerization, but other studies have suggested that three pairs are sufficient (Taylor & Taylor 1993, Winkler *et al.* 1997). Smooth muscle α-actinin was used in these studies. Structures of the rod region of skeletal α-actinin support the four-pair model, where spectrin repeats 2 and 3 mediate the dimerization (Djinovic-Carugo *et al.* 1999, Ylänne *et al.* 2001). Afterwards, the 3D-reconstruction has been accomplished using the four-pair model in smooth muscle α-actinin (Liu *et al.* 2004). The human muscle α-actinins 2 and 3 can form heterodimers (Chan *et al.* 1998), although in low amounts *in vivo*. The four-spectrin repeat α-actinins found in the animal kingdom today most

probably originate from an ancestral α-actinin with only a single spectrin repeat (Virel & Backman 2004).

The C-terminus of α-actinin is composed of two pairs of EF-hands, also called a calmodulin-like domain (CaM). The EF-hand is a paired helix-loop-helix able to coordinate divalent ions, usually Ca^{2+}. A binding of Ca^{2+} changes the conformation from "open" to "closed". (reviewed in Blanchard *et al.* 1989, Broderick & Winder 2002, Broderick & Winder 2005.) The binding of Ca^{2+} to the EF-region probably disturbs the interaction of the neighboring ABD with filamentous actin (Noegel *et al.* 1987). The Ca^{2+}-binding EF-regions of muscle isoforms 2 and 3 are dysfunctional, caused by the absence of crucial ion binding residues (Beggs *et al.* 1992). The structure of the C-terminal EF-hand of α-actinin 2 has been resolved in a complex with the interacting titin peptide (Atkinson *et al.* 2001). Ca^{2+} inhibits the nonmuscle isoform binding to actin (Burridge & Feramisco 1981). Actually, most likely only the first EF-hand of α-actinin 1 can bind Ca^{2+}, whereas the second EF-hand lacks the required residues for Ca^{2+}-coordination (Waites *et al.* 1992). In contrast, both EF-hands of α-actinin 4 may be Ca^{2+}-insensitive (Nikolopoulos *et al.* 2000). Also smooth muscle α-actinin is insensitive to Ca^{2+} (Burridge & Feramisco 1981). The isoform expressed in smooth muscle is a product of alternative splicing of the exons producing the C-terminal part of the first EF-region in α-actinin 1. In contrast to the nonmuscle exon, the smooth muscle exon does not produce a Ca^{2+}-sensitive EF-hand. A transcript containing both smooth and nonmuscle exons has been detected in brain. (Waites *et al.* 1992, Kremerskothen *et al.* 2002 and references therein.)

2.2.2 α-Actinin interactions and functions in muscle

Studies on *Drosophila*-mutants have shown that α-actinin is necessary for muscle function. α-Actinin deficient embryos are able to hatch, but die early at the larval stage due to degeneration of myofibrils characterized by the disruption of Z-disks. (Fyrberg *et al.* 1990, Roulier *et al.* 1992, Fyrberg *et al.* 1998.) These studies suggest that α-actinin is not required for the organization of myofibrils, but it is essential during muscle function. On the other hand, the presence of α-actinin already at the early stages of myofibrillogenesis (Dabiri *et al.* 1997, Sanger *et al.* 2002, Du *et al.* 2003) indicates that α-actinin is also important at the very early stages of sarcomere formation. Interestingly, muscle α-actinins may partially compensate the loss of nonmuscle α-actinin in *Drosophila* (Wahlström *et al.* 2004). A perturbation of endogenous α-actinin function by introducing various α-actinin fragments has severe consequences. Microinjection of α-actinin ABD- and rod -fragments leads to disruption of stress fibers (Pavalko & Burridge 1991). The overexpression of a C-terminally truncated α-actinin causes hypertrophied Z-disks leading eventually to breakdown of myofibrils and stress fibers, whereas the full-length protein does not have such drastic effects (Schultheiss *et al.* 1992, Lin *et al.* 1998). The overexpression of α-actinin fragments may interfere with the essential interactions with α-actinin required to maintain sarcomeric organization. α-Actinin binding partners in muscle are listed in Table 1. The function of the interaction partners in muscle and their interaction site with α-actinin, if known, are indicated.

Already Ebashi and Ebashi in their pioneering studies (Ebashi & Ebashi 1964, Ebashi & Ebashi 1965) found evidence that α-actinin interacts with actin. Later studies have verified that α-actinin binds filamentous actin (Burridge & Feramisco 1981, Mimura & Asano 1986, McGough *et al.* 1994), but does not nucleate actin filaments (Ohtaki *et al.* 1985). The actin binding domain of α-actinin interacts with two adjacent monomers on actin filaments (McGough *et al.* 1994). At the Z-disks, α-actinin crosslinks only antiparallel actin filaments, which come to the Z-disks from opposing sarcomeres (Ishikawa *et al.* 1969). The titin Z-disk portion has two interaction sites with α-actinin: Z-repeats interact with the C-terminal EF-region (Sorimachi *et al.* 1997, Ohtsuka *et al.* 1997b, Gregorio *et al.* 1998, Young *et al.* 1998, Joseph *et al.* 2001) whereas a distinct area of titin interacts with the rod (Young *et al.* 1998). Titin interactions, especially mediated by Z-repeats with the EF-region, may account for the varying widths of the Z-disks by regulating the number of crosslinks mediated by α-actinin (Young *et al.* 1998). Furthermore, titin interaction with the EF-region is controlled by phosphatidylinositol 4,5 bisphosphate (PIP2) (Young & Gautel 2000), which binds to the CH2 of α-actinin (Fukami *et al.* 1992, Fukami *et al.* 1994, Fukami *et al.* 1996). The binding of PIP2 may release the α-actinin EF-region from an autoinhibitory state, where EF-hand is bound to a pseudo-Z-repeat region located between ABD and R1, enabling titin binding (Young & Gautel 2000). In addition to actin and titin, α-actinin also interacts with nebulin (Nave *et al.* 1990) and intermediate filament synemin (Bellin *et al.* 1999, Bellin *et al.* 2001). Hence, α-actinin can be seen as an integrator of several filamentous systems in muscle.

In addition to the filamentous and giant muscle proteins, α-actinin interacts with a growing number of smaller Z-disk components. All the three members of the palladin/myotilin/myopalladin family (reviewed in Otey *et al.* 2005) interact with α-actinin. Myotilin is associated with muscular diseases (reviewed in Otey *et al.* 2005). α-Actinin interacts with muscle LIM protein (MLP) (Louis *et al.* 1997), which has a central role in the Z-disk stretch sensor machinery (reviewed in chapter 2.4). Interestingly, a Q9R mutation in α-actinin 2 found in dilated cardiomyopathy patient disrupts the interaction with MLP (Mohapatra *et al.* 2003). Another evolving group of α-actinin interaction partners are filamin, actinin and telethonin -binding proteins of the Z-disk (FATZ-proteins), also known as myozenin and calsarcins (Faulkner *et al.* 2000, Frey *et al.* 2000, Takada *et al.* 2001, Frey & Olson 2002). Calsarcins interact with calcineurin (Frey *et al.* 2000), which is a calsium-dependent protein phosphatase implicated in hypertrophic signaling (reviewed in Vega *et al.* 2003, Wilkins & Molkentin 2004). The PDZ-LIM proteins' interactions with α-actinin are the topic of chapter 2.3.

The multifunctionality of α-actinin is even more evident when the interactions with the costameric proteins β1-integrin (Otey *et al.* 1990, Otey *et al.* 1993, Kelly & Taylor 2005), vinculin (Wachsstock *et al.* 1987, Kroemker *et al.* 1994, McGregor *et al.* 1994, Bois *et al.* 2005, Kelly *et al.* 2006) and dystrophin (Hance *et al.* 1999) are taken into account. α-Actinin interacts also with a recently found CH-domain containing protein affixin (β-parvin), which is a focal adhesion protein that localizes to costameric regions in muscle (Yamaji *et al.* 2001, Yamaji *et al.* 2004). Affixin interaction with α-actinin is regulated by integrin - integrin linked kinase signaling (Yamaji *et al.* 2004). These costameric connections show that α-actinin is involved in connecting the contractile machinery to the sarcolemma. Indeed, a recent study showed that β1-integrin – actinin interaction is significant in force transmission from the contractile apparatus to the extracellular matrix

in smooth muscle cells (Zhang & Gunst 2006). Furthermore, α-actinin interacts with several kinases (see Table 1 for references) emphasizing that the role of α-actinin function in muscle ranges from a plain structural crosslinker at the Z-disks to a docking site for various signaling cascades.

Table 1. α-Actinin interactions in muscle. PDZ-LIM protein interactions with α-actinin are not included.

Interaction partner	Main function(s) in muscle	α-Actinin binding	
		Binding site	References
Z-disk			
Filamentous actin	Contraction (reviewed in Clark *et al.* 2002)	ABD α-Actinin binds two monomers on actin filaments	(Burridge & Feramisco 1981, Mimura & Asano 1986, McGough *et al.* 1994)
Titin	Multifunctional, for instance ruler in myofibrillogenesis and responsible for elasticity (reviewed in Clark *et al.* 2002) Titin in muscle disorders (Hackman *et al.* 2003)	EF3-4 and R2-R3 EF3-4 controlled by PIP2, which releases α-actinin autoinhibition	(Sorimachi *et al.* 1997, Ohtsuka *et al.* 1997a, Ohtsuka *et al.* 1997b, Gregorio *et al.* 1998, Young *et al.* 1998, Young & Gautel 2000, Joseph *et al.* 2001, Franzot *et al.* 2005)
Nebulin/nebulette	Ruler for thin filament assembly (reviewed in McElhinny *et al.* 2003)		(Nave *et al.* 1990, Moncman & Wang 1999)
Synemin	A novel muscle intermediate filament, which forms heteropolymers with desmin and vimentin, potential connector between myofibrils and sarcolemma (Bellin *et al.* 1999, Bellin *et al.* 2001)	ABD and rod	(Bellin *et al.* 1999, Bellin *et al.* 2001)
CapZ	Caps barbed ends of actin filaments at the Z-disk, involved in regulating thin filament length (reviewed in Littlefield & Fowler 1998)	Rod (55 kDa fragment)	(Papa *et al.* 1999)

Table 2. Continued.

Interaction partner	Main function(s) in muscle	α-Actinin binding	
		Binding site	References
CRP1 (smooth muscle) CRP3/MLP (striated muscle) CRP2 (arteries and fibroblasts) (Louis *et al.* 1997)	Cysteine-rich proteins, with two LIM domains (Weiskirchen *et al.* 1995) MLP at costameres and Z-disk areas (Arber *et al.* 1997), MLP a potential Z-disk stretch sensor (Knöll *et al.* 2002), CRP1 and MLP in muscle development (Arber *et al.* 1994, Crawford *et al.* 1994)	CRP1 to N-term 27 kDa fragment	(Louis *et al.* 1997, Pomies *et al.* 1997, Harper *et al.* 2000)
Myotilin (striated muscle) Myopalladin (striated muscle) Palladin (ubiquitous)	Immunoglobulin C2 domain proteins Implicated in organizing sarcomeres and stretch signaling, myotilin mutations in muscle disorders (reviewed in Otey *et al.* 2005)	Myotilin, R3-R4 – (EF) Myopalladin, EF	(Salmikangas *et al.* 1999, Parast & Otey 2000, Bang *et al.* 2001)
FATZ/calsarcin/ Myozenin	Muscle-specific proteins (Frey *et al.* 2000) implicated in calcineurin-mediated hypertrophic signaling (reviewed in Vega *et al.* 2003, Wilkins & Molkentin 2004) Calsarcin-1 -/- mice develop cardiomyopathy (Frey *et al.* 2004)	R3-R4	(Faulkner *et al.* 2000, Frey *et al.* 2000, Takada *et al.* 2001, Frey & Olson 2002)
Arg binding protein 2 (ArgBP2)	A multidomain scaffolding protein (Rönty *et al.* 2005, and references therein)	ABD-R1	(Rönty *et al.* 2005)
Sarcolemma/ Costamere			
Dystrophin	Connects subsarcolemmal cytoskeleton to sarcolemma (Rybakova *et al.* 2000), Absent in Duchenne muscular dystrophy (DMD) patients (Hoffman *et al.* 1987).	422-894 fragment	(Hance *et al.* 1999)
β1- integrin	β1-integrin important in myoblast fusion, formation of costameres, and in sarcomeric assembly (Schwander *et al.* 2003), β1-integrin – actinin interaction important in force transmission to extracellular matrix in smooth muscle (Zhang & Gunst 2006)	Between R1-R2 (Kelly & Taylor 2005)	(Otey *et al.* 1990, Otey *et al.* 1993, Kelly & Taylor 2005)

Table 2. Continued.

