The ‘Spectraplakins’: cytoskeletal giants with characteristics of both spectrin and plakin families

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Summary

Recent studies have characterised a family of giant cytoskeletal crosslinkers encoded by the *short stop* gene in *Drosophila* and the *dystonin/BPAG1* and *MACF1* genes in mammals. We refer to the products of these genes as spectraplakins to highlight the fact that they share features with both the spectrin and plakin superfamilies. These genes produce a variety of large proteins, up to almost 9000 residues long, which can potentially extend 0.4 μm across a cell. Spectraplakins can interact with all three elements of the cytoskeleton: actin, microtubules and intermediate filaments. The analysis of mutant phenotypes in *BPAG1* in mouse and *short stop* in *Drosophila* demonstrates that spectraplakins have diverse roles. These include linking the plasma membrane and the cytoskeleton, linking together different elements of the cytoskeleton and organising membrane domains.

Key words: Spectrin, Plakin, Cytoskeleton, Adhesion

Introduction

A recently discovered family of cytoskeletal proteins belongs within both the spectrin and plakin superfamilies. These superfamilies consist of proteins that contribute to the linkage between the plasma membrane and the cytoskeleton. Spectrin superfamily members bind and crosslink actin filaments and attach these to membrane receptors. Members of the plakin superfamily were first identified as components of desmosomes and hemidesmosomes, connecting the adhesion receptors to intermediate filaments, but they also can crosslink different cytoskeletal elements. At present we know of two mammalian genes that encode related members of the new family, *BPAG1* and *MACF1*, and a single *Drosophila* gene, *short stop (shot)* (Figs 1 and 2). Each of these is also known by other names, which reflects their independent discovery by different groups: *MACF1* is known as *ACF7*, *MACF*, *MACF7*, *macrophin*, *trabeculin* α and *ABP620; BPAG1* is known as *dystonin* and *MACF2*; *shot* is known as *kakapo*, *kopupu* and *groovin* (Brown et al., 1995a; Bernier et al., 1996; Becker et al., 1997; Prout et al., 1997; Gregory and Brown, 1998; Strumpf and Volk, 1998; Walsh and Brown, 1998; Leung et al., 1999b; Okuda et al., 1999; Sun et al., 1999; Yang et al., 1999; Lee et al., 2000; Leung et al., 2001b; Sun et al., 2001). A single *Caenorhabditis elegans* gene can also be identified in the genomic sequence; this is currently annotated as three genes: ZK1151.1, ZK1151.2 and ZK1151.3 (*C. elegans* Sequencing Consortium, 1998). Because the proteins encoded by these genes share features with both spectrin and plakin superfamily members and may represent the evolutionary precursor of the plakins (see below), we refer to them as spectraplakins to highlight their dual nature. Here we discuss the possible functions of spectraplakins indicated by their sequences and by biochemical and genetic studies, extrapolating from the functions of homologous cytoskeletal proteins.

Spectraplakins in various species were identified independently in quite different contexts. In the fly, the spectraplakin *shot* emerged from genetic screens aimed at identifying genes required for integrin-mediated adhesion (Prout et al., 1997; Gregory and Brown, 1998; Strumpf and Volk, 1998) or axon extension (Van Vactor et al., 1993; Lee et al., 2000). The vertebrate spectraplakin BPAG1/dystonin was identified as a protein targeted by the autoimmune disease bullous pemphigoid in humans (Minoshima et al., 1991) and as the gene affected in the disease dystonia musculorum in mice (Brown et al., 1995a; Guo et al., 1995).

Diverse motifs within spectraplakin isoforms

The full complexity of the genes producing spectraplakins has gradually emerged. The first spectraplakin product characterised, BPAG1e, is a substantial protein of 2649 residues encoded by an 8.7 kb mRNA; yet this appears to be the smallest and simplest of the spectraplakin products (Fig. 3). The previous lack of awareness of the larger spectraplakins is likely to have led to erroneous conclusions being drawn in the older literature about the functions of the smaller spectraplakin isoforms, because with hindsight it seems more likely that some of these functions are performed by the larger isoforms. A complete understanding of which spectraplakin isoforms are made at appreciable levels is yet to come. Analysis of such large RNAs (from 8 to 27 kb) is by necessity achieved by piecing together short cDNAs, but whether the assembled sequence reflects the most abundant mRNA is less clear. Important progress has recently been made in defining the major isoforms by northern analysis (Leung et al., 2001b; Okumura et al., 2002). Examination of the gene structure reveals potential splice donor and acceptor sites that allow one to construct exceptionally large proteins, but at present some of these remain hypothetical.

