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Minireview

Plectin: A Cytolinker by Design

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Plectin is a cytoskeletal protein of > 500 kDa that forms dumbbell-shaped homodimers comprising a central parallel α-helical coiled coil rod domain flanked by globular domains, thus providing a molecular backbone ideally suited to mediate the protein's interactions with an array of other cytoskeletal elements. Plectin self-associates and interacts with actin and intermediate filament cytoskeleton networks at opposite ends, and it binds at both ends to the hemidesmosomal transmembrane protein integrin beta-4, and likely to other junctional proteins. The central coiled coil rod domain can form bridges over long stretches and serves as a flexible linker between the structurally diverse N-terminal domain and the highly conserved C-terminal domain. Plectin is also a target of $p34^{cdc2}$ kinase that regulates its dissociation from intermediate filaments during mitosis.

Key words: Actin / Cytoskeleton / Integrins / Intermediate filaments / $p34^{cdc2}$ Kinase.

Introduction

Plectin belongs to a family of structurally related cytoskeletal linker proteins referred to as plakins (Uitto *et al*., 1996; Ruhrberg and Watt, 1997) or cytolinkers (Wiche, 1998). Desmoplakin (Green *et al*., 1990; 1992), the various isoforms of bullous pemphigoid antigen (BPAG)-1/dystonin (Stanley *et al*., 1981; Sawamura *et al*., 1991; Brown *et al*., 1995a, b; Yang *et al*., 1996), ATC7 (Bernier *et al*., 1996), envoplakin (Ruhrberg *et al*., 1996), and periplakin (Ruhrberg *et al*., 1997) are the other known family members. These proteins have been proposed to preserve the mechanical integrity of the cell by networking intermediate filament(IF)s and linking them to their membrane attachment sites. Plectin, a polypeptide of over 500 kDa, was first identified as a major component of IF preparations obtained from rat glioma C6 cells (Pytela and Wiche, 1980). Contrary to other IF-associated proteins, which were thought to be specific to certain tissues or cell lines, plectin was found to be abundantly expressed in a wide variety of mammalian tissues and cell types (for review see Wiche, 1989). Because of its immunolocalization within dense cytoplasmic network arrays of cultured cells and its association with IFs we postulated that plectin molecules might be involved in network formation of the cytoskeleton, hence its name (Wiche and Baker, 1982; Wiche *et al*., 1982). This idea was supported by the subsequent identification of a variety of interaction partners of plectin, among them components of all three major cytoskeleton-forming protein fiber systems, actin/myosin filaments, IFs, and high molecular-weight microtubule associated proteins (MAPs). However, only recent observations began to deepen our understanding of the essential role played by this protein in the organization and regulation of cytoskeletal networks. Important milestones along the way were the cloning of plectin's cDNA, first reported for rat (Wiche *et al*., 1991), the establishment of the gene structure, the chromosomal localization of the human gene (Liu *et al*., 1996), and the finding that defects in the plectin gene result in epidermolysis bullosa simplex (EBS)-MD, a human autosomal recessive disorder causing severe skin blistering combined with muscular dystrophy (Gache *et al*., 1996; McLean *et al*., 1996; Smith *et al*., 1996). Another recent breakthrough was the generation and phenotypic characterization of plectin-deficient mice generated by targeted gene inactivation (Andrä *et al*., 1997). This minireview will be focussed on the multidomain structure of the molecule and its various interaction partners. For a more comprehensive review on the role of plectin in cytoskeleton organization and dynamics see Wiche (1998). Cytoskeletal cross-linkers in general have recently been reviewed by Bousquet and Coulombe (1996), Ruhrberg and Watt (1997), Fuchs and Cleveland (1998), and Houseweart and Cleveland (1998).

Molecular Structure

Rotary shadowing electron microscopy of purified plectin revealed a dumbbell-like structure comprising a central 200 nm-long rod domain flanked by large globular domains (Figure 1). The visualized structures were likely to consist of two polypeptide chains arranged as a parallel α-helical coiled coil within their rod segments (Foisner and Wiche, 1987; Wiche *et al*., 1991). Molecular mass predictions for full-length single-chain plectin isoforms vary from 507 to 527 kDa depending on several putative first coding exons (Elliott *et al*., 1997).