Interaction partner	Main function(s) in muscle	α-Actinin binding	
		Binding site	References
Vinculin	Focal adhesion protein, in muscle enriched at costameres (Pardo *et al.* 1983)	R4 – N-terminal region of CaM	(Wachsstock *et al.* 1987, Kroemker *et al.* 1994, McGregor *et al.* 1994, Bois *et al.* 2005, Kelly *et al.* 2006)
Affixin	Focal adhesion protein with two CH domains, located in costameres in muscle (Yamaji *et al.* 2001) Implicated in integrin –mediated cell attachment (Yamaji *et al.* 2001, Yamaji *et al.* 2004)	Interacts with α-actinin 2 (residues 300-896), further characterization of α-actinin 1 suggests that both ABD and C-terminal region interact with affixin	(Yamaji *et al.* 2004)
Phospholipids and kinases			
PIP2	Regulates titin-α-actinin interaction (Young & Gautel 2000), a potential regulator of actin crosslinking by α-actinin (chapter 2.2.2.1)	CH2	(Fukami *et al.* 1992, Fukami *et al.* 1994, Fukami *et al.* 1996, Fraley *et al.* 2003)
PIP3	Disrupts interaction between α-actinin and β1-integrin (Greenwood *et al.* 2000)	CH2	(Greenwood *et al.* 2000, Fraley *et al.* 2003)
PI3-kinase	Involved in PIP-synthesis, implicated in hypertrophic growth (reviewed in Vlahos *et al.* 2003)	PIP2 enhances interaction	(Shibasaki *et al.* 1994)
Mitogen- activated protein kinase kinase 1 (MEKK1), a serine-threonine kinase	Involved in response to outside stress signals and in apoptosis programming (for references, see Christerson *et al.* 1999) function in muscle unclear	A fragment without ABD in yeast-two-hybrid screen	(Christerson *et al.* 1999)
Protein kinase N (PKN), a serine-threonine kinase	PKN a downstream target of Rho, which involved e.g. in cell motility and stress fiber formation, Rho regulates PIP2 levels as well (for references, see Mukai *et al.* 1997), function in muscle unclear	R3 and EF-region PKN interaction with α-actinin is PIP2-dependent	(Mukai *et al.* 1997)

Table 2. Continued.

Interaction partner	Main function(s) in muscle	α-Actinin binding	
		Binding site	References
Other			
Phospholipase D	Involved in cardiac function, such as contraction and Ca^{2+}-handling (for references, see Park *et al.* 2000)		(Park *et al.* 2000)
Smitin	A titin-like protein found in smooth muscle (for references, see Chi *et al.* 2005)	R2-R3 and EF-hands, similar to titin	(Chi *et al.* 2005)
Fesselin	Actin-binding smooth muscle protein, regulates myosin ATPase (for references, see Pham & Chalovich 2006)	Rod	(Pham & Chalovich 2006)
α-catenin	Catenins mediate connection of cadherins to actin cytoskeleton in adherens junctions of epithelial cells and in intercalated discs of cardiomyocytes. (Knudsen *et al.* 1995, Nieset *et al.* 1997, for references)	Region between R2 and R3 (residues 479-529), nonmuscle α-actinin	(Knudsen *et al.* 1995, Nieset *et al.* 1997)

2.2.2.1 Regulation of actin binding in muscle

The binding of Ca^{2+}-ions to the EF-region cannot contribute to the regulation of the adjacent actin binding domain of muscle α-actinin isoforms because the EF-hands are dysfunctional (Beggs *et al.* 1992). Phospholipids could offer an alternative way to regulate actin binding to muscle α-actinins. PIP2 binds α-actinin in the second CH domain (Fukami *et al.* 1996, Fraley *et al.* 2003). The phosphatidylinositol (3,4,5)-trisphosphate (PIP3) binding site is also located in the CH2 domain of α-actinin in the same region as the PIP2 site (Fraley *et al.* 2003). Fukami and colleagues (Fukami *et al.* 1992) showed that PIP2 increases the gelation activity of α-actinin. Moreover, α-actinins associated with the cytoskeleton are bound to PIP2, whereas the cytosolic fraction is not (Fukami *et al.* 1994). However, other studies have provided evidence for an opposite effect – both PIP2 and PIP3 actually inhibit the bundling activity of α-actinin (Fraley *et al.* 2003, Corgan *et al.* 2004). It has been shown recently that also epidermal growth factor receptor (EGFR) signaling is involved in the the regulation of the actin bundling by α-actinin (Wahlström *et al.* 2006). α-Actinin also bundles actin filaments together with other proteins, such as myotilin (Salmikangas *et al.* 2003).

At the Z-disks α-actinin crosslinks only antiparallel actin filaments (Ishikawa *et al.* 1969). This is essential for the integrity of sarcomeres. In smooth muscle as well, the actin filaments connected by α-actinin at the dense bodies have opposite polarities (Bond

& Somlyo 1982). However, it has been proposed, based on studying the ability of smooth and striated muscle α-actinins to bundle actin filaments on lipid layers, that they can crosslink filaments in any orientation (Taylor *et al.* 2000). How is the orientation of the crosslinked actin filaments defined? The neck regions between ABD and the rod domain are the most flexible parts of α-actinin (Winkler *et al.* 1997). ABDs of a single α-actinin molecule can rotate 180 to 0 degrees relative to each other (Liu *et al.* 2004), when the 90 degree twist along the rod axis (Ylänne *et al.* 2001) is taken into account. One could expect that the neck region has to be stabilized to restrict the crosslinks only to antiparallel ones. It is shown that PIP2 decreases degradation of α-actinin upon protease treatment affecting the hinge region, in contrast to PIP3 (Corgan *et al.* 2004). Thus, PIP2 may stabilize the neck region. Interestingly, PIP2 binding may regulate both titin and actin interactions with α-actinin. PIP2 may release the EF-hands from an autoinhibitory state allowing titin interaction to the EF-region (Young & Gautel 2000) and simultaneously PIP2 could regulate α-actinin binding to actin. So far there is no direct evidence available of the interaction between muscle EF- and ABD-domains. However, in the 3D reconstruction of the whole skeletal α-actinin molecule (Tang *et al.* 2001) the EF-region is in close proximity to the adjacent ABD. Thus, titin, which has interaction sites on both the EF- and rod regions (see Table I for references) may also contribute to the regulation of the neck region flexibility. At which stage is the orientation of actin filaments restricted? I-Z-I bodies, which arise as independent assemblies in myofibrillogenesis at very early stages (reviewed in chapter 2.1.3), already resemble the Z-disks. This raises the possibility that nebulin, the ruler of thin filament assembly (reviewed in McElhinny *et al*. 2003), which is shown to bind α-actinin (Nave *et al.* 1990) may control the orientations of crosslinks in the maturing I-Z-I bodies. The four and half LIM domain protein 3 (FHL3) is a Z-disk actin-binding protein, which apparently does not interact directly with α-actinin, but inhibits the actin bundling by α-actinin (Coghill *et al.* 2003) indicating that it may be involved in regulation of actin crosslinking at the Z-disk. The overall complexity of the Z-disk suggests that the regulation of actin crosslinking is the sum of several events occurring in the course of myofibrillogenesis.

2.2.3 Features of nonmuscle α-actinins

The binding partners of nonmuscle α-actinins have been recently reviewed by Otey and Carpen (Otey & Carpen 2004). The objective of this chapter is to give a short overview of the functional properties of nonmuscle α-actinins. Several studies have suggested that α-actinin 4 is involved in kidney disease and tumorigenesis. α-Actinin 4 deficiency in mice leads to a glomerular kidney disease characterized with proteinuria and a change in morphology of podocytes (Kos *et al.* 2003). Focal and segmental glomerulosclerosis (FSGS) is a kidney disease found in humans. FSGS associates with point mutations (K228E, T232I and S235P) in the ABD of α-actinin 4. Interestingly, these mutations lead to an increase in actin binding affinity. α-Actinin 4 is expressed in human kidney, whereas the other nonmuscle actinin isoform, α-actinin 1, is not. (Kaplan *et al.* 2000.) However, in mice both isoforms are detected in kidney (Kos *et al.* 2003). In the K228E α-

actinin 4 mutant, α- actinin 4 has a tendency to aggregate (Yao *et al.* 2004) indicating that increased actin binding in high-speed pelleting assays (Kaplan *et al.* 2000) may partially be a secondary effect, and may not necessarily reflect an increased binding to actin. The kidney disease caused by a mutated α-actinin 4 is probably due to both loss of function of α-actinin 4 and accumulation of eventually toxic aggregates of the mutant protein (Yao *et al.* 2004).

α-Actinin 4 has been implicated as a tumor suppressor in neuroblastoma cells. The high expression of α-actinin 4 reduces colony formation of tumor cells (Nikolopoulos *et al.* 2000, Menez *et al.* 2004) indicating that α-actinin 4 may affect the metastatic potential of tumor cells. Interestingly, the inhibition of phosphatidylinositol 3 (PI3) -kinase leads to a translocation of α-actinin 4 to nuclei. The cytosolic localization of α-actinin 4 may indicate a higher metastatic potential of cancer cells, in contrast to the nuclear localization. (Honda *et al.* 1998.) Also α-Actinin 1 may act as a tumor suppressor affecting cell motility of fibroblasts (Gluck *et al.* 1993, Gluck & Ben-Ze'ev 1994). In addition in kidney disease and tumorigenesis, α-actinin 4 may have a role in endocytosis (Araki *et al.* 2000).

2.3 PDZ-LIM proteins

The mammalian PDZ-LIM protein family has seven members (Fig. 4). As a common feature, all members of this family have conserved PDZ and LIM domains. The actinin-associated LIM protein (ALP) (Xia *et al.* 1997), C-terminal LIM domain protein with a molecular weight of 36 kDa (CLP36, also known as CLIM and Elfin) (Wang *et al.* 1995, Kotaka *et al.* 1999, Bauer *et al.* 2000, Kotaka *et al.* 2001), reversion-induced LIM protein (RIL) (Kiess *et al.* 1995, Bashirova *et al.* 1998), Mystique, also known as PDZ and LIM domain protein 2 (Pdlim2) (Torrado *et al.* 2004, Loughran *et al.* 2005) have one N-terminal PDZ domain and one C-terminal LIM. The Z-band alternatively spliced PDZ-containing protein (ZASP/Cypher/Oracle) (Faulkner *et al.* 1999, Zhou *et al.* 1999, Passier *et al.* 2000), Enigma (Wu & Gill 1994) and Enigma homology protein (ENH) (Kuroda *et al.* 1996) have one N-terminal PDZ domain and three C-terminal LIM domains. The name ZASP/Cypher will be used in this thesis. The members of the PDZ-LIM protein family are also known as the ALP/Enigma family, ALP defining the subclass with a single LIM domain, and Enigma the subclass with three LIM domains (McKeown *et al.* 2005).

Of the seven PDZ-LIM family members, ALP, ZASP/Cypher and CLP36 have a conserved ZASP/Cypher-like motif (ZM) (SMART prediction (Schultz *et al.* 1998) accession number SM 00735, IPR006643, Fig. 4) in the internal region. This motif is composed of 26-27 conserved residues. In *Caenorhabditis elegans* a single gene encodes all members of the ALP/Enigma family (McKeown *et al.* 2005) containing also the ZM-motif. Neither the structural or functional characteristics of the internal region are known. In contrast, the PDZ and LIM domains are conserved folds found in numerous other proteins mediating various functions. The sequence identity within the family in the conserved domains is high, being higher within subfamilies. For instance, the PDZ domain of human ALP (af039018) has a 62% identity with human CLP36 PDZ

(nm_020992), whereas the PDZ domain identity between human ALP (af039018) and ZASP/Cypher (aj133767) PDZ is 54%. The identity in the LIM domains is also high, 66% between ALP and CLP36. In contrast, the identity in the internal region is only 30% between human ALP and CLP36.