To be able to refer to the domain structure of the different

spectraplakin gene products in a consistent manner, we have adopted the following nomenclature (see also Fig. 3). Running from N- to C-terminus, the main domains are abbreviated by a letter: 'C' for each calponin-homology domain, a small 'p' for the plakin domain that is present in all known isoforms, a capital 'P' for the plectin-repeat domain ('P' in *shot* and *MACF1*, 'P1' or 'P2' in *BPAG1*), 'S' for the spectrin-repeat domain and 'G' for the GAS2 domain. The four known N-terminal starts of translation are labelled 1, 2, 3 and e. In many cases the precise N-terminus is not known; so we indicate alternatives separated with a slash, for example, 1/2CCpPSG, 3C/epPSG, or 1/2/C/3/C/epPSG.

Spectrin superfamily motifs

The first sets of cDNAs pieced together for both *shot* and *MACF1* encode the 1CCpSG isoform, which overall has features characteristic of the spectrin superfamily (Fig. 1) (Gregory and Brown, 1998; Strumpf and Volk, 1998; Leung et al., 1999b). Proteins in this superfamily can be defined by two features: (1) an N-terminal actin-binding domain; and (2) a section of α-helical spectrin repeats. The completion of the *C. elegans* and *Drosophila* genome sequences (*C. elegans* Sequencing Consortium, 1998; Adams et al., 2000) has revealed the minimal set of members of the spectrin superfamily conserved in worms, flies and humans: one each of α-actinin, dystrophin and spectraplakin, and two β-spectrins. Two other members, *Drosophila* MSP-300 (Rosenberg-Hasson et al., 1996) and mammalian Nuance (Zhen et al., 2002), are similar but probably not orthologues, and there does not appear to be a worm protein similar to either. β- and α-spectrin appear to have arisen by the division of a single gene into two parts (Thomas et al., 1997), and so an α-spectrin should be included in the minimal set. As expected from the increase in genome size, mammals contain more copies of each of these proteins. For example, they have two spectraplakins, MACF1 and BPAG1, and two dystrophins, dystrophin and utrophin (not shown in figure) (Roberts and Bobrow, 1997).

The actin-binding domain (ABD) found close to the N-terminus in all members of the spectrin superfamily consists of two calponin-homology domains. This type of ABD is not restricted to this family; more distantly related versions are found in other types of actin-binding proteins, such as filamin (Korenbaum and Rivero, 2002), but the lack of spectrin repeats excludes these other proteins from the spectrin superfamily. The structure of the ABD in dystrophin and utrophin has been solved, revealing a bundle of α-helices arranged in an extended head-to-tail dimer (Keep et al., 1999; Norwood et al., 2000).

Four different N-termini, the result of different transcription start sites, have been characterised in the spectraplakins Shot and BPAG1 (Fig. 3) and three in MACF1. The first two start sites produce different short peptides preceding the ABD: 1=ShotA, BPAG1n1, MACF1 isoform 1; 2=ShotB, BPAG1n2, MACF1 isoform 2 (Brown et al., 1995b; Bernier et al., 1996; Yang et al., 1996; Gregory and Brown, 1998; Lee et al., 2000) (N.H.B., unpublished). Comparable differences in the plectin sequence preceding the ABD alter its interaction with the integrin β4 cytoplasmic tail (Geerts et al., 1999), but it is not yet known whether these different N-termini alter the function of spectraplakins. The other two start sites produce forms lacking part or all of the ABD (Bernier et al., 1996; Yang et al., 1999; Lee et al., 2000). Transcription start 3 (used in ShotC, BPAG1n3 and MACF1 isoform 3) splices halfway into the ABD, producing a protein that has a single calponin-homology domain: 3CpSG. In BPAG1n3, this change destroys the actin-binding ability but uncovers microtubule-binding activity (Yang et al., 1999). Use of transcription start e results in a protein completely lacking the calponin-homology domains of the ABD (BPAG1e and ShotD). The alternative start sites also provide tissue-specific expression, as has been shown for both *BPAG1* and *shot* (Yang et al., 1996; Lee et al., 2000).