The 214 kDa C-terminal globular domain (Gln2759-Ala4687), encoded by a single very large (> 6 kb) exon (exon 32), consists of a tail preceded by 6 repeats, each of 28 to 39 kDa, and a short segment (Gln2759-Thr2785) link-

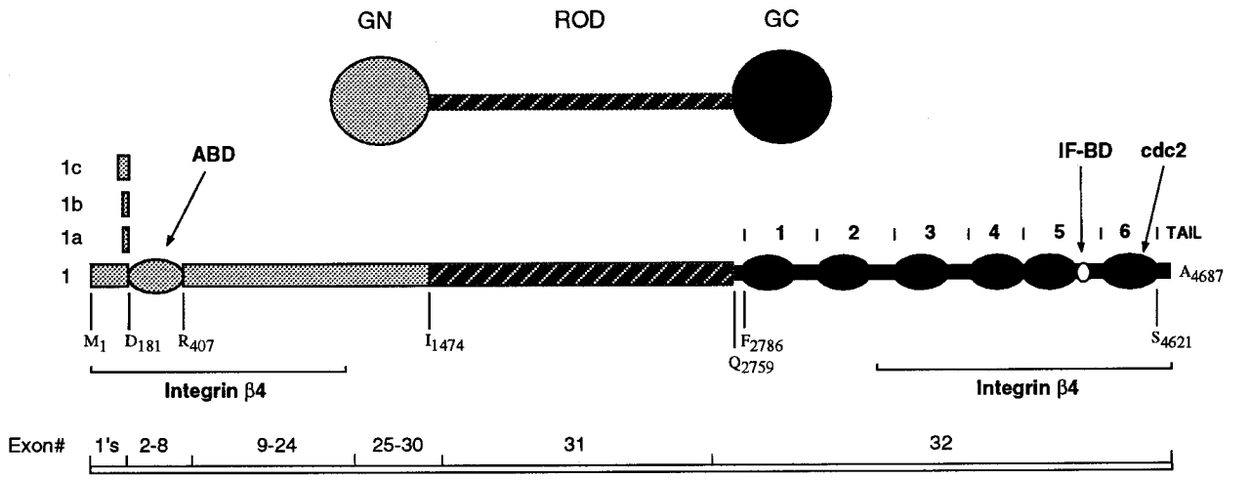

Fig. 1 Schematic Representation and Exon Allocation of Plectin's Subdomains.
Upper part: model of the plectin molecule as visualized by electron microscopy (not drawn to scale). GN, N-terminal globular domain; GC, C-terminal globular domain. Center and lower part: organization and exon allocation of subdomains. Note that differential splicing of alternative first coding exons (exons 1–1c) into a common exon 2 generates transcripts encoding isoforms of slightly different mass and with distinct N-termini. Numbered amino acid residues below the middle part indicate the start of individual subdomains (numbers according to rat plectin, GenBank accession no. P30427). The following segments are indicated: N-terminal globular domain (M1-I1474, grey); rod (I1474-Q2759, hatched); C-terminal globular domain (Q2759-A4687, black); N-terminus (M1-D181); actin binding domain (D181-R407); linker between rod and C-terminal repeat 1 (Q2759-F2786); C-terminal tail (S4621- A4687). The extent of the α-helical coiled coil rod domain was predicted using the *COILS* program (Lupas *et al*., 1991). Segments corresponding to the C-terminal sequence repeats 1–6 are indicated, their highly conserved core regions are depicted as ellipsoids. Note the different lengths of regions downstream of cores: ~ 90 amino acid residues (repeats 1–3), 7 (repeat 4), 106 (repeat 5), and 14 (repeat 6). Segments to which functions have been assigned are indicated: ABD, actin binding domain; IF-BD, intermediate filament binding domain; cdc2, phosphorylation site for $p34^{cdc2}$ kinase; integrin β4, regions harboring one or more binding sites for integrin β4. Note that the predicted domain borders of the rod do not coincide with the ends of the segment encoded by exon 31, but reside in segments encoded by flanking exons 30 and 32. Furthermore, the N-terminal border of the highly conserved ABD coincides with the junction between exon 1 and exon 2, while its C-terminal end corresponds to codons in the center of exon 8. The N-terminal binding site(s) for integrin β4 have been located to a segment encoded by exons 1–24. Also note that the lengths of exons and the sizes of the corresponding protein segments are only roughly proportional.

