Plectin repeats and modules: strategic cysteines and their presumed impact on cytolinker functions

Lubomír Janda,[1] Jiří Damborský,[2] Günther A. Rezniczek,[1] and Gerhard Wiche[1]*

Summary

Plectin, a member of the cytolinkers protein family, plays a crucial role in cells as a stabilizing element of cells against mechanical stress. Its absence results in muscular dystrophy, skin blistering, and signs of neuropathy. The C-terminal domain of plectin contains several highly homologous repeat domains that also occur in other cytolinkers. Secondary structure analysis revealed that the building block of these domains, the PLEC repeat, is similar to the ankyrin repeat. We present a model that attempts to explain how the C-terminal domain, which comprises ~1900 amino acid, could be stabilized to maintain its structural integrity even under extensive mechanical stress. In this model, larger solenoid modules formed from PLEC repeats can be disulfide-bridged via conserved cysteines. Our hypothesis suggests that this process could be mediated by cytoplasmic NOS-generated products, such as the radical peroxynitrite. Reinforcement of molecular structure would provide a rationale why during exercising or physical stress radicals areformed without necessarily being deleterious. This article contains supplementary material that may be viewed at the BioEssays website at http://www.interscience.wiley.com/jpages/0265-9247/suppmat/23/v23_11.1064. *BioEssays* 23: 1064–1069, 2001. © 2001 John Wiley & Sons, Inc.

Introduction

Plectin, a large cytoskeletal linker protein (> 500 kDa), is expressed as numerous differentially spliced variants that are abundant in a wide variety of mammalian tissues and cell types.[1] As the prototype of a growing family of proteins, known as plakins or cytolinkers,[2,3] that probably all arose from a common ancestor gene, plectin plays an essential role in cytoskeleton network organization with consequences for structural integrity and resistance of cells and tissues against mechanical stress. This concept is strongly supported by the finding that the hereditary disease epidermolysis bullosa simplex (EBS)-MD, characterized by severe skin blistering and muscular dystrophy, is caused by defects in the plectin gene,[4] and by the similar phenotype of plectin knockout mice.[5]

Structurally, plectin consists of a ~200 nm long central coiled-coil rod flanked by globular N- and C-terminal domains (Fig. 1A). In the C-terminal domain binding sites for intermediate filament (IF) subunit proteins, integrin β4 and α-spectrin/fodrin have been identified, as well as a unique cdk (cyclin-dependent kinase) $p34^{cdc2}$ phosphorylation site.[2] The structure of this domain is dominated by six highly homologous ~300 amino acid residues-long repeat domains (RDs),[6] which also occur in other cytolinker protein family members, such as desmoplakin [7] (three RDs), the epithelial and neuronal isoforms of BPAG1/dystonin [8] (two RDs), and envoplakin [9] (one RD). Additional structures shared by these proteins are the central α-helical rod and an N-terminal globular domain. Periplakin [10] has its N-terminal globular domain and its rod domain in common with plectin, but lacks C-terminal repeat domains. In contrast, epiplakin, a recently described protein, consists exclusively of repeat domains[11] (thirteen RDs). Another plectin-related protein, ACF7/MACF [12] and its *Drosophila* orthologue kakapo,[13] resembles plectin only in its N-terminal domain structure, but differs in rod and C-terminal domains.

In view of plectin's enormous size (> 500 kDa) and exposure to mechanical stress forces, the question arises how the molecule maintains its structural integrity. While dimerization and/or tetramerization of the polypeptide chains via their α-helical rod domains probably is an important factor, the mode of stabilization of the globular N- and C-terminal domains is not resolved. In this article, we suggest a model of plectin's C-terminal domain structure, which is largely based on secondary structure analysis. Furthermore, we present a hypothetical mechanism how nitric oxid synthase (NOS)-mediated disulfide bridge formation involving strategically located cysteines may stabilize this structure.