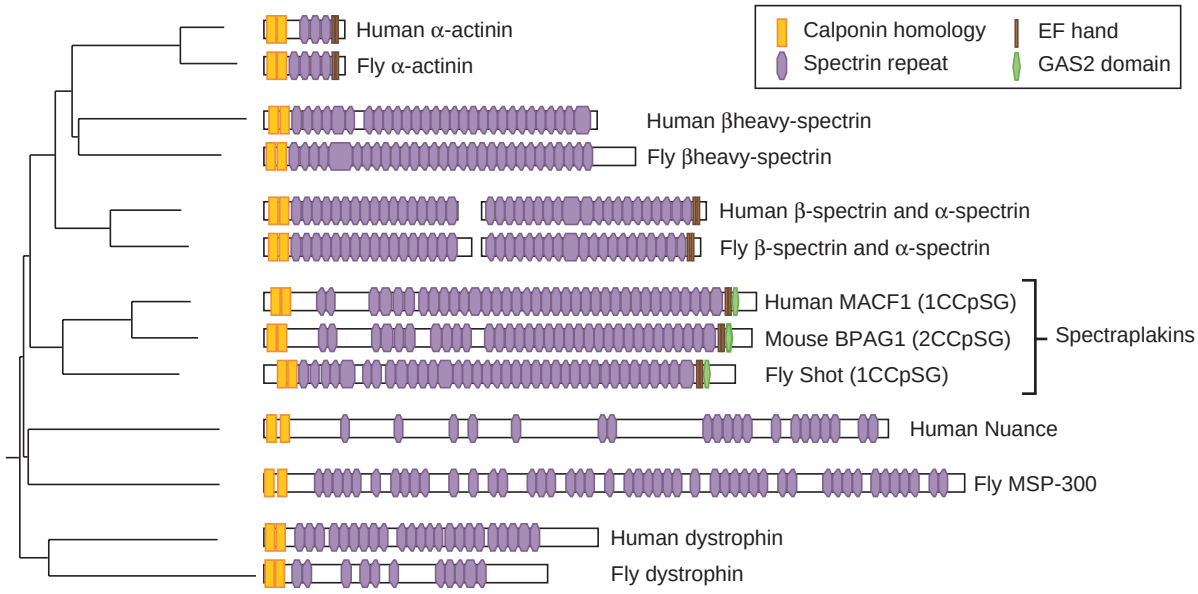

Fig. 1. The spectrin protein superfamily. Depicted is a selection of members of the spectrin family of proteins, comparing human and fly orthologues for each member, and in the case of spectraplakins showing both mammalian genes (*MACF1* and *BPAG1*). The spectrin family members are arranged in a phylogenetic tree on the basis of the sequences of their calponin-homology type actin-binding domains. Protein domains detected by SMART analysis are shown in coloured boxes (Schultz et al., 1998).

The second characteristic feature of the members of the spectrin superfamily is a series of spectrin repeats (also called dystrophin repeats). Each repeat is between 110 and 120 residues long and forms a three-helix bundle (Yan et al., 1993; Pascual et al., 1997). The row of α-helical spectrin repeats in spectrin family members is thought to form an extended rod-like structure whose function is to separate the different functional domains at either end of the molecule. For example, in dystrophin the repeats separate the N-terminal ABD from the C-terminal membrane-interaction domain (Brown and Lucy, 1997). A form of Shot lacking the rod domain still has some activity in vivo, because it is able to rescue certain phenotypes (Lee and Kolodziej, 2002a). However, deletion of a few repeats of dystrophin is sufficient to cause a mild form of muscular dystrophy (Palmucci et al., 1994), and so the absolute length of the rod can be essential for optimal function.