ing it to the rod. All 6 repeats contain a core region comprising multiple copies of a tandemly repeated 19 (Wiche *et al*., 1991), or 2 × 19 (Green *et al*., 1992) amino acid residue motif. The striking homology between the central regions of the 6 repeats suggests that they may be the result of a series of gene duplication events (Wiche *et al*., 1991). The differences in length of some of the repeats is due to the insertion of differentially long sequences between the C-terminal ends of the repeats' core regions and the beginning of the following repeat (Figure 1). These parts of the polypeptide chain are likely to form looplike extensions that are more accessible to potential interaction partners than the core regions, which probably play a more structural role. One of plectin's most important molecular interfaces so far characterized, its IF-binding domain, has in fact been mapped to such a region, namely the one interspersed between the highly repetitive core domains of repeats 5 and 6 (see below).

The C-terminal tail region, spanning the last 67 residues of the plectin protein (Ser4621-Ala4687), has a calculated pI of 11.4 due to its relatively high content of arginines. This region also contains a high proportion of serines and threonines, which as potential phosphoacceptors may play a role in neutralizing positive charges in this region and thereby may control some of the molecule's interactions. In addition, the tail region contains a GSRX motif (tandemly repeated four times), which is also found in other cytolinker family members. The functional significance of this motif as well as that of the tail region as a whole remains unclear.

While the entire 214 kDa C-terminal globular domain of plectin and the predominant part of the 150 kDa central rod domain are encoded by two exons of unusually large size [> 6kb (exon 32) and > 3kb (exon 31), respectively], over 30 exons (including several alternative first coding exons) ranging in size from 62 bp (exon 2) to 541 bp (exon 1) encode plectin's 154–170 kDa N-terminal globular domain (Liu *et al*, 1996; Elliott *et al*., 1997). The analysis of transcripts by RACE and RNase protection assays led to the identification of several isoforms with alternative first coding exons, that showed significant variation in expression levels in different tissues (Elliott *et al*., 1997; P. Fuchs *et al*., unpublished results). In all cases characterized so far, the various first exons have been found to be spliced into the same downstream exon (exon 2), which is the first of 7 exons encoding a highly conserved actin binding domain (ABD), extending from Asp181-Pro406. Plectin shares this domain with a large number of proteins that have been identified as genuine actin binding proteins, such as dystrophin, spectrin, and α-actinin.

The tripartite molecular structure of plectin, comprising an N-terminal globular domain with several putative sub-

domains, a central α-helical coiled coil rod, and a C-terminal globular domain consisting of highly conserved repeats, is shared with all other cytolinker family members, except for periplakin which lacks a globular C-terminal domain (Ruhrberg *et al*., 1997).

The C-Terminal Domain Mediates Cell Cycle-Dependent Linkages to IFs and Contains an Integrin Beta-4 Binding Site

Vimentin was the first direct binding partner of plectin to be characterized (Pytela and Wiche, 1980; Wiche *et al*., 1982). Plectin was shown to colocalize with IFs of the vimentin type in cultured cells, to copurify and associate with vimentin IFs assembled *in vitro*, and to bind to solid phase-immobilized forms of the IF subunit protein (Foisner *et al*., 1988). These early experiments fell short of indicating which of plectin's several molecular domains harbor IF interaction sites. Fab fragments generated from monoclonal antibodies recognizing epitopes on plectin's α-helical rod domain partially inhibited plectin-vimentin interaction *in vitro*, suggesting that this domain was responsible for binding, or otherwise optimized the interaction of the two proteins (Foisner *et al*., 1991a). The crucial role of the C-terminal globular domain of plectin in IF inter-

Table 1 Interaction Partners of Plectin.