Plectin modules contain five ankyrin-like repeats with a predicted tertiary structure of a solenoid

In plectin, all six RDs share a strongly conserved central core region, hereafter referred to as the plectin (PLEC) module. The

[1]Institute of Biochemistry and Molecular Cell Biology, University of Vienna, Austria
[2]National Centre for Biomolecular Research, Masaryk University, Czech Republic
Funding agency: The Austrian Science Research Fund ; Grant numbers: P14520, S611.
*Correspondence to: Dr. Gerhard Wiche, Institute of Biochemistry and Molecular Cell Biology, University of Vienna, Dr. Bohrgasse 9, 1030 Vienna, Austria. E-mail: wiche@abc.univie.ac.at

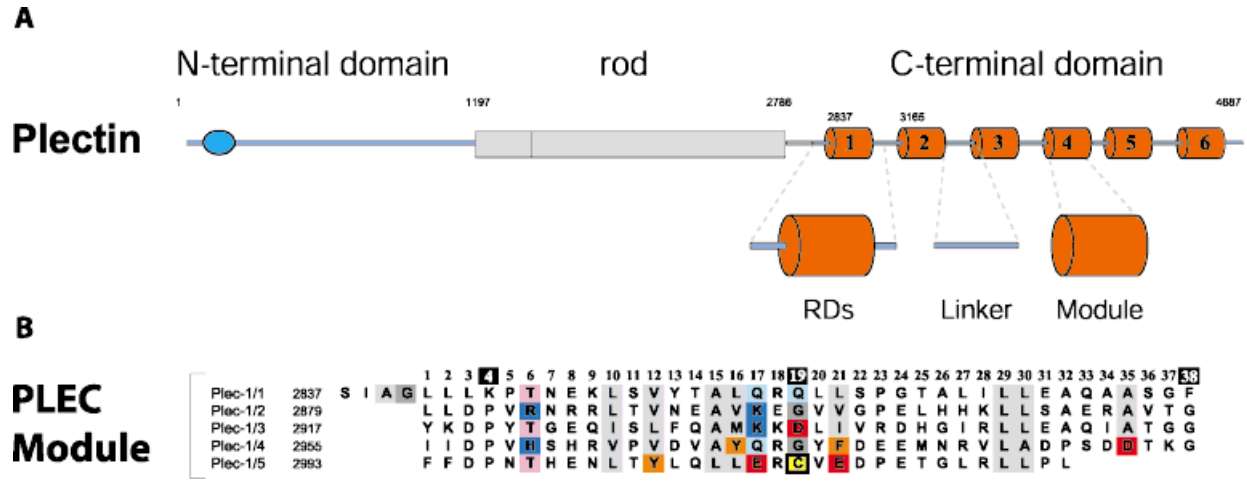

Figure 1. Schematic diagram of plectin's subdomain structure (**A**) and sequence alignment of rat PLEC repeats 1-5 contained in PLEC module 1 (**B**). **A:** N-terminal domain with its actin-binding domain (blue ellipsis), central rod, and C-terminal domain containing six RDs are indicated. Core regions of RDs forming PLEC modules are connected by linkers of different lengths. **B:** Numbers assigned to names specify module and repeat. Numbers preceding amino acid sequences specify position of first amino acid residues shown (database accession number: X59601), headline numbers indicate position within repeats. Conserved hydrophobic residues in both repeats are shown in light gray and the hydrophobic turn-forming amino acid glycine in dark gray. Positively charged amino acids are shown in dark blue, asparagine and glutamine in light blue, negatively charged amino acids in red, aromatic amino acids in orange, hydroxy-amino acids in pink, and cysteines in yellow.