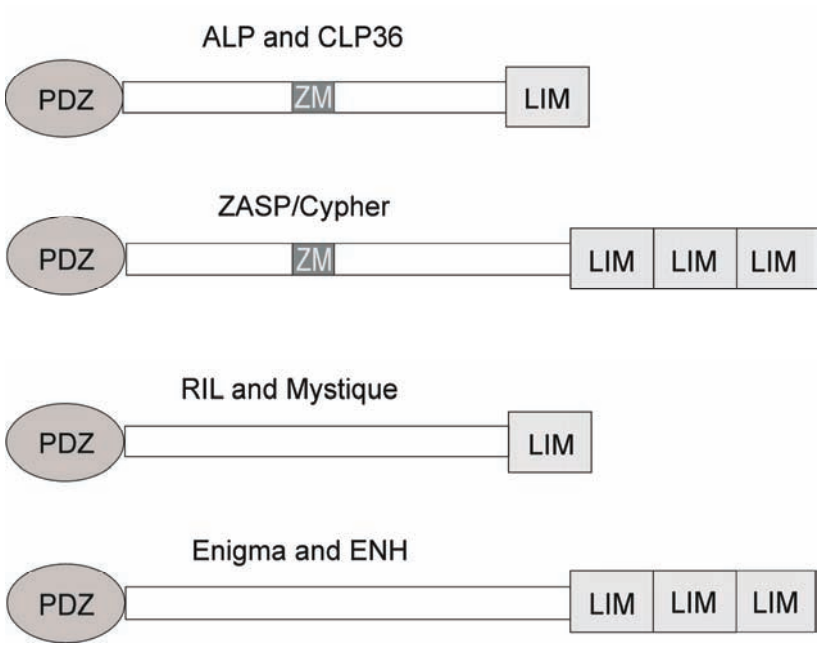

Fig. 4. Domain composition of PDZ-LIM proteins. All members have one PDZ and one to three LIM domains, but only ALP, CLP36 and ZASP/Cypher have a ZM-motif in the internal region.

2.3.1 The PDZ domain

The acronym PDZ comes from the first three proteins (Kennedy 1995), in which this domain was identified. These are the postsynaptic-density protein (PSD-95) (Cho *et al.* 1992), discs large protein (Dlg) (Woods & Bryant 1991) and zona occludens protein (ZO-1) (Itoh *et al.* 1993). PDZ domains are found in a large variety of organisms, but primarily in metazoans. The PDZ domain proteins can be classified into three groups based on other domains present in the same proteins. The first group consists of proteins that contain only PDZ domains. Membrane-associated guanylate kinase (MAGUK) -proteins form the second group. MAGUK proteins have one or more PDZ domains with Src-homology 3 (SH3) and guanylate-kinase-like (GuK) domains, also some additional domains may exist. The third group is composed of PDZ domain proteins with other than GuK or SH3 domains. PDZ-LIM proteins belong to the third group. (Nourry *et al.* 2003.) A similar classification has been reviewed previously by Harris and Lim (Harris & Lim 2001).

2.3.1.1 Structure and function

The basic PDZ fold is 80-90 residues. Typically, the PDZ domains bind to a C-terminal peptide, but PDZ domains can also bind internal motifs, other PDZ domains and lipids. The C-terminal recognition by the PDZ domain can be divided into 3 classes based on the target sequence. (reviewed in Nourry *et al.* 2003.) The basic PDZ domain is a globular fold composed of six β-sheets and two α-helices, which form a hydrophobic pocket for the C-terminal peptide (Doyle *et al.* 1996, Daniels *et al.* 1998, Tochio *et al.* 1999). The PDZ domain structures of ZASP/Cypher (Au *et al.* 2004) and ALP (1V5L in the Protein data bank (PDB)) resemble closely the basic PDZ fold. The fold and the interaction mode of PDZ domains resemble those of phosphotyrosine-binding (PTB) and pleckstrin homology (PH) domains, although the sequence similarity is low (Nourry *et al.* 2003). PDZ domains of separate proteins can form heterodimers in a head-to-tail manner (Brenman *et al.* 1996, Tochio *et al.* 2000). The requirement for dimerization is a β-finger structure, which can dock to the PDZ binding groove mimicking the C-terminal peptide (Hillier *et al.* 1999, Tochio *et al.* 2000). The PDZ domain can interact with a peptide without a free carboxy-terminus (Chevesich *et al.* 1997), if the internal peptide conformation mimics a C-terminal peptide, which can fit into the hydrophobic pocket of the PDZ domain (Gee *et al.* 1998). As an example of lipid interactions, the PDZ domains of several proteins, such as syntenin, can bind PIP2 (Zimmermann *et al.* 2002).

The rare existence of canonical PDZ-domains in bacteria and yeast indicates that this domain has evolved in response to multicellularity. PDZ domains are often found in multidomain protein complexes mediating protein-protein interactions, for instance clustering receptors at neuronal junctions. They are involved in directing signalling molecules to defined cellular compartments. (for reviews, see Harris & Lim 2001, Nourry *et al.* 2003, Kim & Sheng 2004.) In general, the specificity of the PDZ-C-terminal ligand recognition is high (Nourry *et al.* 2003). In some cases the interaction can be inhibited by phosphorylation of the target peptide motif (for references, see Kim & Sheng 2004). PIP2 also competes for binding to the peptide (Zimmermann *et al.* 2002). Oligomerization of PDZ domains may stabilize the adjacent domain enabling the interaction (Feng *et al.* 2003). Membrane-associated guanylate kinase inverted 1 -protein (MAGI-1), which is a MAGUK protein, may have a similar mechanism. The PDZ5 interaction with the C-terminus of α-actinin 4 is enhanced by the PDZ4 domain, whereas the PDZ4 alone does not interact with α-actinin (Patrie *et al.* 2002).

2.3.2 The LIM domain

The acronym LIM comes from the first three proteins in which this domain was found. These are the *Caenorhabditis elegans* cell-lineage protein (LIN-11) (Freyd *et al.* 1990), rat insulin gene-enhancer binding protein (Isl1) (Karlsson *et al.* 1990) and a protein required in specification of mechanosensory neurons in *C. elegans* (MEC-3) (Way & Chalfie 1988). LIM domains are found in eukaryotes, but they are absent in prokaryotes (Kadrmas & Beckerle 2004). Most recently human LIM proteins have been classified into four broad categories based on sequence similarity within the LIM domain and the

presence of other domains in the same polypeptide (Kadrmas & Beckerle 2004): 1) Nuclear LIM proteins, some of them containing a homeodomain, 2) proteins that are composed solely of LIM domains, 3) LIM proteins associated with the actin cytoskeleton, which have other domains, for instance PDZ or SH3 and 4) LIM proteins containing catalytic domains, such as kinases. PDZ-LIM proteins belong to the third group in this classification. Interestingly, the divergence in the three LIM domain subclasses of the PDZ-LIM family is reasonably high considering that in other similar proteins the LIM domains are quite similar. A rough classification separates LIM proteins into nuclear and cytoplasmic proteins (reviewed in Dawid *et al.* 1998).

2.3.2.1 Structure and function

LIM domains are zinc-finger motifs composed of 55 residues. They have 8 conserved residues, mostly cysteine or histidine, at defined positions, which are responsible for the zinc-ion coordination. Conserved residues 1-4 bind one zinc-ion, and residues 5-8 the other one. LIM domains recognize a vast number of target motifs, and they can also form homo- and heterodimers. (reviewed in Dawid *et al.* 1998, Kadrmas & Beckerle 2004.) Structural studies have shown that a single LIM domain consists of two zinc-fingers, each composed of two antiparallel β-hairpins (Perez-Alvarado *et al.* 1994, Perez-Alvarado *et al.* 1996, Konrat *et al.* 1997). It is not clear whether the zinc-fingers are directly involved in interactions. Structural studies have demonstrated that the interaction surfaces depend on the specific binding partners. Moreover, a single LIM domain can bind several targets or more than one LIM is required to bind one target. Originally structural similarities to GATA-type zinc-fingers, which can bind both protein and DNA raised the question of whether the zinc-fingers of LIM domains could also bind DNA. Direct interactions of LIM domains with DNA have not been proven yet, but several studies have argued that it might be possible. (reviewed in Kadrmas & Beckerle 2004.)
LIM domain proteins have multiple functions, which can be separated into four categories (Kadrmas & Beckerle 2004): 1) Multiple LIM domains can act as adaptors or a scaffold bringing multiprotein complexes together, 2) LIM domains may act as inhibitors competing for protein-protein interactions, 3) LIM domains can be involved in intramolecular autoinhibition, and 4) LIM domains can target signaling proteins to their proper cellular location. LIM proteins may also have various combinations of these four functions.

LIM domain proteins are found in various cell compartments, and their location may change, for instance CRPs (Weiskirchen *et al.* 1995) can shuttle between the nucleus and actin cytoskeleton (Arber & Caroni 1996, Jain *et al.* 1996, Chang *et al.* 2003), zyxin shuttles from nuclei to focal adhesions upon stretching (Cattaruzza *et al.* 2004) and FHL-proteins shuttle between the cytoplasm and nucleus (Muller *et al.* 2002). PDZ-LIM proteins do not fall precisely to any of the four categories, although they have similarities. PDZ-LIM proteins act as localizers, for instance, but the localization to the cytoskeleton in most cases is mediated by the PDZ domain, whereas the LIM domains interact with protein kinase C (PKC) isoforms.

2.3.3 Proteins containing the ZM-Motif: ALP, CLP36, ZASP/Cypher

ALP, CLP36 and ZASP/Cypher are the three ZM-motif-containing PDZ-LIM proteins. Two ALP variants have been found both in chicken and rat. A 36 kDa size protein is enriched in cardiac and smooth muscle, whereas a 40 kDa size variant is found in skeletal muscle. (Xia *et al.* 1997, Pomies *et al.* 1999.) These correspond to the splice variants of the internal ZM-motif containing exons 4 and 6 of human ALP (paper I). Similar splicing may occur also in Atlantic salmon (Andersen *et al.* 2004). Two ALP genes have been found in zebrafish with one or two ZM-motif-encoding exons (te Velthuis *et al.* 2006). The single ALP/Enigma gene in *C. elegans* encodes one ALP (ALP-1A) and three Enigma isoforms (ALP-1B, ALP-1C, ALP-1D) (McKeown *et al.* 2005).

ZASP/Cypher (Faulkner *et al.* 1999, Zhou *et al.* 1999, Passier *et al.* 2000) is the family member with one PDZ and three LIM domains. Faulkner and colleagues (Faulkner *et al.* 1999) found four splice variants in human, later the number increased to six (Vatta *et al.* 2003) including three similar transcripts found previously (Faulkner *et al.* 1999). Thus, it appears that in human, there are seven splice variants of ZASP/Cypher. The ZASP/Cypher variant 1 lacks the terminal LIM domains, due to the stop codon in exon 9 (Vatta *et al.* 2003). As in ALP, the ZM-motif is alternatively spliced from exon 4 or from exon 6 (paper II). Also a transcript containing both exons 4 and 6 (Vatta *et al.* 2003) and a transcript (Faulkner *et al.* 1999) lacking the ZM-motif exons have been reported. In mice, there are six variants altogether, three with ZM-exon 6 and three with ZM-exon 4 (Zhou *et al.* 1999, Passier *et al.* 2000, Huang *et al.* 2003). Thirteen splice variants have been found in zebrafish (van der Meer *et al.* 2006).

No expression of CLP36 (Wang *et al.* 1995, Kotaka *et al.* 1999, Bauer *et al.* 2000, Kotaka *et al.* 2000, Kotaka *et al.* 2001) splice variants have been characterized to date.

2.3.3.1 Expression and cellular localization

ALP is expressed in striated muscle and in tissues enriched in smooth muscle, such as arteries. ALP is absent in undifferentiated mouse C2C12 myoblasts, but it is highly induced after fusion of myoblasts to myotubes. (Xia *et al.* 1997, Pomies *et al.* 1999.) In cultured chicken cardiomyocytes ALP co-localizes with α-actinin already at the Z-bodies during differentiation (Henderson et al. 2003). In mature Z-disks ALP co-localizes with α-actinin (Xia *et al.* 1997, Pomies *et al.* 1999, Henderson *et al.* 2003) and with α-actinin, vinculin, desmin and catenin at intercalated discs in mouse heart (Pashmforoush *et al.* 2001, Henderson *et al.* 2003). ALP and α-actinin also co-localize along stress fibers in a spotted pattern and in lamellipodia in chicken embryonic fibroblasts. An overlapping localization is also observed with vinculin in the ends of stress fibers terminating in focal adhesions. (Pomies *et al.* 1999.) In *C. elegans*, ALP-1 proteins localize to dense bodies, dense plaques, cell-cell junctions and are detected also in nuclei (McKeown *et al.* 2005).

The strongest CLP36 expression is detected in muscle, especially in developing heart (Kotaka *et al.* 1999, Kotaka *et al.* 2001, Vallenius *et al.* 2004), where CLP36 localizes at the Z-disks with α-actinin and at the intercalated discs with vinculin (Kotaka *et al.* 2000). However, CLP36 is also expressed in several nonmuscle tissues, such as lung, liver,

placenta and testis (Wang *et al.* 1995, Kotaka *et al.* 1999, Kotaka *et al.* 2001, Vallenius & Mäkelä 2002). In mouse, CLP36 is expressed at high levels in skin and intestinal epithelial cells (Vallenius *et al.* 2000). CLP36 is also detected in endometrial decidual cells during pregnancy (Miehe *et al.* 2005). In cultured nonmuscle cells and C2C12 myoblasts, CLP36 localizes on stress fibers (Bauer *et al.* 2000, Vallenius *et al.* 2000, Kotaka *et al.* 2001), but not to focal contacts (Bauer *et al.* 2000).