Subcellular structure/protein	Method	References
Intermediate filaments		
Vimentin	CP	Pytela and Wiche, 1980
	CP, IF	Wiche *et al*., 1982
	CS, EM, BO, IE	Foisner *et al*., 1988
	IE, IF	Wiche *et al*., 1993
	BA, EM, IF	Nikolic *et al*., 1996
	IE, IF	Svitkina *et al*., 1996
Cytokeratins	BO	Foisner *et al*., 1988
	IF	Wiche *et al*., 1993; Nikolic *et al*., 1996
	IE, IF	Rezniczek *et al*., 1998
Desmin	BA, EM	F. Steinböck, unpubl. results
GFAP	BO	Foisner *et al*., 1988
Neurofilament proteins 210/160/70	BO	Foisner *et al*., 1988
	IF	Errante *et al*., 1994
Microfilaments	IE, IF	Seifert *et al*., 1992; Foisner *et al*., 1995; Svitkina *et al*., 1996
Actin	BA, IF	Andrä *et al*., 1998
Microtubules	EM	Pytela and Wiche, 1980
	CP, IE	Koszka *et al*., 1985
	IE, IF	Svitkina *et al*., 1996
MAP1 and MAP2	BO	Herrmann and Wiche, 1987
Subplasma membrane skeleton	IE, IF	Wiche *et al*., 1983
Fodrin	BO	Herrmann and Wiche, 1987
Desmosomes		
Desmoplakin	IE, IF, IP	Eger *et al*., 1997
Hemidesmosomes	IE, IF	Wiche *et al*., 1984
	EM, IF	Andrä *et al*., 1997
Integrin β4	BA, BO, IF, IE	Rezniczek *et al*., 1998
Nuclear lamina		
Lamin B	BO, PP	Foisner *et al*., 1991b
Phosphokinases		Herrmann and Wiche, 1983
Ca^{2+}/calmodulin-dependent kinase	PP	Herrmann and Wiche, 1987
PKA and PKC	PP	Foisner *et al*., 1991b
$p34^{cdc2}$ kinase	PP	Foisner *et al*., 1996; Malecz *et al*., 1996
Signaling molecules		
PIP2	BA	Andrä *et al*., 1998

Abbreviations: BA, Eu^{3+}-based quantitative *in vitro* binding assay; BO, blot overlay; CP, copurification; CS, cosedimentation; EM, electron microscopy; IE, immunoelectron microscopy; IF, immunofluorescence microscopy; IP, immunoprecipitation; PP, phosphorylation *in vitro* and/or *in vivo*.

action was revealed only when plectin cDNA expression constructs became available. In transient transfection experiments using COS and PtK2 cells it was observed that the expressed C-terminal domain proteins associated with the vimentin and cytokeratin filament networks and eventually caused their collapse into perinuclear aggregates. Mutant proteins corresponding to other parts of the plectin molecule, such as the rod, the N-terminal globular domain, or both, but lacking the C-terminal domain, neither colocalized nor affected the IF networks (Wiche *et al*., 1993). Subsequently, plectin's IF-binding site was mapped to a ~50 amino acid residue-long sequence located between the highly conserved core regions of plectin's C-terminal repeats 5 and 6, using a combination of different *in vivo* and *in vitro* assays, including cell transfection, targeted mutagenesis, quantitative *in vitro* binding assays using recombinant plectin mutant proteins expressed in bacteria, and electron microscopy of networks reconstituted *in vitro* from vimentin IFs and plectin mutant proteins. A short stretch of amino acids (Lys4262-Arg4280) with all the characteristics of a bipartite nuclear localization signal (NLS) turned out to be an essential part of this binding site (Nikolic *et al*., 1996).

Apart from vimentin, several other types of IF subunit proteins have been shown to interact with plectin, including desmin, GFAP, type I and type II cytokeratins, as well as the nuclear IF protein lamin B (Table 1). Dissociation constants of $K_D \approx 1\,\mu M$ were measured for the interaction of both vimentin and desmin with plectin's repeat 5; in the case of cytokeratins these values were considerably higher. The plectin-binding affinities of recombinant IF proteins (vimentin, cytokeratins) expressed in bacteria were indistinguishable from those of 'native' IF proteins isolated from cultured cells. Rising concentrations of plectin repeat 5 led to increased interlinking of all types of IFs assembled *in vitro*. However, at high molar ratios of plectin repeat 5 to IF protein (1:1, or above), filaments started to disassemble, apparently due to a destabilizing effect of the plectin protein. Cytokeratin IFs showed higher resistance against depolymerization than vimentin filaments, and the same was true for native compared to recombinant IFs (F.A. Steinböck, unpublished results).