six modules are connected to each other by less-conserved linker sequences of variable lengths (Fig. 1A). The module itself is composed of a 38-residues motif (2x 19-residues)[6] tandemly repeated five times (Fig. 1B), which has been defined in the databases (SMART, http://smart.embl-heidelberg.de/ and Pfam, http://www.sanger.ac.uk/cgi-bin/Pfam/, respectively) as the PLEC domain,[14] or plectin repeat,[15] hereafter called PLEC repeat. To identify other proteins with a similar motif, a threading search for remote homologues of the PLEC repeat was conducted, using the methods Bioinbgu,[16] (3D-PSSM v2.5.1,[17] HMM SAM-T99,[18] and GenTHREADER v2.0.[19] Bioinbgu revealed a strikingly similar composition of secondary elements in the PLEC repeats of plectin (consensus score of 6.4) and the ankyrin (ANK) repeats[20] of cyclin-dependent kinase inhibitor $p18^{Ink4c}$ (Fig. 2),[21] the three-dimensional crystal structure of which is known. Similar to the ANK repeat motif,[22,23] PLEC repeats have a hairpin–helix–loop–helix ($\beta_2\alpha_2$) structure. In the ANK repeat motif, two glycine residues (at positions 15 and 27) terminate the two α-helices, while a third glycine (at position 4) terminates the β-hairpin structure (see Fig. 2C in the supplementary material on the *BioEssays* website at http://www.interscience.wiley.com/jpages/0265-9247/suppmat/23/v23_11.1064. The PLEC repeat has only two highly conserved glycine residues (at positions 19 and 38) terminating the α-helices, whereas the β-hairpin structure is terminated by the proline in position 4 (Fig. 1B; and see Fig. 2B in the supplementary material at *BioEssays* website http://www.interscience.wiley.com/jpages/0265-9247/suppmat/23/v23_11.1064. A highly conserved proline is present also in the ANK motif (at position 7), but there it initiates the first α-helix.[21] Stacking of multiple ankyrin repeats results in a conserved tertiary structure, known as a solenoid, which is held together by a network of reciprocal hydrogen bond interactions.[23] The similarity of the PLEC motif to the ANK repeat, reported here for the first time, qualifies plectin (and related cytolinkers) as a new solenoid protein family member.

Strategic cysteines in plectin's C-terminus: a model

We postulate that solenoid PLEC modules contain a basic structural unit comprising five PLEC repeats arranged in tandem helices. The structure of the unit is stabilized through numerous contacts between the repeats, similar to ANK repeats.[22,23] Typically, two small hydrophobic amino acids (alanine and glycine) participating in β-hairpin formation are present in front of the first PLEC repeat in each module. Furthermore, our model proposes that the cysteines found in all of the six modules play a crucial role in preserving and reinforcing the overall tertiary structure of plectin's C-terminal domain. They are all located either outside, close to, or at the very borders of the α-helices forming the PLEC repeats (see Fig. 2B in the supplementary material on the *BioEssays* website at http://www.interscience.wiley.com/jpages/0265-

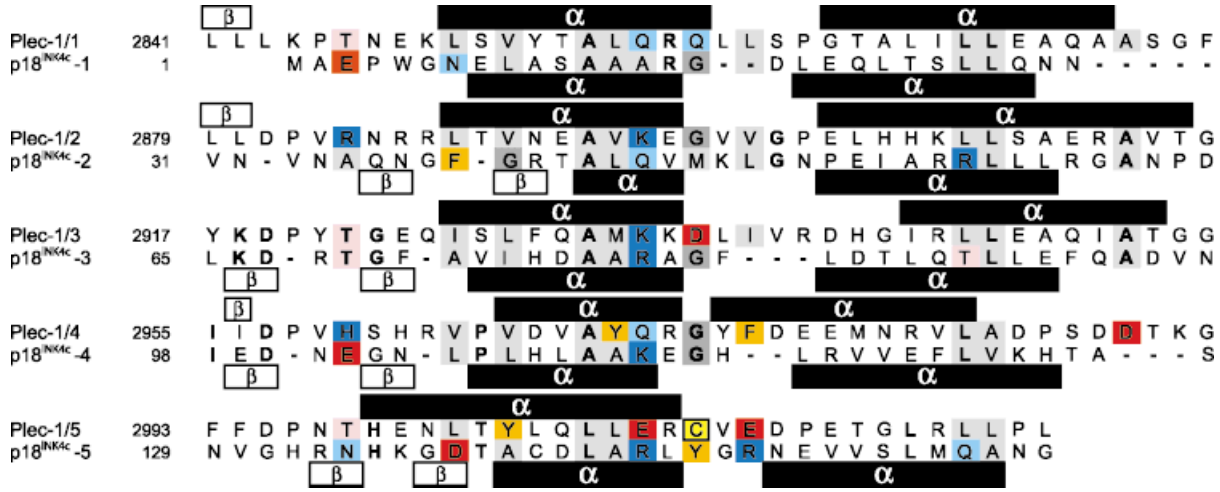

Figure 2. Sequence alignment of PLEC repeats 1–5 contained in rat PLEC module 1 and ANK repeats 1–5 of mouse cyclin-dependent kinase inhibitor $p18^{INK4c}$ (GI1168870). Numbers assigned to names indicate module (plectin) and repeat numbers (plectin and $p18^{INK4c}$), numbers preceding sequences indicate positions of first amino acid residues shown (according to databases). Amino acids are highlighted in color as specified in Figure 1. Residues identical in both sequences are shown in bold; black and white horizontal bars represent predicted α-helical and β-strand secondary structures (JPred program[39]), respectively; //, omission of 4 residues in $p18^{INK4c}$ sequence; dashes, sequence gaps introduced for better alignment.