The greatest diversity within the spectrin superfamily occurs at the C-terminus. Several members of the superfamily have a pair of EF hands, although some are too divergent to be detected by domain-profiling programmes, such as SMART (Schultz et al., 1998). The EF hands of α-actinin and spectrin bind Ca^{2+} (Witke et al., 1993; Lundberg et al., 1995) and are essential for some aspects of Shot function (Lee and Kolodziej, 2002a). Dystrophin and the related protein utrophin contain a domain that interacts with the transmembrane protein dystroglycan and the cytoplasmic protein syntrophin (Koenig et al., 1988). The C-terminus of MSP-300 contains a leucine zipper not found in other family members (Rosenberg-Hasson et al., 1996), whereas the spectraplakins are the only ones to contain a GAS2 domain. There are GAS2 domains in the protein products of five genes in the human genome: *BPAG1*, *MACF1*, *GAS2* (growth arrest specific protein 2), *GAR17* (GAS2 related) and *GAR22* (Lander et al., 2001; Venter et al., 2001). Similarly, the genomes of *Drosophila* and *C. elegans* each have two genes encoding proteins containing this domain: the spectraplakin gene and a shorter gene (CG3973 and M116.5, respectively) (*C. elegans* Sequencing Consortium, 1998; Adams et al., 2000). GAS2 is a small protein that was identified through its upregulation upon growth arrest in cultured cells (Brancolini et al., 1992). Recent SMART analysis shows that GAS2 and GAR22 also share with the spectraplakins a calponin-homology domain; M116.5 contains four such domains (K.R., S.L.G. and N.H.B., unpublished). Deletions or protease-cleavage products of the GAS2 protein that contain the GAS2 domain give dramatic, apoptosis-like rearrangements of the actin cytoskeleton in cell culture (Brancolini et al., 1995). Despite these effects on actin, experiments described below indicate that the GAS2 domain binds microtubules. Analysis of current cDNA sequences in the database (K.R., S.L.G. and N.H.B., unpublished) shows that each spectraplakin gene has two alternatively spliced versions of the sequence near the GAS2 domain; however, the natures of these variants differ between the species, and there are not yet data on how these changes alter function.

Plakin superfamily motifs

The plakins are a family of proteins that share two features: (1) a plakin domain at the N-terminus; and (2) a set of short repeats recently termed plectin repeats (Schultz et al., 1998), which are an internal repeat within the so-called plakin-repeat domain (Leung et al., 2001a). In addition to the spectraplakins, the plakins include plectin, desmoplakin, envoplakin, periplakin and epiplakin (Ruhrberg and Watt, 1997; Leung et al., 2001a; Leung et al., 2002). Because *Drosophila shot* and its *C. elegans* orthologue are the only genes in these organisms that have a plakin domain or plectin repeats, it seems likely that a spectraplakin gene was the progenitor of the plakin family, which subsequently expanded in vertebrates (Leung et al., 2001a).

Despite being a defining feature of plakins, the plakin domain appears to be derived from a series of spectrin repeats, since SMART and other domain-recognition programmes are able to detect divergent spectrin repeats within the plakin domain (Fig. 2). However the plakin domains are much more similar to each other than to the spectrin repeat region of spectrin superfamily members, which suggests that the divergence has given rise to a new function that has been conserved. The plakin domain lies either at the N-terminus or just following the ABD and contains sites that mediate direct or indirect binding to adhesion receptors. The plakin domain of BPAG1e (epP1) binds directly to the transmembrane protein BPAG2 [BP180 (Hopkinson and Jones, 2000)] and that of