There is evidence that plectin's interaction with IFs, and presumably other binding partners, is regulated by phosphorylation (see Table 1). Of particular interest was the observation that plectin is a target of $p34^{cdc2}$ kinase, and, during M-phase, becomes phosphorylated at a unique site (threonine 4542) in plectin's C-terminal repeat 6, not far from the IF binding site located in the C-terminal end of repeat 5. At the same time, plectin dissociates from cytoplasmic vimentin network structures, becomes more soluble, and is found diffusely distributed throughout the cytoplasm (Figure 2). Apparently the protein loses its cytolinker functions and may act as a regulatory element in the dramatic rearrangement of the cytoskeleton during M-phase (for a more detailed discussion see Foisner, 1997). In addition, it has been observed that the extend of *in vitro* interaction between plectin and lamin B is significantly decreased upon phosphorylation of plectin by either protein kinase A (PKA) or C (PKC), whereas plectin's binding to vimentin is differentially affected by these kinases (Foisner *et al*., 1991b).

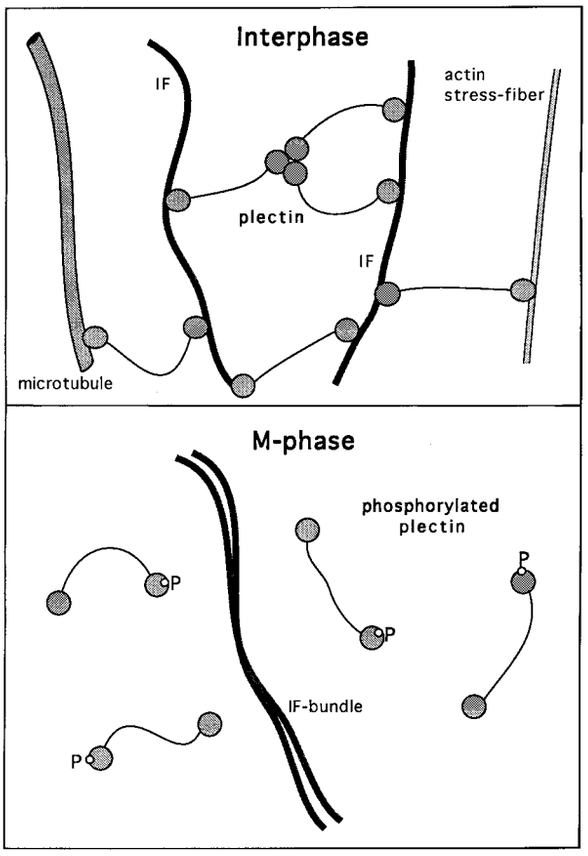

Fig. 2 Schematic Model of Plectin Function during Mitosis. During interphase plectin forms cytoskeletal network arrays by interlinking IFs, microtubules and actin filaments (upper panel). Upon phosphorylation by M-phase-activated $p34^{cdc2}$ kinase at a unique C-terminal target site (Thr4542), plectin dissociates from IFs which at the same time become bundled, while actin stress fibers and cytoplasmic microtubule networks disassemble. The depicted plectin structures are representative of polypeptides arranged as dimers (parallel) or tetramers (antiparallel dimers), without showing the second phosphorylation site of the tetrameric structure.

The C-terminal globular domain of the molecule also plays a role in the binding of plectin to integrins. A direct interaction between plectin and the β4 subunit protein of the hemidesmosomal α6β4 integrin receptor has recently been demonstrated (Rezniczek *et al*., 1998), and there is evidence for interaction with other types of integrin subunit proteins (unpublished data). *In vitro* and *in vivo* binding assays using cDNA expression constructs corresponding to various parts of plectin and integrin β4 showed that both the N- and the C-terminal globular domains of plectin contained integrin β4-binding sites. For optimal C-terminal binding plectin's repeat 6 domain turned out to be indispensable, but not sufficient. Interestingly, also for integrin β4 at least two separate plectin-binding sites were found within its cytoplasmic domain,