9247/suppmat/23/v23_11.1064; Fig. 3A). This prompts us to suggest that these cysteine residues are instrumental in binding PLEC modules to each other independent of their linker sequences (Fig. 3A).

We propose a circular arrangement of plectin modules 1–5, with module 6 positioned in the center (Fig. 3A,B). In this model, consecutive modules are orientated antiparallel to each other. The linker sequence between modules 5 and 6 (Fig. 3A, blue loop) contains the IF-binding region,[24] whereas the tail of module 6 harbors a microtubule-binding site.[25] Thus this is a region of the molecule exposed to great mechanical stress, and this could be the reason why more than half of all the cysteines present in the entire C-terminal domain of rat plectin (7 of 13) reside within modules 5 and 6 (6 Cys) and their connecting segment (Cys_{4316}) (see Fig. 2B in the supplementary material at *BioEssays* website at http://www.interscience.wiley.com/jpages/0265-9247/suppmat/23/v23_11.1064; Fig. 3A). If covalent disulfide bridges indeed play a role in increasing the rigidity of the structure, as we predict, the only arrangement where all cysteines would be optimally placed is the one presented in our model (module 6 in center of modules 1–5). Any other arrangement would be less compact due to fewer contacts with the neighboring solenoids. Having less interfaces, solenoids will be held together by weaker interactions. This would make the molecule less resistant against mechanical stress and could have dramatic effects on the integrity and resistance of cells and tissues for transient time periods (e.g., in actively contracting muscle). Consistent with the proposed central position of module 6, we find that it contains slightly more (2–7%) hydrophobic residues than the other modules.

A comparison of the protein sequences of human and rat plectin showed 93% identity.[26] Surprisingly, the C-terminal part of human plectin contains four additional cysteines (in total 17), 30% more than the rat orthologue. It will be interesting to test whether this correlates with different mechanical properties or a more compact structure of human compared to rodent plectin.

On the ultrastructural level, single plectin molecules are visualized predominantly as dumbbells (Fig. 3C) with an average contour length of close to 200 nm. These structures represent either dimers formed by two polypeptide chains arranged in parallel,[2] or tetramers where two parallel dimers are arranged antiparallel.[27] The diameter of the globular end domains visualized by electron microscopy (~9 nm) correlates well with the diameter of a wire model of plectin's C-terminal domain (Fig. 3B). The C-terminal parts of the two polypeptide chains forming the dimer under certain conditions may also generate interchain disulfide bridges, leading to a further increase in the resistance of the molecule against mechanical stress.

A number of observations are consistent with the proposed solenoid modular model of plectin's C terminus. (1) Recombinant proteins corresponding to individual modules in vitro formed aggregates of fairly homogeneous sizes (5–10 nm) and shapes, the larger ones resembling those formed from