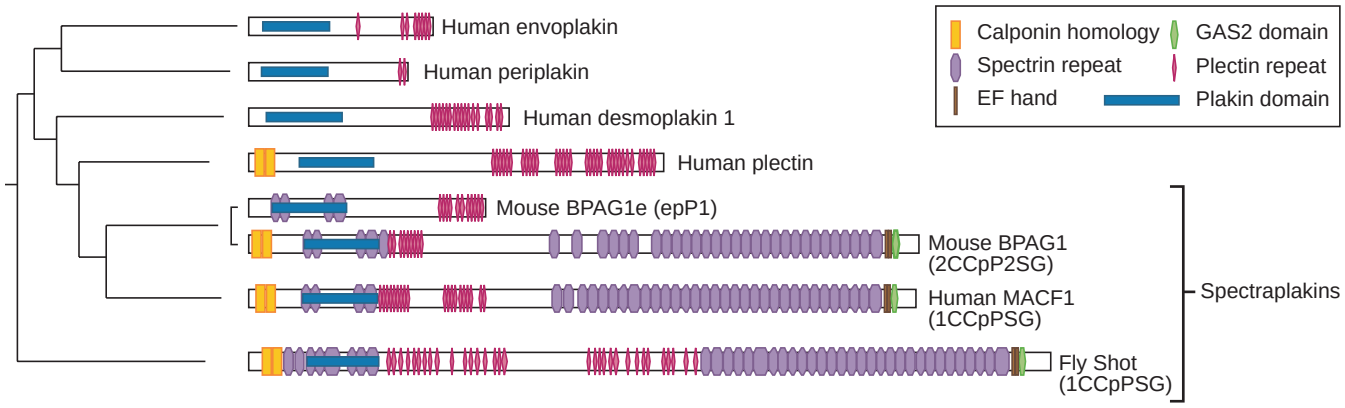

Fig. 2. The plakin protein superfamily. Depicted are all known members of the plakin family of proteins containing the plakin domain, arranged in a phylogenetic tree on the basis of the sequences of plakin domains (blue bar). Protein domains detected by SMART analysis are shown in coloured boxes (Schultz et al., 1998).

plectin binds to the β4 integrin subunit (Rezniczek et al., 1998). The plakin domain of desmoplakin binds to plakoglobin and plakophilin, which in turn bind to the desmosomal cadherins, desmoglein and desmocollin (Smith and Fuchs, 1998). Thus, in contrast to the spectrin repeat domain, the plakin domain is not a spacer rod but an important interaction domain.

Defining the beginning and end of a plectin repeat is not straightforward, as these repeats frequently occur in tandem. Recent structural analysis has revealed a structural repeat of 38 residues that forms a β-hairpin followed by two antiparallel α-helices (Choi et al., 2002). 4.5 of these repeats form a complex globular domain; desmoplakin contains three such domains, and plectin contains six (Janda et al., 2001). The only function known for these repeats is to bind intermediate filaments (Ruhrberg and Watt, 1997; Leung et al., 2002), but there are two reasons to think that they have additional functions. The first is that the *shot* gene has an extensive array of plectin repeats, but the *Drosophila* genome lacks genes encoding cytoplasmic intermediate filaments (Adams et al., 2000). The second is that the arrangement of the plectin repeats in spectraplakins, particularly MACF1 and the invertebrate proteins, is not organised into the groups of 4.5 repeats that are so well conserved in the other plakins. This suggests that they do not form the same kind of globular domain.

In addition, in spectraplakins the plectin repeats are found not only at the C-terminus, as in the other plakins, but also in the middle of larger protein isoforms (Figs 2 and 3). The plectin repeats in *C. elegans*, *Drosophila* and *MACF1* are all contained within a single large exon, whereas in the *BPAG1* gene they are encoded in two sets of exons (P1 + P2). The *BPAG1* gene product BPAG1e (epP1) that has C-terminal plectin repeats is the major epithelial isoform (Leung et al., 2001b). Evidence from ESTs shows that the *C. elegans spectraplakin* gene also produces an isoform that has the plectin repeats at the C-terminus (wormbase: http://www.wormbase.org/). The *BPAG1* gene produces another isoform that has the plectin repeats in the middle of the protein, but only the second set [1/2C/3/C/eP2SG (Leung et al., 2001b)]. The *MACF1* and *shot* genes appear only to produce isoforms in which the plectin repeats are in the middle of the protein (1/2C/3/C/epPSG), judging from northern analysis for *MACF1* (Gong et al., 2001), and genomic sequence and EST evidence (Rubin et al., 2000) for *shot* (Fig. 3) (K.R., S.L.G. and N.H.B., unpublished). Given that *Drosophila* lacks cytoplasmic intermediate filaments but *C. elegans* has them (Goldman, 2001), it is attractive to speculate that the spectraplakin plectin repeats have a novel, at present unknown, function when in the middle of the protein and bind intermediate filaments when at the C-terminus.