enabling multiple interactions between the two molecules. A tight interaction of plectin and integrin β4 is consistent with the phenotype of plectin-deficient EBS-MD patients (Gache *et al*., 1996; McLean *et al*., 1996; Smith *et al*., 1996) and plectin gene knockout mice (Andrä *et al*., 1997), which suggested an important role of plectin in the stabilization and mechanical strengthening of hemidesmosomal junctions. This major IF anchoring and cell adhesion device of epidermal basal keratinocytes uses the integrin receptor α6β4 as its major transmembrane element to provide a link between the extracellular matrix and the cytoskeleton (see also Niessen *et al*., 1997; and Schaapveld *et al*., 1998).

The N-Terminal Domain Binds to Actin, Affects Microfilament Dynamics, and Contains an Alternative Integrin Beta-4 Binding Site

The high-level expression of plectin in muscle tissue and its association with microfilament attachment sites, such as dense plaques of smooth muscle, Z-lines, and intercalated disc structures of skeletal and cardiac muscle, were early indicators that plectin might play a role in the organization of the actin-based cytoskeleton (Wiche *et al*., 1983; Zernig and Wiche, 1985). Furthermore, immunogold electron microscopy of whole mount cytoskeletons obtained from cultured cells showed that plectin molecules formed thin connecting bridges between cytoplasmic actin/myosin networks and vimentin IF arrays (Foisner *et al*., 1995; Svitkina *et al*., 1996). The N-terminal domain of plectin became the top candidate for mediating such interactions when a highly conserved actin binding domain (ABD) of the CH-CH type (Stradal *et al*., 1998), as found in spectrin and related proteins, was shown to reside near plectin's N-terminus by cDNA sequence analysis (Liu *et al*., 1996; McLean *et al*., 1996; Elliott *et al*., 1997). Andrä *et al*. (1998) recently demonstrated this site to be fully functional, using *in vitro* and *in vivo* approaches.

Based on binding data obtained with purified G actin and recombinant plectin fragments containing the ABD, a dissociation constant ($K_D = 3.2 \times 10^{-7}$ M) very similar to that of other actin binding proteins was determined. Furthermore, it was shown that plectin's binding to actin is dependent on the signaling molecule phosphaditylinositol-4,5-biphosphate (PIP2), which apparently binds within or close to plectin's ABD (Andrä *et al*., 1998).

The biological significance of the plectin-actin interaction was revealed in studies of primary fibroblast and astroglia cell cultures obtained from plectin (–/–) mice. Andrä *et al*. (1998) found that the actin cytoskeleton, including focal adhesion contacts, was developed more extensively in plectin-deficient cells than in wild-type cells. The plectin-deficient cells failed to show characteristic short-term rearrangements of their actin cytoskeletons in response to extracellular stimuli activating the rho/rac/cdc42 signaling cascades. As a consequence, cell motility, adherence, and shear stress resistance were altered, and morphogenic processes were delayed. Moreover, the actin stress fiber phenotype of plectin-deficient fibroblasts could be reversed to a large degree by transient transfection with expression plasmids encoding full-length plectin or plectin fragments containing the N-terminal ABD. These results revealed a novel role of plectin as a regulator of cellular processes involving actin filament dynamics, that clearly goes beyond its previously proposed roles as a mere scaffolding protein or mechanical stabilizer of cells.

The N-terminal globular domain of plectin harbors also one or more binding sites for the integrin α6β4 subunit protein β4 (Rezniczek *et al*., 1998; see also preceding chapter); their precise location and exon allocation awaits further analysis. According to amino acid sequence analysis several additional putative functional sites may reside in the N-terminal globular domain, including a nuclear localization signal in the segment encoded by exon 1, an ER targeting sequence within the segment corresponding to exons 8 and 9, phosphorylation sites for PKA (segments corresponding to exons 2 and 16), as well as some potential interaction sites for PKC and casein kinase 2 (unpublished data).

An intriguing aspect of plectin's N-terminal domain regarding its structural and potentially functional diversity is the large variety of differentially spliced transcripts exhibiting alternative first coding exons. It will be challenging to determine whether different protein products corresponding to all these isoforms are indeed expressed in different tissues and cells, and if so, whether they affect functional properties of the protein.