ZASP/Cypher is primarily expressed in cardiac and skeletal muscle (Faulkner *et al.* 1999, Zhou *et al.* 1999, Passier *et al.* 2000). During embryogenesis, ZASP/Cypher is first expressed in the developing heart and gradually the expression expands to other striated muscles (Zhou *et al.* 1999). In mice, ZM-exon 6 containing variants are skeletal muscle specific, whereas ZM-exon 4 variants are restricted to cardiac muscle (Huang *et al.* 2003). A similar expression pattern has been detected in zebrafish, except that the ZM-exon 6 variant was found both in skeletal and cardiac muscle (van der Meer *et al.* 2006). A similar specificity has not been reported in humans. In muscle, ZASP/Cypher is targeted to the Z-disks (Faulkner *et al.* 1999) where it co-localizes with α-actinin (Zhou *et al.* 1999, Zhou *et al.* 2001). Co-localization of ZASP/Cypher and α-actinin has been detected in the Z-bodies as well (Wang *et al.* 2005).

2.3.4 Proteins lacking the ZM-motif: RIL, Mystique, Enigma and ENH

Two splice variants of RIL exist in brain. The short form lacks the LIM domain (Bashirova *et al.* 1998.). Three variants of Mystique have been found, of which Mystique 3 does not have a LIM domain (Loughran *et al.* 2005). No expression of Enigma (Wu & Gill 1994) splice variants have been characterized to date. Altogether, four splice variants of ENH exist (ENH1-4). Three of them are short forms (ENH2-4), where the compositions of the internal exons vary, and the LIM domains are excluded. The short forms have a region with homology to glycosyl hydrolase. (Nakagawa *et al.* 2000, Niederlander *et al.* 2004.)

2.3.4.1 Expression and cellular localization

RIL is expressed nonmuscle tissues, such as brain and lung and in a variety of cultured cells lines (Kiess *et al.* 1995, Bashirova *et al.* 1998, Schulz *et al.* 2004, Vallenius *et al.* 2004). RIL localizes in dendritic spines in cultured neurons (Schulz *et al.* 2004) and along stress fibers (Vallenius *et al.* 2004). Mystique is expressed in nonmuscle cells, such as epithelial cells and lung (Torrado *et al.* 2004, Loughran *et al.* 2005). Mystique co-localizes with α-actinin on stress fibers (Torrado *et al.* 2004) and also with integrin and α-actinin at focal contacts (Loughran *et al.* 2005).

Enigma is expressed in brain and skeletal muscle, where it localizes mostly in the Z-disk region (Guy *et al.* 1999). In cultured cells, Enigma is located in the cytoplasm and membrane ruffles rich in actin filaments (Borrello *et al.* 2002, Barres *et al.* 2005). ENH is expressed in skeletal and cardiac muscle (Kuroda *et al.* 1996). The distribution pattern of different isoforms is specific. The short ENH4 is expressed only in skeletal muscle,

ENH3 both in skeletal and cardiac, ENH2 in skeletal and ENH1 in cardiac muscle. (Niederlander *et al.* 2004.) ENH co-localizes with α-actinin at the Z-disks (Nakagawa *et al.* 2000, Niederlander *et al.* 2004). ENH is expressed also in various cancer cell lines (Nakagawa *et al.* 2000).

2.3.5 PDZ-LIM proteins as linkers

Due to their domain composition, PDZ-LIM proteins are regarded as linkers between cytoskeletal elements and signaling cascades. There is an increasing body of evidence that the PDZ-LIM proteins indeed have such a pivotal role. The cytoskeletal connection is probably required to target the signaling components, such as kinases, to their proper cellular location. The interaction partners of PDZ-LIM proteins are summarized in Table 2.

2.3.5.1 Cytoskeletal connections

Of the seven PDZ-LIM family members, six interact directly with α-actinin. The location of this interaction is indicated in Table II. Xia and colleagues (Xia *et al.* 1997) used a yeast-two-hybrid screen to show that the PDZ domain interacts with the rod domain of α-actinin. To date, other interaction partners of ALP have not been reported. CLP36 also interacts with α-actinin (Bauer *et al.* 2000, Kotaka *et al.* 2000, Vallenius *et al.* 2000), but the exact location of the interaction site is uncertain. In a yeast-two-hybrid screen, a fragment lacking the PDZ domain of CLP36 interacted with the EF-region of α-actinin 2 (Kotaka *et al.* 2000). Using the same method Bauer and colleagues (Bauer *et al.* 2000) reported that the internal region between PDZ and LIM domains binds to the spectrin repeats 2 and 3. In immunoprecipitation analysis, the full CLP36 interacted with α-actinin, but this interaction was abolished when the 24 N-terminal residues of CLP36 were removed (Vallenius *et al.* 2000). α-Actinin spectrin repeats were sufficient to bind full-length CLP36. Vallenius and colleagues (Vallenius *et al.* 2000) concluded that the PDZ domain interacts with the spectrin repeats of α-actinin. The third ZM-motif containing the PDZ-LIM protein ZASP/Cypher has been shown to interact with the C-terminal region of α-actinin via the PDZ domain (Faulkner *et al.* 1999, Zhou *et al.* 2001).

The RIL PDZ binds to the C-terminus of α-actinin (Schulz *et al.* 2004.) However, Vallenius and co-workers (Vallenius *et al.* 2004) reported interactions of RIL with the α-actinin rod domain, but with a lower affinity when compared to CLP36. RIL was shown to regulate stress fiber dynamics (Vallenius *et al.* 2004). In addition to α-actinin, Mystique appears to bind several other cytoskeletal components, for example filamin (Torrado *et al.* 2004). Mystique is important in cell adhesion and motility of epithelial cells (Loughran *et al.* 2005). The PDZ domain of ENH interacts with α-actinin and actin (Nakagawa *et al.* 2000, Niederlander *et al.* 2004). Enigma was shown to interact with skeletal β-tropomyosin via the PDZ domain (Guy *et al.* 1999), but not with α-actinin, although Enigma localized to the Z-lines. Enigma is also involved in regulation of the actin cytoskeleton (Barres *et al.* 2005). It has been suggested based on homology modelling with ZASP/Cypher PDZ domain structure that all PDZ-LIM proteins would

have a similar PDZ fold able to bind the C-terminus of α-actinin (Au *et al.* 2004). ALP PDZ (1V5L in PDB) has a structure similar to that of ZASP/Cypher PDZ.

2.3.5.2 Signaling connections

All the members with three LIM domains interact with PKC isoforms via the LIM domains, with varying specificities. The LIM domains of ZASP/Cypher interact with PKC isoforms α, β1, ζ, γ, σ and ε (Zhou *et al.* 1999), whereas the LIM domains of ENH interact with PKC βI, γ and ε, but not with α, σ and ζ (Kuroda *et al.* 1996). Enigma interacts via the LIM domains with PKC isoforms α, β1 and ζ, but not with γ, σ or ε (Kuroda *et al.* 1996). Both ZASP/Cypher and ENH are phosphorylated by PKC βI (Kuroda *et al.* 1996, Zhou *et al.* 1999). PKCs are serine-threonine kinases, which are involved in hypertrophic growth in heart (for reviews, see Simpson 1999, Vlahos *et al.* 2003.) ZASP/Cypher has also a linkage to calcineurin-mediated hypertrophic signaling (for reviews, see Vega *et al.* 2003, Wilkins & Molkentin 2004) via its interaction to calsarcins (Frey & Olson 2002), which bind calcineurin (Frey *et al.* 2000). CLP36 interacts with CLP36 interacting (Clik1) kinase, whereas ALP does not (Vallenius & Mäkelä 2002). CLP36 interaction leads to translocation of Clik1 from the nucleus to stress fibers. The function of Clik1 is not known.

PDZ-LIM proteins have been shown to interact with several receptors. As examples, the second LIM domain of Enigma interacts with Ret/ptc2 (Durick *et al.* 1996, Wu *et al.* 1996). C-Ret is a proto-oncogene encoding a receptor tyrosine kinase. Ret/ptc2 is a fusion protein as a result of chromosomal translocation. Ret/ptc2 is composed of N-terminal two-thirds of type Iα regulatory subunit of cyclic AMP-dependent protein kinase (RI) and the entire tyrosine kinase domain of c-Ret. This fusion protein is found in papillary thyroid carcinoma. (for references, see Durick *et al.* 1996.) Interaction with Enigma is required for the mitogenic activity of Ret/ptc2 (Durick *et al.* 1996). A further study indicated that a subcellular location by Enigma is one of the requirements for Ret/ptc2 mitogenic signaling (Durick *et al.* 1998). Enigma interacts also with the wild-type Ret receptor and Ret oncoproteins carrying mutations associated with multiple endocrine neoplasia type 2 (MEN2) cancer syndromes (Borrello *et al.* 2002).

RIL has been implicated in receptor clustering in neurons, which is a common function of PDZ domains. In cultured neurons, overexpression of RIL enhances accumulation of α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors at dendritic spines by connecting them to the actin cytoskeleton (Schulz *et al.* 2004). The interaction of RIL with the AMPA receptor is mediated by the LIM domain (Schulz *et al.* 2004). Phosphorylation may regulate RIL function; the LIM domain of RIL is phosphorylated *in vivo,* and RIL can be dephosphorylated by protein tyrosine phosphatase (PTP-BL), which binds the LIM domain of RIL. (Cuppen *et al.* 1998.) An internal binding of RIL PDZ to the RIL LIM domain has been detected (Cuppen *et al.* 1998, Cuppen *et al.* 2000) indicating that RIL may oligomerize.

Table 2. Interactions of PDZ-LIM proteins.

PDZ-LIM protein	Interaction partner	References
ZM-motif		
ALP	α-Actinin,	(Xia *et al.* 1997, Pomies *et al.*
	PDZ to rod (Xia *et al.* 1997)	1999)
CLP36	α-Actinin,	(Bauer *et al.* 2000, Kotaka *et*
	Internal to rod (Bauer *et al.* 2000)	*al.* 2000, Vallenius *et al.* 2000)
	PDZ to rod (Vallenius *et al.* 2000)	
	129-256 (a part of the internal region + LIM) to	
	EF-region (Kotaka *et al.* 2000)	
	Clik1 (CLP36 interacting kinase)	(Vallenius & Mäkelä 2002)
ZASP/Cypher	α-Actinin, PDZ to the C-terminal region (Faulkner	(Faulkner *et al.* 1999, Zhou *et*
	et al. 1999, Zhou *et al.* 2001)	*al.* 1999, Zhou *et al.* 2001)
	PKCα, β1, ζ, γ, σ and ε	(Zhou *et al.* 1999)
	Calsarcins/FATZ/myozenin	(Frey & Olson 2002)
No ZM-motif		
RIL	α-Actinin, PDZ to C-terminus (Schulz *et al.* 2004),	(Schulz *et al.* 2004, Vallenius
	full RIL to rod (Vallenius *et al.* 2004)	*et al.* 2004)
	AMPA glutamate receptor subunit	(Schulz *et al.* 2004)
	Protein tyrosine phosphatase (PTP-BL)	(Cuppen *et al.* 1998, van den
		Berk *et al.* 2004)
	Forms heterodimers with ALP	(van den Berk *et al.* 2004)
	Internal binding of RIL PDZ to RIL LIM domain	(Cuppen *et al.* 1998, Cuppen *et*
		al. 2000)
	Thyroid receptor interacting protein 6 (TRIP6)	(Cuppen *et al.* 2000)
Mystique	α-Actinin, probably via PDZ (Loughran *et al.*	(Torrado *et al.* 2004, Loughran
	2005), filamin A, myosin VI and myosin heavy	*et al.* 2005)
	polypeptide 9	
Enigma	β-tropomyosin	(Guy *et al.* 1999)
	insulin receptor (InsR)	(Wu & Gill 1994, Wu *et al.*
		1996)
	Ret/ptc2	(Durick *et al.* 1996, Wu *et al.*
		1996)
	Ret oncoproteins	(Borrello *et al.* 2002)
	Adaptor protein with PH and SH2 domains (APS)	(Barres *et al.* 2005)
	PKCα, β1 and ζ	(Kuroda *et al.* 1996)
ENH	α-Actinin via PDZ (Nakagawa *et al.* 2000)	(Nakagawa *et al.* 2000,
	actin	Niederlander *et al.* 2004)
	PKCβI, γ and ε	(Kuroda *et al.* 1996)

2.4 Muscle disorders and the role of the Z-disk as a stretch sensor

A myopathy is a general term for disorders where muscle tissue is affected. Myopathies are neuromuscular disorders characterized by muscle weakness caused by dysfunction of muscle fibers. The age of onset varies from infancy to late adulthood, and the severity ranges from minor disabilities to fatality. There is a wide spectrum of both acquired and inherited myopathies. The muscular dystrophies are a group of inherited diseases characterized by progressive weakness and degeneration of the skeletal muscles. (for recent updates, http:/www.ninds.nih.gov/disorders/myopathy/myopathy.htm and http://www.ninds.nih.gov/disorders/md/md.htm.) The objective of this chapter is to give a short overview of defects in sarcomeric proteins that lead to muscle disorders and discuss about the role of the Z-disk as an intrinsic stretch sensor machinery of cardiomyocytes.