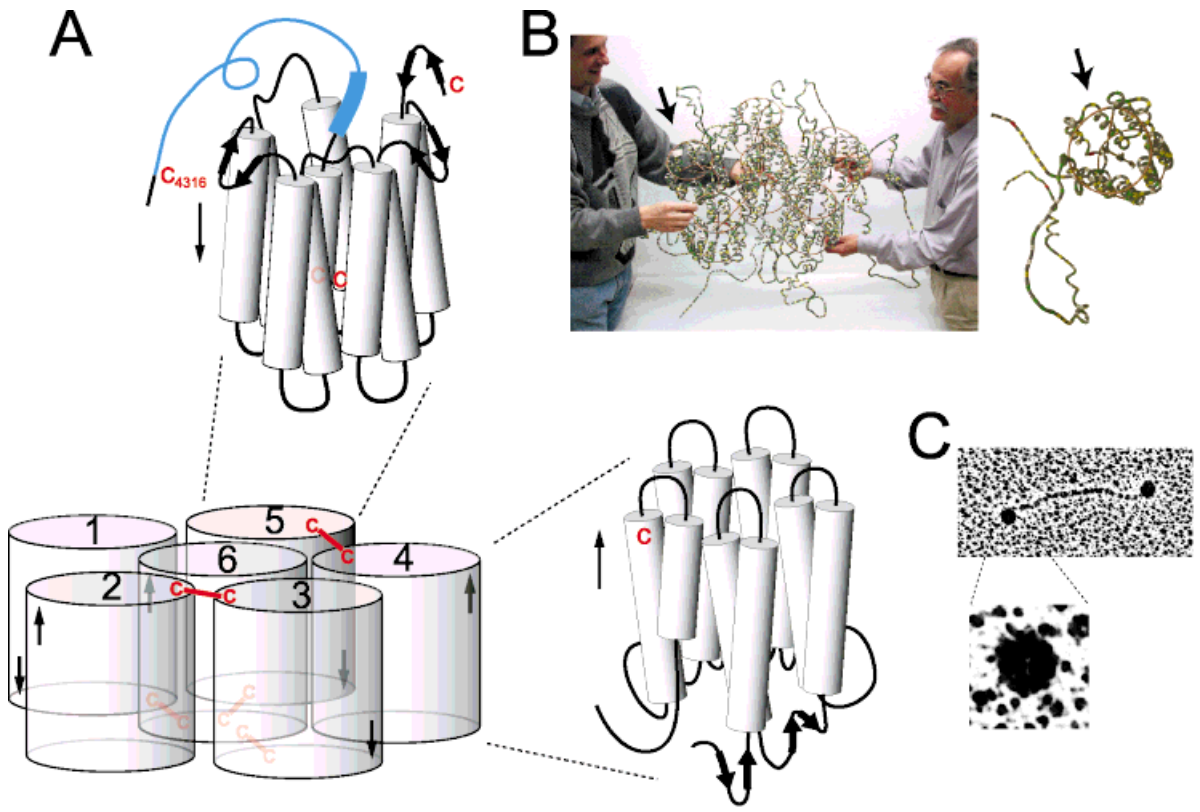

Figure 3. Predicted structure and electron microscopy of plectin's C-terminal domain. **A:** Schematic diagram of solenoid PLEC modules 1–6 arranged as compact 6-cylinder structure stabilized by disulfide bridges. Note antiparallel orientation of consecutive cylinders (arrows). Each module is formed of five $\beta_2\alpha_2$-PLEC repeats folded into cylindrical shape as shown for modules 4 and 5. Helix motifs of PLEC repeats are depicted as low-diameter cylinders connected by loops. Module-forming PLEC repeats are linked by β-hairpins (black arrows). According to secondary structure predictions, the most pronounced β-strand structures are the ones in front of the first PLEC repeat helix and those between helices 2/3, and 6/7. Linker sequences between modules (varying in length from 50 to >200 residues) are not shown, except for the IF/vimentin-binding region (blue line) containing an essential bipartite NLS-like basic residues cluster[24] (blue box). **B:** Hypothetical wire model built at a scale of 1:50 000 000 (1 Å = 0.5 cm). Arrows mark module 5. The module and its linker segment containing the IF-binding site are shown in detail next to the photograph. **C:** Rotary shadowing electron microscopy of a single plectin molecule and enlargement of one of its globular head domains. Head domains have 9 nm-diameters on average, as visualized by negative staining electron microscopy.[27]

modules 1–6 of the native molecule[24] (see Fig. 3C). (2) These oligomers were unusually stable towards reducing reagents, such as DTT or β-mercaptoethanol, even in the presence of urea, as demonstrated by PAGE. However, when similar modules were analyzed in which the cysteines had been mutagenized to serines oligomer formation could no longer be observed (unpublished data). This suggests that, in principle, the modules are capable of forming homo-oligomers as well as hetero-oligomers stabilized by highly resistant disulfide bonds. Heterooligomerization of PLEC modules may also play a role in interactions between different cytolinker protein family members.