The Central Rod Domain Generates a Flexible Linker Spanning over Long Distances

Plectin's large central rod domain (Ile1474-Ser2758) is characterized by the presence of long stretches of heptad repeats distinctive of α-helical coiled coils (McLachlan and Stewart, 1975). Furthermore, as found by Fourier analysis, there is a staggered strict period of 10.4 for acidic as well as basic residues (Wiche *et al*., 1991), which shows that the rod is built of regions with alternating positive and negative charges. These structural properties of the rod domain suggest that it would be energetically most favorable for the protein to form parallel homodimers. Indeed, the existence of plectin in solution as a dimeric species has been confirmed by gel permeation chromatography, indicating a molecular weight of $\sim 1.1 \times 10^6$ (Weitzer and Wiche, 1987). It is conceivable that besides homodimers, heterodimers containing plectin isoforms with distinct N-termini encoded by alternative first coding exons could be formed. This may lead to increased functional diversity and potential for differential regulation of plectin.

The regular heptad repeat found in the rod sequence is punctuated several times by small regions which probably disrupt the coiled coil conformation locally. Such regions may represent areas of local flexibility enabling bends or

kinks to form in the otherwise rigid coiled coil conformation (Wiche *et al*., 1991). In fact, structures with strictly linear rod sections were hardly observed on the ultrastructural level. Rotary shadowing of specimens revealed bending to various degrees including complete loop formation due to self-interaction of the globular end domains (Foisner and Wiche, 1987). Thus, the nearly 200 nm-long rod of plectin appears to be ideally suited to connect binding partners of plectin over relatively long distances in a flexible way.

Due to its α-helical nature and surface charge the rod domain is highly antigenic. When the whole molecule was used as an immunogen to raise monoclonal antibodies, out of close to 20 different antibodies obtained, all, except for one, were reactive with epitopes residing along the rod domain, as determined by epitope mapping using a variety of methods (Wiche *et al*., 1991; Foisner *et al*., 1994; M. Stöcher, unpublished data). Furthermore, contrary to other molecular domains, it proved difficult to generate large quantities of the rod in recombinant form in bacteria. Generally, a cascade of shorter polypeptides were obtained in addition to the full length protein, as detected by immunoblotting analysis (F.A. Steinböck, unpublished data).

Summary and Outlook

Plectin's molecular properties, abundant expression in different types of cells and tissues, and strategic cytoarchitectural locations make it a cytolinker protein par excellence. Due to its tripartite molecular structure this protein seems perfectly designed to interlink cytoskeletal filaments and to connect them to junctional or anchoring structures by forming wide-streched bridges. The globular N-terminal and C-terminal domains, each of them harboring several subdomains, enable direct, and in some cases even multiple-site contacts to a variety of specific interaction partners. In addition, molecular target sites for kinases, including cell cycle-regulated $p34^{cdc2}$ kinase, and binding sites for signaling molecules, such as polyphosphoinositides, provide a broad spectrum of possible ways how plectin's versatile functions in the organization and dynamics of the cytoskeleton could be regulated. Finally, due to its long and flexible α-helical rod domain and its ability to self-interact, plectin is able to form cytoskeletal bridging elements of quite contrasting molecular shape, such as extended linear or bent dumbbells, or globules with extending filamentous protrusions or loops.

Future important research aims include the 3-dimensional structure analysis of molecular subdomains of plectin and of interfaces with its binding partners at the atomic level using X-ray diffraction of protein crystals. Furthermore, it will be of interest to elucidate biochemically the mechanism of plectin-induced IF disassembly and to localize plectin-binding sites on IF-subunit proteins, like vimentin, desmin, and cytokeratins. The biological significance of plectin transcript diversity is likely to become another focus of research. In particular, it will be interesting to investigate whether plectin isoforms encoded by differentially spliced transcripts have distinct functions or properties, such as binding activities, protein turnover, or cellular targeting. We expect these studies to provide further insights into plectin's role as cytolinker and putative regulator of viscoelastic properties of cells, and eventually they may contribute to a better understanding of how cytoskeletal network elements, in particular cytolinkers, influence and control morphogenesis and differentiation of cells.

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