Defects in the filamentous systems of striated muscle can cause muscular disorders. The giant titin molecule carries mutations in several muscle diseases, such as limb girdle muscular dystrophy type 2J (LGMD2J) and human skeletal tibial muscular dystrophy (TMD) (reviewed in Hackman *et al.* 2003, Guglieri *et al.* 2005). Mutations in nebulin, skeletal actin isoform and thin filament associated proteins tropomyosin and troponin associate with nemaline myopathies, which are characterized with the presence of nemaline bodies of Z-disk origin (Agrawal *et al.* 2004, and references therein). Also the Z-disk components may cause muscular diseases. Myotilin, one of the α-actinin-binding proteins, is mutated in limb-girdle muscular dystrophy 1A (LGMD1A) and in myofibrillar myopathy (MFM) (reviewed in Otey *et al.* 2005). Other forms of MFM are associated with mutations in desmin, αB-crystallin (Selcen & Engel 2005, for references) and ZASP/Cypher (see below). Both LGMD1A and MFM are characterized with the streaming of the Z-disks (Otey *et al.* 2005). An additional example of Z-disk component in muscle disease is telethonin (titin capping protein, T-cap), which is associated with LGMD2G (reviewed in Guglieri *et al.* 2005). Titin and telethonin are involved in the stretch sensor machinery of cardiac muscle, as discussed below.

Cardiomyopathies are often associated with defects in sarcomeric proteins (for reviews, see McNally *et al.* 2003, Chang & Potter 2005, Morita *et al.* 2005). Cardiac diseases are characterized by changes in the cardiac wall thickness and the volumes of the heart chambers. In hypertrophic cardiomyopathy the cardiac walls thicken due to an increase in myofiber size. (Vlahos *et al.* 2003.) Dilated cardiomyopathy is characterized by enlarged chambers and thinned walls. The degeneration of myofibers leads to impaired contractile function. (Schönberger & Seidman 2001.) As examples of sarcomeric defects, an actin mutation likely to disrupt the connection to the Z-disks and intercalated discs, may cause dilated cardiomyopathy (Olson *et al.* 1998), whereas an actin mutation in the M-line portion associates with hypertrophic cardiomyopathy (Mogensen *et al.* 1999).

The cardiac muscle has an ability to sense increased mechanical loads, such as caused by hypertension (Lorell & Carabello 2000) or valvular defects (Epstein & Davis 2003). Cardiac cells can response to the increased demand by hypertrophic growth, which is a result of induction of gene expression required to increase myofiber size (reviewed in Vlahos *et al.* 2003). As a result of dysfunctional stretch sensing, cell death pathways may be induced leading to dilated cardiomyopathy and heart failure, as suggested by Knöll

and colleagues (Knöll *et al.* 2002). However, hypertrophic growth is often accompanied by deleterious side effects as well, such as defects in Ca^{2+}-handling and fibrosis (Vlahos *et al.* 2003). How is the hypertrophic response induced? Calcium-Calcineurin pathway has a central role in the hypertrophic growth. Activation of calcineurin by Ca^{2+}- leads to translocation of nuclear factor of activated T cells (NF-AT) to nuclei and induction of hypertophic gene program. There are also other hypertrophic signaling cascades mediated by phosphoinositide 3-kinases (PI3K), mitogen-activated protein -kinases (MAPs) and PKC isoforms. There is some evidence of interplay between calcineurin and PKC-signaling. These signaling pathways are largely triggered by external components, such as hormones and growth factors. For instance, the renin-angiotensin system, which regulates blood volume, is involved in the induction of PKC-mediated hypertrophic growth. (For reviews, see Simpson 1999, Vega *et al.* 2003, Vlahos *et al.* 2003, Wilkins & Molkentin 2004.)

Interestingly, recent findings strongly suggest that the Z-disk possesses a stretch sensor (for reviews, see Epstein & Davis 2003, Pyle & Solaro 2004, Hoshijima 2006.) Muscle LIM protein (MLP), which is a member of cysteine-rich protein (CRP) –family (Weiskirchen *et al.* 1995), forms a complex together with titin and telethonin that is a potential candidate for stretch sensing. MLP is an α-actinin-binding (Louis *et al.* 1997) Z-disk protein (Arber *et al.* 1997), and MLP-deficient mice develop dilated cardiomyopathy shortly after birth (Arber *et al.* 1997). MLP deficiency changes the elastic properties of titin by affecting the telethonin interaction with titin. In the absence of MLP, the stretch sensor machinery is dysfunctional – the survival by hypertrophic growth is impaired. (Knöll *et al.* 2002.) This suggests that MLP has a structural role in the stretch sensing machinery. However, MLP translocates to the nucleus under mechanical pressure (Ecarnot-Laubriet *et al.* 2000), which suggests that MLP is also involved in the induction of genes required for the hypertrophic response. Furthermore, MLP is required for formation of the calcineurin-calsarcin –complex (reviewed in Vega *et al.* 2003, Wilkins & Molkentin 2004), at the Z-disk and induction of downstream hypertrophic signaling (Heineke *et al.* 2005). An increasing body of evidence indicates that other cellular compartments in addition to the Z-disks, such as costameres and intercalated discs, contribute also to stretch sensing (reviewed in Hoshijima 2006).

2.4.1 PDZ-LIM proteins in muscle disorders

A deficiency in ALP in mice leads to an embryonic dilated cardiomyopathy in the right ventricle (Pashmforoush *et al.* 2001), but does not affect skeletal muscle (Jo *et al.* 2001). Thinning of the right ventricular wall is observed at embryonic days 11.5-12.5 (Pashmforoush *et al.* 2001). ALP deficiency, however, is not lethal. Dilation of the right ventricle occurs in 20-30% of ALP -/- mice (Lorenzen-Schmidt *et al.* 2005). The heart defect indicates that ALP is important when the muscle is under high biomechanical stress, as the right ventricle of the developing embryo is in the uterus. The heart phenotype has been confirmed later by analysis of ALP-knockout mice hearts in response to hypoxia (Lorenzen-Schmidt *et al.* 2005). Indeed, an ALP deficiency is associated with decreased contraction of the right ventricle. The increase in the wall thickness

(hypertrophic growth) in response to hypoxia was lower in ALP knockout mice in contrast to the wild type. (Lorenzen-Schmidt *et al.* 2005.) The lack of a phenotype in skeletal muscle indicates that other PDZ-LIM proteins may compensate for the loss of ALP in skeletal muscle. An overexpression of ALP enhances sarcomeric organization in cardiomyocytes and ALP enhances the ability of α-actinin to crosslink actin filaments (Pashmforoush *et al.* 2001). The ALP gene is located on chromosome 4q35 close to the locus mutated in fascioscapulohumeral muscular dystrophy (Xia *et al.* 1997). The linkage of this with ALP was not supported in the analysis of ALP expression in a small subgroup (3 persons) of patients (Bouju *et al.* 1999).

ZASP/Cypher-deficient mice die with severe disruption of sarcomeric organization both in skeletal and cardiac muscle (Zhou *et al.* 2001). The majority of the ZASP/Cypher -/- mice died within 24 hrs after birth. The heart ventricles were dilated, and muscles weakened. The evidence of Z-disk disruption in ZASP/Cypher deficient mice (Zhou *et al.* 2001) earlier in cardiac muscle than in skeletal muscle supports the conclusion that ZASP/Cypher is required to maintain sarcomeric integrity when muscle is functional. The ZASP/Cypher mice could be rescued from lethality with knock-in of either the short (no LIM domains) or long ZASP/Cypher skeletal isoform, although some symptoms of myofiber abnormalities were detected (Huang *et al.* 2003). A knock-down of ZASP/Cypher in zebrafish leads to somite deformation and thinning of the heart ventricular walls. The phenotype could be rescued with the short ZASP/Cypher that does not have the LIM domains. (van der Meer *et al.* 2006.) Two recent studies have suggested that mutations in the ZASP/Cypher gene associate with cardiomyopathy (Vatta *et al.* 2003) and myofibrillar myopathy (Selcen & Engel 2005). The majority of these point mutations are located on exons 4 and 6. A D626N mutation (Arimura *et al.* 2004, Xing *et al.* 2006) in the third LIM domain in cardiomyopathy patients has been shown to increase the binding affinity to PKCs.

3 Aims of the present work

During the course of this work, verification of the PDZ domain interaction with the α-actinin rod domain prior to structural studies of ALP and CLP36 evolved into a detailed analysis of the interaction site of these proteins with α-actinin. The identification of an internal interaction site in CLP36 and ALP guided the research towards ZASP/Cypher, which has point mutations in the internal region associated with muscular diseases.

The specific aims of the study were:

1. To map the interaction sites between ALP, CLP36 and α-actinin
2. To study whether ZASP/Cypher has an internal interaction site similar to that found in ALP and CLP36 and if patient mutations affect this interaction.
3. To analyze the structure of the ALP internal region that is responsible for the interaction with α-actinin

4 Materials and methods

4.1 Generation of constructs

Various fragments of human ALP, chicken ALP, human CLP36, human ZASP/Cypher and human α-actinin 2 proteins were generated. For protein expression, a modified pET24d (Novagen, Merck Biosciences, Schwalbach, Germany) and modified pET8c (Novagen, Djinovic-Carugo *et al.* 1999) vectors were used. In both vectors, a Histidine tag was introduced into the N-terminus of the insert. The His-tag could be removed with tobacco etch virus (TEV) protease (Invitrogen). For cell culture studies, Myc-pcNDA3 (described in paper I), pEGFP-C1, pEGFP-N1 and pEYFP-C1 (Clontech, BD Biosciences) and pELGFP (a gift from M. Way, Cancer Research UK, London, UK Frischknecht *et al.* 1999) vectors were used. For details, see papers I-III.

4.2 Protein expression and purification

For protein expression, plasmids were transformed in the *Escherichia coli* BL21(DE3) strain. Protein expression was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Expression was induced for 3 hrs in +37 °C, except for the ZASP/Cypher proteins, where expression was induced at +15 °C for 20 hrs. To produce ^{15}N and ^{15}N-^{13}C labeled ALP, the induction time at +37 °C was 8 hrs in M9-media. His-tag affinity chromatography using nickel nitrilotriacetic acid-agarose (Qiagen) was used as a first purification step. The His-tag was removed with TEV protease (Invitrogen) and the proteins were further purified with anion exchange and size exclusion chromatographies. For details, see papers I-III.

4.3 Interaction analysis

Surface plasmon resonance (SPR) analysis (SPR Biacore 3000 system, Biacore, Uppsala, Sweden) was used to study ALP, CLP36 and ZASP/Cypher interactions with α-actinin. SPR methodology (reviewed in Schuck 1997) allows the interactions to be followed in real-time and can be also applied to other molecules in addition to proteins. In this study, the ligand protein was covalently immobilized on CM5 chips. The analyte protein was injected in the fluid phase. The effect of an analyte protein to the reference channel (without ligand, but otherwise a similar immobilization) was subtracted from the response on the ligand channel. For details, see papers I-III.

To analyze whether the ALP fragment containing the PDZ domain and the internal region could affect the ability of α-actinin to bind actin filaments, a high speed actin pelleting assay was used. Purified α-actinin and ALP proteins were added to polymerized actin, and ultracentrifugation was performed. For details, see paper I.

4.4 Cell culture

Localization of green fluorescent protein (GFP) - or Myc -tagged ALP and ZASP/Cypher fragments on stress fibers was analyzed. Chinese hamster ovarian (CHO-K1, ATCC CCL-61 American Tissue culture collection, Manassas, VA) and mouse myogenic cells C2C12 (ATCC CRL-1772) were used. Both form adherent layers when cultured on plastic. The constructs of interest were transfected to CHO and C2C12 cells. After transfection, CHO cells were plated on fibronectin for 4 hrs and fixed in 4 % paraformaldehyde and 0.5 % Triton X-100 and stained with the antibodies of interest. The protocol for C2C12 was similar, except that the cells were fixed and stained directly after transfection without plating on fibronectin. An Olympus BX51 microscope was used for detection. For details, see paper I.