It will be difficult to gather experimental backing for our model due to the nature of plectin and cytolinkers in general, especially their large size and limited solubility. However, recombinant PLEC modules generated individually or in certain combination (with and without mutated cysteine residues) should be useful for biochemical and crystallographic analyses. Although the question of how modules are positioned relative to each other can hardly be addressed by crystallography mainly due to limitations in solubility of specimens, it should be possible to measure fluorescence emission and CD spectra of wild-type and mutated PLEC modules under various conditions of denaturation to examine differences in chemical stability. Also, low resolution NMR should provide useful information about gross structural features.

Putative role of oxidants in structure preservation

The following considerations are in line with the view that disulfide bridges play a role as strengthening elements of cytoskeletal structures, particularly of those involving plectin and related cytolinkers.

(1) It seems likely that during evolution there has been an increased requirement for stable cytoskeletal protein structures that nevertheless are capable of undergoing dynamic changes. This demand could be satisfied by amino acids, such as cysteine, which can form strong (a disulfide bridge is $> 30-90$ times stronger than ionic or hydrogen bonds) and easily reversible covalent linkages.

(2) Cysteines normally are in their reduced state in the cytoplasm. It is not unusual, however, to find intramolecular disulfides resistant to DTT reduction[28,29] or pairs of intermolecular disulfides upon oxidation by NO and other oxidants.[30] If radicals (such as peroxynitrite) are present, thiol groups are preferentially oxidized (10^6 and 10^3 times more efficient than nucleic acids and other amino acids, respectively).[31] The reaction of cysteine with peroxynitrite is particularly fast (rate constant 10^{-3} M^{-1}, i.e., 1,000 times faster than its reaction with H_2O_2).[32]

(3) Peroxynitrite is generated from NO, the product of the nitric oxid synthase (NOS)-mediated conversion of arginine to citrulline, and superoxide O_2^- ($NO + O_2^- \rightarrow OONO^-$). The isoform of NOS expressed in skeletal muscle, neuronal NOS, is part of the utrophin/dystrophin-associated glycoprotein complex,[33] and, consequently, it is closely associated with the sarcolemma. Moreover, since peroxynitrite is 70 times more soluble in nonpolar solvents than in polar solvents and 90% of its reaction with O_2 occurs in the membrane,[34,35] it can be expected that this compound is predominantly present close to the cytoplasmic face of the plasma membrane, where plectin and other constituents of cytoskeleton–plasma membrane junctional complexes are concentrated. In fact, plectin has recently been found to colocalize with the utrophin-associated glycoprotein complex and to directly bind to more than one of its components (unpublished data). Thus, highly reactive oxygen intermediates, such as peroxynitrite, known to be generated in actively contracting muscle at increased levels,[36] could very effectively (in a locally restricted area) lead to the formation of intramolecular and intermolecular disulfide bridges via oxidation of cysteines in plectin and related cytolinker proteins.

(4) The importance of disulfide bridges in PLEC modules is underlined by the fact that all but one of the cysteines found in modules 4–6 of plectin are conserved in the three modules of desmoplakin. Furthermore, based on structural evidence, a possible role of disulfide bridge formation in increasing sarcomeric resistance under oxidative stress conditions has recently been suggested for another muscle protein, the giant polypeptide titin.[37] Similarly, a role of disulfide bonding in stabilization of cytoskeletal structures has been proposed for cytokeratins.[38]

To test the putative role of NOS in disulfide bridge formation in plectin molecules, several experimental approaches seem feasible. They range from colocalization studies of NOS and plectin isoforms in cells and tissues to quantification of SH groups in plectin, with and without stimulation of NOS, by chemical titration or fluorescence emission spectroscopy.

In conclusion, our hypothesis provides a rationale for the association of NOS, a potentially hazardous enzyme, with membrane-bound protein complexes in muscle fibers and other types of cells exposed to mechanical stress, including nerves. This goes beyond the previously proposed role of NOS in signaling. The development of larger muscle tissues and complex nervous systems very likely required the establishment of mechanisms capable of mechanically strengthening the cytoskeleton upon stress without compromising its dynamics. Rapid formation of disulfide bridges in cytolinker proteins, such as plectin, coupled to NOS-catalyzed formation of peroxynitrate could have satisfied this demand.

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