To study the localization of ALP and ZASP/Cypher fragments in mature sarcomeres, differentiated C2C12 myoblasts were used. These murine cells originate from satellite cells, which differentiate into myofibers under serum-deprived conditions (Burattini *et al.* 2004 and references therein). After transfection, the cells were allowed to differentiate in 2 % horse serum media for 5 days. The cells were then fixed and stained with an antibody against sarcomeric α-actinin. For details, see paper I.

To determine how stress fiber disruption would affect ZASP/Cypher co-localization with α-actinin, 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) (I2764, Sigma-Aldrich) was used to disrupt the stress fibers. ML-7 inhibits the non-muscle myosin 2A light chain kinase and the lack of phosphorylation blocks the myosin motor activity in smooth muscle (Somlyo & Somlyo 1994). Transfected CHO or C1C12 cells were plated on fibronectin after transfection and ML-7 was added. At selected time points ML-7 was removed by washing the cell layers with serum- and antibiotic -free culture media. For details, see paper II.

To characterize the stability of ZASP/Cypher point mutants, GFP-fusions were transfected into CHO cells. The cells were lysed in 10% sodium dodecyl sulfate (SDS) 24 hrs after transfection, and protein concentration was estimated. Lysates were subjected to

SDS-polyacrylamide gel electrophoresis and transferred to ECL nitrocellulose membranes (Amersham Pharmacia Biotech). A GFP-antibody (632375, BD Biosciences) and ZASP/Cypher antibody (a gift from Georgine Faulkner, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) were used for detection. For details, see paper II.

Live cell microscopy of ALP internal fragment and α-actinin was performed by Pirta Hotulainen (Institute of Biotechnology, Helsinki University, Finland). For details, see paper III.

4.5 Circular dichroism

To analyze the folding of ALP fragments circular dichroism (CD) measurements were performed using a JASCO J-715 spectrometer (JASCO Corporation, Hachioji City, Tokyo, Japan). Purified ALP proteins were diluted to 0.15 mg/ml for measurement. The far-UV spectrum (195-250 nm) was measured at 20 °C. The effect of buffer alone was subtracted prior to the calculation of molecular ellipticity. For details, see paper III.

4.6 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) analysis of the ALP internal fragment and α-actinin rod domain was performed by Nanna Alho, Sampo Mattila (Department of Chemistry, Oulu University, Finland) and Perttu Permi (Institute of Biotechnology, Helsinki University, Finland). For details, see paper III.

5 Results

5.1 ALP/CLP36 and α-actinin have two interaction sites (I)

Previous studies have shown that the PDZ-LIM proteins ALP and CLP36 interact with α-actinin, but the exact location of the interaction sites is not clear (Table 2 in chapter 2.3.5). A number of constructs of ALP, CLP36 and α-actinin 2 were generated to characterize the interaction using SPR detection. Purified ALP and CLP36 fragments were used as the ligands and α-actinin 2 fragments as analytes. As a result, the internal fragments of ALP and CLP36 were shown to interact with the rod domain of α-actinin and the PDZ domains of ALP and CLP36, bound to the α-actinin C-terminus. Deletion of the four C-terminal residues (ESDL) from α-actinin 2 abolished the interaction. Moreover, a C-terminal 10-mer peptide of α-actinin 2 bound to the PDZ domain. (Table II and Fig. 2, paper I.) This fits with the consensus ligand sequence of group I PDZ domains, which recognize an S/T-X-Φ-COOH (X = any amino acid, Φ = hydrophobic amino acid) C-terminal sequence (Harris & Lim 2001).

A general function of α-actinin is to bind filamentous actin. Therefore we tested whether ALP would affect the ability of α-actinin to bundle actin filaments *in vitro*. The estimated stoichiometry of 1.6 for ALP:α-actinin indicates that two ALP molecules interact with a single α-actinin dimer. When the affinities between α-actinin and actin were estimated in the presence and absence ALP, similar affinities (around 1 μM) were obtained. (Fig. 3, paper I.) Thus, the ALP fragment containing both interaction sites does not affect the ability of α-actinin to bind filamentous actin.

5.1.1 The ALP ZM-motif is required for Z-disk targeting (I)

The presence of the two interaction sites between ALP and α-actinin was further tested at the cellular level. GFP- and Myc-tagged fragments of ALP were generated and transfected into cultured CHO cells. After transfection, stress fiber formation was facilitated by plating the cells on fibronectin. The full-length ALP and a fragment lacking the LIM domain (residues 1-284) showed a striated pattern on stress fibers. The internal

fragment of ALP (112-284) was also sufficient for localization on these α-actinin containing structures. The PDZ domain (1-112) alone did not localize, although an earlier study has shown that a L78K mutation in the PDZ domain disrupts the interaction to α-actinin in a yeast-two-hybrid screen (Xia *et al.* 1997). ALP did not localize on focal adhesions with vinculin, in contrast to a previous study by Pomies and colleagues (Pomies *et al.* 1999). (Fig. 4, paper I.)

At the time we found that the internal region of ALP and CLP36 interacted with the rod domain of α-actinin, we noticed in the SMART database that, of the Z-disk PDZ-LIM proteins, only ALP, CLP36 and ZASP/Cypher have a conserved 26-residue motif in the internal region termed the ZASP/Cypher-like (ZM) motif. In ALP and ZASP/Cypher the ZM-motif is alternatively spliced from exons 4 or 6. Next we wanted to test whether this motif is required for localization of ALP in the cell. To study the localization in myofibrils, C2C12 myoblasts, which fuse into myotubes and show sarcomeric organization, were used. Indeed, the ALP PDZ+ZM (1-232) fragment localized to the Z-disks, but a fragment lacking the ZM-motif (PDZ-ZM, 1-182) did not (Fig. 7, paper I). Analyses of various deletion fragments of the ALP internal region further confirmed that sequences close to the ZM-motif are involved in targeting ALP to the α-actinin containing structures. An ALP fragment of 120 residues containing the ZM-motif was sufficient for localization. (Fig. 6, paper I.) Furthermore, ALP PDZ+ZM (1-232) interacted with the rod domain and with the C-terminal EF3-4 fragment of α-actinin in SPR measurement, indicating the presence of the two interaction sites in this fragment.

In ALP, the ZM-motif is located either on exon 4 or on exon 6. The human ALP construct that we used contained the exon 4. We used also chicken ALP corresponding to the exon 6 variant of human ALP (78% identity in the entire protein), and showed that both of these internal fragments localize to the Z-disks (Fig. 7, paper I). This result is in accordance with an earlier study by Henderson and co-workers (Henderson *et al.* 2003), who showed that a L78K mutation in a full-length ALP does not prevent localization to the Z-disks. The PDZ domain did not localize to the Z-disks (Fig. 7, paper I). Thus, our cell biological analysis showed that the internal region of ALP containing the ZM-motif was important in targeting ALP in α-actinin -containing structures in cells.

5.2 Internal interaction site also in ZASP/Cypher (II)

The next step in this study was to test whether the PDZ-LIM protein ZASP/Cypher, which contains a ZM-motif, also has the internal interaction site. Several point mutations in the internal region of ZASP/Cypher are associated with dysfunction of cardiac and skeletal muscle (Vatta *et al.* 2003, Selcen & Engel 2005). Our hypothesis was that these mutations may affect the interaction with α-actinin.

A previous study has shown that the PDZ domain of ZASP/Cypher interacts with the C-terminus of α-actinin (Zhou *et al.* 2001), but there is no clear evidence for the presence of an internal interaction site in ZASP/Cypher. The ZM-motif containing exons of ZASP/Cypher are spliced either from exon 4 or from exon 6 (Vatta *et al.* 2003, Fig. 1, paper II) as in ALP. A number of human ZASP/Cypher constructs of both internal splice variants were generated and their localization in cultured C2C12 myoblasts and CHO

cells was tested. As in ALP, the ZM-motif containing fragments of ZASP/Cypher exon 4 and exon 6 variants with sizes of approximately 130 residues were sufficient for targeting the fragments to stress fibers. The shortest of these ZASP/Cypher fragments corresponded to the ALP splice variant 4 and 6 fragments with respect to the location of the ZM-motif. (Fig. 3, paper II). The ZASP/Cypher PDZ domain alone (1-84) did not localize in C212 cells (Fig. 2, paper II) or in CHO cells (unpublished), in contrast to a previous study, where the ZASP/Cypher PDZ domain localized to the Z-disks in cardiomyocytes (Zhou *et al.* 2001). ZASP/Cypher GFP-fusions did not localize with vinculin in focal contacts (unpublished). The localization of endogenous ZASP/Cypher in myoblasts was analyzed with the ZASP/Cypher antibody. The behavior of the endogenous ZASP/Cypher was similar to that of the GFP-fusions: co-localization with α-actinin on stress fibers was evident, but not in focal contacts (unpublished).

To further confirm the presence of an internal interaction site, an SPR measurement was performed. A partially purified ZASP/Cypher variant 1 protein (1-283) containing the PDZ domain and part of the internal region including the ZM-motif interacted with the rod domain of α-actinin and competed with the ALP internal fragment in the interaction to the rod domain of α-actinin (Fig. 7, paper II). This result indicates that the ZASP/Cypher internal region interacts with the rod and that the ZASP/Cypher and ALP interaction sites on the rod overlap.

5.2.1 Release of ZASP/Cypher co-localization with α-actinin (II)

To study how the ZASP/Cypher and α-actinin co-localization is regulated, we used an ML-7 inhibitor to disrupt stress fibers. The ZASP/Cypher internal exon 4 (112-298) and ZASP/Cypher variant 1 GFP-fusions were tested (Fig. 4, paper II). Transfected cells were allowed to attach to fibronectin prior to ML-7 treatment. ZASP/Cypher and α-actinin co-localization was released with stress fiber disassembly leading to an accumulation of α-actinin in the cell periphery, where α-actinin partially co-localized with vinculin. Neither the ZASP/Cypher exon 4 internal fragment (112-298) nor the ZASP/Cypher variant 1 were in these peripheral areas. Endogenous ZASP/Cypher was also followed using the ZASP/Cypher antibody. A similar behavior was observed in the presence of ML-7 as with the transiently transfected ZASP/Cypher GFP-fusions (unpublished). The ALP GFP-fusion also behaved in a similar manner (unpublished).

5.2.2 Analysis of patient mutations (II)

Two studies have suggested that mutations in the ZASP/Cypher gene are associated with cardiomyopathy and myofibrillar myopathy (Vatta *et al.* 2003, Selcen & Engel 2005). The majority of these mutations are located in exons 4 and 6, some of them residing in the ZM-consensus sequence (Fig. 1, paper II). Therefore, the effect of the point mutations on the co-localization of ZASP/Cypher with α-actinin was tested. Point mutations were generated in ZASP/Cypher exon 4 (112-298) and ZASP/Cypher variant 1 GFP-fusion constructs, and all the point mutations found in exons 4, 6, 7, and 9 were tested.

Localization was analyzed both in CHO and C2C12 cells and the pattern of the mutants was similar to the wild type. (Fig. 5, paper II.)The mutants did not affect the cytoskeleton of transfected cells, as evaluated by comparison of actin cytoskeleton between transfected and non-transfected cells.

The next aim was to study whether the point mutations would affect the stability of ZASP/Cypher proteins. Transfected CHO cells were lysed in 10 % SDS and subjected to Western analysis using the GFP antibody for detection. The amount of the GFP-size degradation product compared to the full-size GFP-fusion was estimated in each sample. This analysis showed that the point mutations did not significantly destabilize the ZASP/Cypher protein. (Fig. 6, paper II.)

5.3 ALP – Rod interaction (III)

In papers I and II we had shown that the internal region of ALP, CLP36 and ZASP/Cypher interacted with the α-actinin rod domain. The ZM-motif containing fragments of ALP and ZASP/Cypher were also sufficient for co-localization with α-actinin. To study the ALP internal region and α-actinin rod interaction more carefully with NMR spectroscopy, several ALP fragments were generated to obtain an optimal fragment for protein purification. The ALP fragment (residues 107-273) could be purified in high amounts and size exclusion chromatography showed a single oligomeric state. CD analysis indicated that this fragment is mostly unfolded in solution. (Fig. 1, paper III.) However, co-localization of this fragment with α-actinin on stress fibers and a direct interaction to the rod domain in SPR measurement verified that ALP (107-273) is functional (Fig. 2 and Fig. 3, paper III).

^{15}N- and ^{15}N-^{13}C-labelled ALP proteins were produced for NMR experiments. ^{15}N-ALP (107-273) appeared in NMR as a largely unfolded protein. However, titration of ALP with the α-actinin rod domain induced a signal dispersion indicating that the rod domain can stabilize the unstructured ALP. Surprisingly, similar stabilization of ^{15}N-^{13}C-ALP was obtained with externally added aspartic acid enabling the backbone assignment (Alho *et al.* 2006). The comparison of the assigned data to the α-actinin titration data enabled the intepretation how the interaction with the rod domain may affect the ALP internal region. First, NMR analysis showed that ALP (107-273) has some partially structured areas located on residues 164-186, 200-206, 214-233 and 256-267 that already appear without the rod domain and without aspartic acid. Second, the titration of ALP with the rod domain increased stabilization on areas 137-167 and 221-249. (Fig. 4, paper III.)

The next goal was to determine whether these structured areas are involved in a direct interaction with the rod domain. Our previous studies of ALP localization suggested that residues 151-218 containing the ZM-motif (residues 183-209) would define the minimal localization/interaction area (151-232 paper I, 151-218 unpublished). Interestingly, this area possesses several structured areas detected in NMR. Five 20-22 residue overlapping peptides of region 151-218 were designed and their interactions with the α-actinin rod domain were tested using SPR. Of these five peptides, the peptide 3 (PLEMELPGVKIVHAQFNTPMQL, residues 175-196) containing the N-terminal half of

the ZM-consensus interacted with the α-actinin spectrin repeats 1-4 with an affinity of 3 μM, whereas the other four peptides did not show interaction with this fragment. The peptide 3 did not interact with α-actinin spectrin repeats 2 and 3, whereas ALP (107-273) did. Altogether, these results suggest that the folding of ALP, on areas surrounding the peptide 3 region in particular, is increased by the interaction with the rod domain resulting to an extended interaction surface on the rod domain, and that the ZM-motif partially defines the interaction area. (Fig. 5 and Fig. 6, paper III.)

6 Discussion

6.1 Two interaction sites

The first aim of this study was to map the interaction sites of the Z-disk PDZ-LIM domain proteins ALP and CLP36 with α-actinin. Using recombinant proteins purified from bacteria in SPR analysis we showed that the PDZ domain of ALP and CLP36 recognizes the C-terminal ESDL-sequence of α-actinin. This is a recognition sequence of group I PDZ domains (Harris & Lim 2001). A deletion of this sequence abolishes the interaction. Our results are in agreement with previous results showing that the PDZ domains of ZASP/Cypher and RIL interact with the C-terminus of α-actinin (Zhou *et al.* 2001, Schulz *et al.* 2004). Furthermore, Au and colleagues (Au *et al.* 2004) proposed that all PDZ-LIM proteins would have a fold similar to ZASP/Cypher PDZ that contains the binding site of the α-actinin C-terminus. Indeed, the ALP PDZ domain has a fold in solution (1V5L in PDB) similar to that of ZASP/Cypher PDZ. In contrast to our results, the ALP and CLP36 PDZ domains have been earlier suggested to interact with the α-actinin rod domain in yeast-two hybrid screens and immunoprecipitation analyses (Xia *et al.* 1997, Vallenius *et al.* 2000). This discrepancy may be due to different experimental systems. We did not observe any interaction of purified ALP or CLP36 PDZ domains with the rod domain of α-actinin in our SPR assay.

In addition to the PDZ domain interaction with the C-terminus we found that the internal regions of ALP, CLP36 and ZASP/Cypher interact with the rod domain of α-actinin. Only these three proteins contain the conserved stretch of 26-27 residues, termed the ZM-motif, in their internal region. In ALP and ZASP/Cypher the ZM-motif is alternatively spliced either from exon 4 or exon 6. Finer mapping of the interaction site showed that, in ALP, sequences close to the ZM-motif are required for the interaction. The binding site was mapped to residues 175-196 (ALP peptide 3, PLEMELPGVKIVHAQFNTPMQL) containing the N-terminal half of the ZM-motif. This peptide bound to α-actinin rod domain spectrin repeats 1-4, but not to repeats 2-3, whereas the ALP internal fragment (107-273) interacts with both suggesting that residues 175-196 do not cover the entire interaction area with the rod (Fig. 5). A further characterization with circular dichroism spectrosopy revealed that the ALP internal region

is mostly unfolded in solution, whereas partially structured areas were found by NMR spectroscopy close to the peptide 3 sequences. Interaction with α-actinin further stabilized the structure around peptide 3, supporting its involvement in the interaction. Thus, our results suggest that ALP internal region occupies an extended interaction surface on the rod domain and that the ZM-motif is involved in the interaction. This type of interaction mode, where a largely unstructured protein is stabilized upon interaction, is relatively uncommon, but it has been detected. For instance, the cytoplasmic tail of cadherin is unstructured by itself, but it occupies an extended interaction surface upon binding on β-catenin (Huber *et al.* 2001, Huber & Weis 2001). A previous study has indicated that the internal region of CLP36 interacts with the rod domain (Bauer *et al.* 2000), but in this study the site was not mapped in more detail. From the high-speed pelleting assay, we obtained an estimate of 2:1 stoichiometry, indicating that two ALP molecules interact with a single α-actinin dimer (Fig. 5). A similar ratio has been suggested for CLP36 with α-actinin (Vallenius *et al.* 2000)

We tested the localization of both interaction sites in cultured CHO and mouse C2C12 cells. These cells form stress fibers, which resemble the organization of myofibrils (reviewed in chapter 2.1.5). Furthermore, C2C12 cells differentiate into myotubes, which enabled us to follow the localization in an actual sarcomeric system. The PDZ domains of ALP and ZASP/Cypher did not localize either on stress fibers or Z-disks. This result differs from those of other studies, in which different cell culture models have been used. The ZASP/Cypher PDZ domain was shown to localize to the Z-disks of cultured cardiomyocytes (Zhou *et al.* 2001) and the PDZ domain of *C. elegans* ALP-1 protein localized to dense bodies, which are analogues of Z-disks, in the nematode body wall muscle (McKeown *et al.* 2005). It is possible that the C-terminal interaction site is exposed only under certain conditions or other molecules may mask the interaction site of endogenous α-actinin. In our study the internal regions of ALP and ZASP/Cypher containing the ZM-consensus sequence were mainly responsible for co-localization with α-actinin in cultured cells. ALP or ZASP/Cypher did not co-localize with α-actinin at focal contacts either in CHO or C2C12 cells, in contrast to a previous study indicating ALP localization at focal contacts in chicken embryo fibroblasts (Pomies *et al.* 1999). Both internal exon 4 and exon 6 splice variants of ALP showed a striated pattern on stress fibers and localized to the Z-disks. Internal fragments of ALP and ZASP/Cypher were sufficient for co-localization with α-actinin already at the Z-bodies (unpublished). Moreover, the ALP fragment containing the PDZ domain and the ZM-motif (1-232) localized to the Z-disks, whereas a construct lacking the ZM-motif (1-182) did not. The shortest localizing internal fragments of ALP were around 120 residues containing the ZM-motif. Similar size (130 residues) fragments of ZASP/Cypher exon 4 and exon 6 splice variants containing the ZM-motif were sufficient for localization on stress fibers. These results suggest that both the exon 4 and exon 6 ZM-motif containing fragments are able to mediate the interaction with α-actinin in cells. However, our results do not rule out the possibility that internal regions of ALP and ZASP/Cypher may also interact with other Z-disk components. Of the seven PDZ-LIM proteins, ALP, CLP36, ZASP/Cypher, ENH, and Enigma are found at the muscle Z-disk and the nonmuscle PDZ-LIM members RIL and Mystique show a striated pattern on stress fibers (reviewed in chapters 2.3.3.1 and 2.3.4.1). All, except Enigma, are shown to interact with α-actinin (chapter 2.3.5.1). To conclude, it is not clear whether the ZM-motif is a signature for the internal α-actinin

interaction site, or whether the internal domains of other PDZ-LIM proteins could also interact with the α-actinin rod.

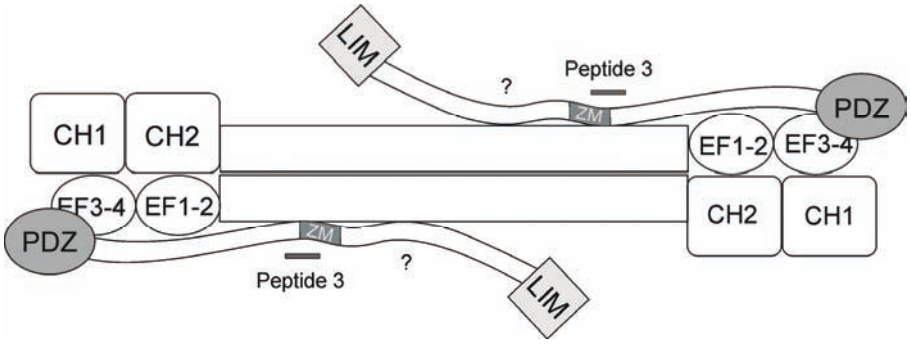

Fig. 5. Two ALP molecules can interact with a single α-actinin dimer. ALP has two interaction sites with α-actinin: the PDZ domain binds to the C-terminus, whereas the internal region interacts with the rod domain. ALP peptide 3 (residues 175-196) containing the N-terminal half of the ZM-motif bound to the R1-R4 fragment but not to R2-R3. The ALP internal fragment (107-273) interacted with both suggesting that residues 175-196 do not define the entire interaction area on the rod domain. Question marks denote possible other interaction surfaces on the rod.

6.2 PDZ-LIM proteins and the actin cytoskeleton

Several point mutations in exons encoding ZASP/Cypher internal regions have been found in patients with cardiomyopathy and myofibrillar myopathy (Vatta *et al.* 2003, Selcen & Engel 2005). Some of these mutations are located in the ZM-motif (Fig. 1, paper II). We hypothesized that these mutations might interfere with binding to the α-actinin rod domain. We followed the behavior of the GFP-tagged ZASP/Cypher mutants in cells. First, we did not find any evidence that these mutations affected ZASP/Cypher co-localization with α-actinin. Second, the transient overexpression of mutant ZASP/Cypher proteins did not markedly affect the organization of the actin cytoskeleton either in nonmuscle or muscle cells. Third, the mutations did not destabilize the ZASP/Cypher proteins. Vatta and co-workers (Vatta *et al.* 2003) suggested that the D117N substitution in ZASP/Cypher variant 1 would disrupt the actin cytoskeleton of C2C12 myoblasts. In our study, the D117N mutant did not affect the cytoskeleton either in C2C12 myoblasts or in CHO cells. The co-localization with α-actinin on stress fibers was similar to the wild-type ZASP/Cypher. Moreover, the D117N mutation did not affect the binding to the rod domain. The average age of onset of disease in patients carrying ZASP/Cypher mutations is reasonably high, 54 years in myofibrillar myopathy patients (Selcen & Engel 2005), whereas a ZASP/Cypher deficiency in mice is lethal (Zhou *et al.* 2001). However, single amino acid substitutions may require a longer time to cause myopathy in patients, in contrast to the total absence of ZASP/Cypher protein in knockout mice. Minor effects may remain undetected in short term cell-culture analysis.

The instability and insolubility of recombinant ZASP/Cypher proteins prevented a detailed interaction analysis of all the mutant proteins with α-actinin at the protein level.

A general function of α-actinin is to bind filamentous actin. In addition to PIP2 and Ca^{2+}-ions as regulators of actin binding, α-actinin -interacting proteins may affect actin binding (chapters 2.2.1 and 2.2.2.1). The nonmuscle PDZ-LIM protein RIL enhances α-actinin binding to filamentous actin (Vallenius *et al.* 2004). Furthermore, the overexpression of RIL induces thick irregular stress fibers, whereas CLP36 does not. We tested whether ALP fragment containing the PDZ domain and the internal region would affect the ability of α-actinin to bind actin filaments in a high-speed pelleting assay. ALP did not interfere with α-actinin binding to actin. An earlier study has demonstrated that ALP enhances the ability of α-actinin to crosslink actin filaments (Pashmforoush *et al.* 2001) in a low speed sedimentation assay. This suggests that one explanation of the dilated cardiomyopathy in ALP deficient mice (Pashmforoush *et al.* 2001) might be the impairment of actin crosslinking at the Z-disks. It is possible that the two interaction sites on α-actinin may regulate the flexible hinge region of α-actinin and thus, restrict the orientations of actin filaments to antiparallel ones as in the Z-disks (Ishikawa *et al.* 1969). On the other hand, this would predict that ALP would reduce the crosslinking of actin polymers *in vitro*. Furthermore, Pashmforoush and colleagues (Pashmforoush *et al.* 2001) showed that overexpression of ALP stabilizes sarcomeres. It would be interesting to study whether this stabilizing effect requires both interaction sites.

After we had shown that the ZM-motif containing PDZ-LIM protein ZASP/Cypher also has the internal interaction site with the rod, we wanted to explore further how the interaction between ZASP/Cypher and α-actinin is regulated in cultured cells. The stress fibers of transfected cells were disrupted with the ML-7 inhibitor. The results of these experiments suggested that the intact actin cytoskeleton is required for the interaction. The co-localization of ZASP/Cypher (both variant-1 and the internal fragment) with α-actinin was released when stress fibers were disrupted. Similar observations were made with ALP (unpublished). In contrast, a somewhat similar experimental protocol for CLP36 in an earlier study has shown the opposite effect. Pretreatment of platelets with the actin polymer disruptive agent (cytochalasin), does not affect the amount of α-actinin coimmunoprecipitated with CLP36, suggesting that their interaction is independent of the actin cytoskeleton (Bauer *et al.* 2000, Wang *et al.* 2005). The accumulation of α-actinin in the cell periphery after stress fiber disassembly may also suggest that other interactions are competing with ZASP/Cypher and ALP in binding to α-actinin in the periphery, where α-actinin partially co-localizes with vinculin. This is also suggested by the absence of ALP and ZASP/Cypher in focal contacts without subjecting cells to ML-7 treatment.

6.3 Future perspectives

The tremendous variety in α-actinin interaction partners shows that at muscle Z-disks, α-actinin is not solely a crosslinker of actin filaments, though this function is essential in muscle (Fyrberg *et al.* 1990, Roulier *et al.* 1992, Fyrberg *et al.* 1998). A number of molecules bind α-actinin (for references, see Table 1 in chapter 2.2.2). Interestingly,

several interaction partners bind to the same regions recognized by the PDZ-LIM proteins ALP, CLP36 and ZASP/Cypher (Fig. 6).

The costameric proteins vinculin and β1-integrin, and the recently found affixin, bind to the rod-EF –region and thus are potential competitors that could explain the absence of ALP and ZASP/Cypher in focal contacts as well as the distinct pools of α-actinin after predisposition of cells to ML-7 treatment. It is also possible that additional proteins that are not expressed in CHO or C2C12 cells are required for targeting ALP and ZASP/Cypher in these locations. Titin, the giant multifunctional sarcomeric protein, interacts with the rod and with the EF-region of α-actinin (for references, see Table 1) resembling the interaction of ALP, CLP36 and ZASP/Cypher with α-actinin. Structural studies suggest that the interaction sites of ZASP/Cypher PDZ and titin on the EF-region do not overlap (Atkinson *et al.* 2001, Au *et al.* 2004). Hence, titin and ZASP/Cypher can bind the α-actinin EF-region simultaneously. In contrast, several PDZ-LIM proteins may compete in the binding to the C-terminus and ALP, CLP36 and ZASP/Cypher potentially compete for the internal interaction site on the rod as our biochemical interaction data on ALP and ZASP/Cypher indicate.

A deficiency of ALP in mice leads to cardiomyopathy (Pashmforoush *et al.* 2001). The phenotype of ZASP/Cypher knockout mice is more severe, both cardiac and skeletal muscles are affected (Zhou *et al.* 2001). As a common feature, both these mice have dilated cardiomyopathies. In ZASP/Cypher knockouts, both ventricles are enlarged, whereas in ALP knockouts, only the right ventricle is affected. In mouse heart, ALP is primarily localized at intercalated discs (Pashmforoush *et al.* 2001), whereas ZASP/Cypher expression does not differ between intercalated discs and Z-disks (Zhou *et al.* 1999, Zhou *et al.* 2001). This difference may explain why ZASP/Cypher cannot compensate for the loss of ALP in cardiac muscle, as suggested by Zhou and co-workers (Zhou *et al.* 2001). Pashmforoush and colleagues (Pashmforoush *et al.* 2001) proposed that the right ventricle dilation in ALP deficient mice might be a result of impairment of the catenin-actinin complex at the intercalated disc. Interestingly, the α-catenin interaction site is located on the rod (Nieset *et al.* 1997), as is the internal interaction site between ALP and α-actinin. It is tempting to speculate that ALP, the internal interaction site in particular, may regulate the interaction between α-actinin and α-catenin.

The effects of ALP and ZASP/Cypher deficiencies in mouse hearts are manifested when muscle is functional and encounters increased workloads. For instance, in ZASP/Cypher deficient mice, the Z-disks of cardiac muscle, which starts to contract at embryonic day 8, are disrupted earlier than in skeletal muscle. These studies indicate that ZASP/Cypher and ALP are required to maintain the sarcomeric organization during contraction. MLP deficient mice also have dilated cardiomyopathy (Arber *et al.* 1997). However, a later study indicates that MLP together with titin, and telethonin acts as a stretch sensor of the Z-disks (Knöll *et al.* 2002). Arber and colleagues (Arber *et al.* 1997) mentioned that in MLP deficient mice the ALP expression is enhanced and ALP-/- and MLP-/- deficient mice have more severe phenotypes. The response to hypoxia, in terms of cardiac wall thickness, in ALP -/- mice is lower that in wt mice (Lorenzen-Schmidt *et al.* 2005), which indicates that hypertrophic growth as a compensatory mechanism to increased workload is impaired.

ZASP/Cypher is implicated in stretch signaling via its linkages to calcineurin and PKC isoforms. Both of these are important in hypertrophic growth (Simpson 1999, Vega *et al.*

2003, Vlahos *et al.* 2003, Wilkins & Molkentin 2004). Calsarcins, also termed myozenin and FATZ-proteins (Faulkner *et al.* 2000, Takada *et al.* 2001) interact with calcineurin (Frey *et al.* 2000). A predisposition of calsarcin-deficient mice to pressure overload enhances calcineurin activation and leads to hypertrophy of cardiac muscle (Frey *et al.* 2004). The calsarcin interaction site is located on the rod region (R3-R4) of α-actinin (Faulkner *et al.* 2000, Takada *et al.* 2001). The similar location of the internal interaction site on the rod raises the possibility that ZASP/Cypher, ALP and CLP36 may regulate calsarcin-mediated calcineurin signaling in muscle. Calsarcins also interact with ZASP/Cypher (Frey & Olson 2002), but the exact interaction site is not known. It might be that the patient point mutations in ZASP/Cypher may affect this interaction. The LIM domains of ZASP/Cypher interact with PKCs, whereas no interaction partners of ALP LIM have been found. The binding of ZASP/Cypher to the main Z-disk component α-actinin may target PKC signaling to the proper cellular location. In conclusion, α-actinin, the rod domain in particular, may act as a docking site of hypertrophic signaling cascades at the muscle Z-disks. The actual role of the ZM-motif containing PDZ-LIM proteins in these signaling events remains to be solved.

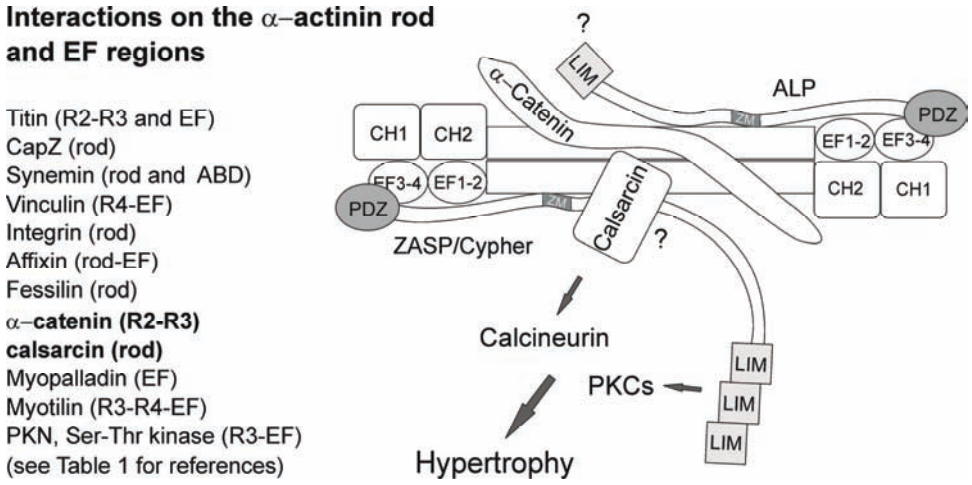

Fig. 6. The rod region of α-actinin is a docking site for a number of proteins. Only ALP, ZASP/Cypher, calsarcin and α-catenin are indicated in the schematic view. The ALP internal region may regulate α-catenin interaction with α-actinin at the intercalated discs. ZASP/Cypher may target and regulate hypertrophic signaling cascades at the Z-disks. Interaction partners of the ALP LIM domain and the interaction site between ZASP/Cypher and calsarcin are not known (indicated with question marks).

7 Conclusions

The Z-disks connect the adjacent sarcomeres in muscle. In addition, the Z-disks are evolving as signaling centers that possess an intrinsic stretch sensor. α-Actinin is essential for providing strength to the Z-disks by crosslinking actin filaments. The PDZ-LIM proteins are a family of α-actinin interacting proteins. Three members of the family, ALP, CLP36 and ZASP/Cypher, have a conserved ZM-motif in the internal region, and all localize to the Z-disks. The first aim of this work was to localize the exact interaction sites between ALP and CLP36 with α-actinin. Two interaction sites were found: The PDZ domain interacted with the C-terminus of α-actinin and the internal region bound to the rod domain. The presence of an internal interaction site in the third ZM-motif containing member ZASP/Cypher was also verified. The ZM-motif in ALP was shown to partially mediate the interaction to the rod, but it does not cover the entire interaction area. Thus, it is possible that the PDZ-LIM proteins that do not have a ZM-motif, may also have an internal interaction site with α-actinin. The patient point mutations in the ZASP/Cypher internal region did not affect the co-localization with α-actinin or the stability of the ZASP/Cypher protein.

This study clarifies the interaction sites of PDZ-LIM proteins with α-actinin. It appears that recognition of the α-actinin C-terminus via the PDZ domain is a general mechanism in the PDZ-LIM family. A previous study has indicated that the CLP36 internal region may interact with the rod. The presence of an internal interaction site in all the ZM-motif containing members was verified and characterized in more detail. The internal region was necessary and sufficient for co-localization of ALP and ZASP/Cypher with α-actinin on stress fibers and at the Z-disks. However, ALP and ZASP/Cypher did not localize with α-actinin on focal adhesions, which are analogues of muscle costameres with respect to the protein composition. It is possible that other interaction partners on the rod domain, such as vinculin or integrin, occupy the interaction areas in focal adhesions.

α-Actinin, the rod domain in particular, binds to many structural and signaling molecules at the muscle Z-disks. However, it is unlikely that the interacting molecules on the rod domain act independently – there must be regulation and competition among the binding partners. The apparent extended nature of the ALP internal interaction site on the rod suggests that it may regulate several other interactions occurring on the rod. What roles do the PDZ-LIM proteins play in muscle? ALP and ZASP/Cypher deficiency in

mice affects muscle tissues. The hypertrophic compensatory mechanism to increased workloads on cardiac muscle is impaired in ALP deficient mice. In ZASP/Cypher knock-out mice the heart ventricles are dilated and both cardiac and skeletal muscles are characterized by the disruption of the Z-disks. ZASP/Cypher may compensate for the loss of ALP in skeletal, but apparently not in cardiac muscle. ALP expression is high in the intercalated discs where it co-localizes with catenin, which is important in connecting contractile machinery to the sarcolemma and to adjacent cardiomyocytes. It is possible that ALP regulates the rod – catenin interaction and may thus explain the weakening of the cardiac walls in ALP deficient mice. ZASP/Cypher interacts with calsarcin and PKC isoforms, which are both important in hypertrophic growth. ZASP/Cypher may target PKC signaling to the Z-disks. The calsarcin interaction site is also located on the rod domain, thus it can be potentially regulated by ZASP/Cypher. ALP and CLP36 may also contribute to this regulation.

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I Klaavuniemi T, Kelloniemi A & Ylänne J (2004) The ZASP-like motif in actinin-associated LIM protein is required for interaction with the α-actinin rod and for targeting to the muscle Z-line. J Biol Chem 279: 26402-10.

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III Klaavuniemi T, Alho N, Hotulainen P, Kelloniemi A, Permi P, Mattila S & Ylänne J, Characterization of the interaction between actinin-associated LIM protein (ALP) and the rod domain of α-actinin. Manuscript.

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