Plectin and the spectraplakins are the only plakins to have an ABD at the N-terminus (Fig. 2). It remains possible that plectin is a third spectraplakin, and, if so, we would predict that it has exons downstream that encode spectrin repeats and a GAS2 domain, but this genomic sequence is not yet available.

Summary of spectraplakin gene products

From the sequence of the genes, we can deduce that the spectraplakins have a series of five protein domains: (1) a choice of four N-termini, which may include an ABD; (2) a plakin domain, related to spectrin repeats, that appears to be present in all spectraplakin isoforms; (3) a plectin-repeat-containing segment, which may be at the C-terminus or within the protein; (4) a long rod composed of spectrin repeats; and (5) a C-terminal interaction domain that contains two EF hands and a GAS2 domain. So far three major variations have been observed: (1) the epP1 form of BPAG1, consisting of plakin plus plectin repeats; (2) the 1/2CCpSG forms of all three genes, which contain an ABD, plakin and spectrin repeats and a GAS2 domain; and (3) the 1/2CCpP1SG or 1/2CCpPSG forms containing all domains. It is still somewhat unclear which N-terminal variants are combined with each of the C-terminal possibilities (Fig. 3). The combination of ABDs and the short plectin-repeat-containing C-terminus (1/2CCpP1 or 3CpP1, formerly known as BPAG1n), however, seems to be a minor form of the *BPAG1* gene product rather than the main nervous system variant (Brown et al., 1995a; Yang et al., 1996; Leung et al., 2001b). Splicing together in silico all of the exons downstream of the alternative starts in *BPAG1*, *MACF1* and *shot* produces 27 kb mRNAs encoding proteins of >8500 residues with a molecular mass of almost 1000 kDa (Figs 2 and 3). If these proteins have an extended structure similar to dystrophin and spectrin, they would have a length of >400 nm.

Spectraplakins interact with all three cytoskeletal elements: actin, microtubules and intermediate filaments

The large size and modularity of spectraplakins and the known functions of some of the protein domains incorporated suggest a multitude of possible interaction partners and, potentially, different sets of binding partners for different spectraplakin isoforms. Homo- or hetero-dimerisation of some members of both spectrin and plakin protein families adds another level of complexity (Chan et al., 1998; Geerts et al., 1999).

The ABDs of mouse MACF1, BPAG1 and *Drosophila* Shot bind to actin in vitro (Yang et al., 1996; Andra et al., 1998; Leung et al., 1999b; Karakesisoglou et al., 2000; Lee and Kolodziej, 2002a). The dissociation constants of these domains for actin are comparable for MACF1 (0.35 μM), Shot (0.022 μM), BPAG1 (0.21 μM), plectin (0.32 μM) and dystrophin (0.13 μM) (Ohanian et al., 1984; Jarrett and Foster, 1995; Yang et al., 1996; Andra et al., 1998). In the case of dystrophin and plectin, three specific subdomains in the tandem calponin-homology domains mediate actin binding (Fontao et al., 2001). In addition, the ABD interacts with other proteins. In plectin and BPAG1, part of this domain mediates homo- and hetero-dimerisation, and in plectin the actin-binding site also binds to the cytoplasmic tail of the β4 integrin (Geerts et al., 1999). When the ABD alone of MACF1 is expressed in mouse keratinocytes, it associates with actin, but the longer 1CCpSG isoform of MACF1 containing this ABD associates with microtubules rather than actin (Karakesisoglou et al., 2000). MACF1 is predominantly found at the peripheral ends of microtubules in these cells. When a similar long form of Shot is expressed in vertebrate fibroblasts, it associates with aggregates of actin and microtubules, and, if the ABD is deleted, the protein just associates with microtubules (Lee and Kolodziej, 2002a). However, in the converse situation, when the microtubule-binding domain of Shot is deleted, the association of the modified protein with actin filaments is substantially reduced. These results suggest that some

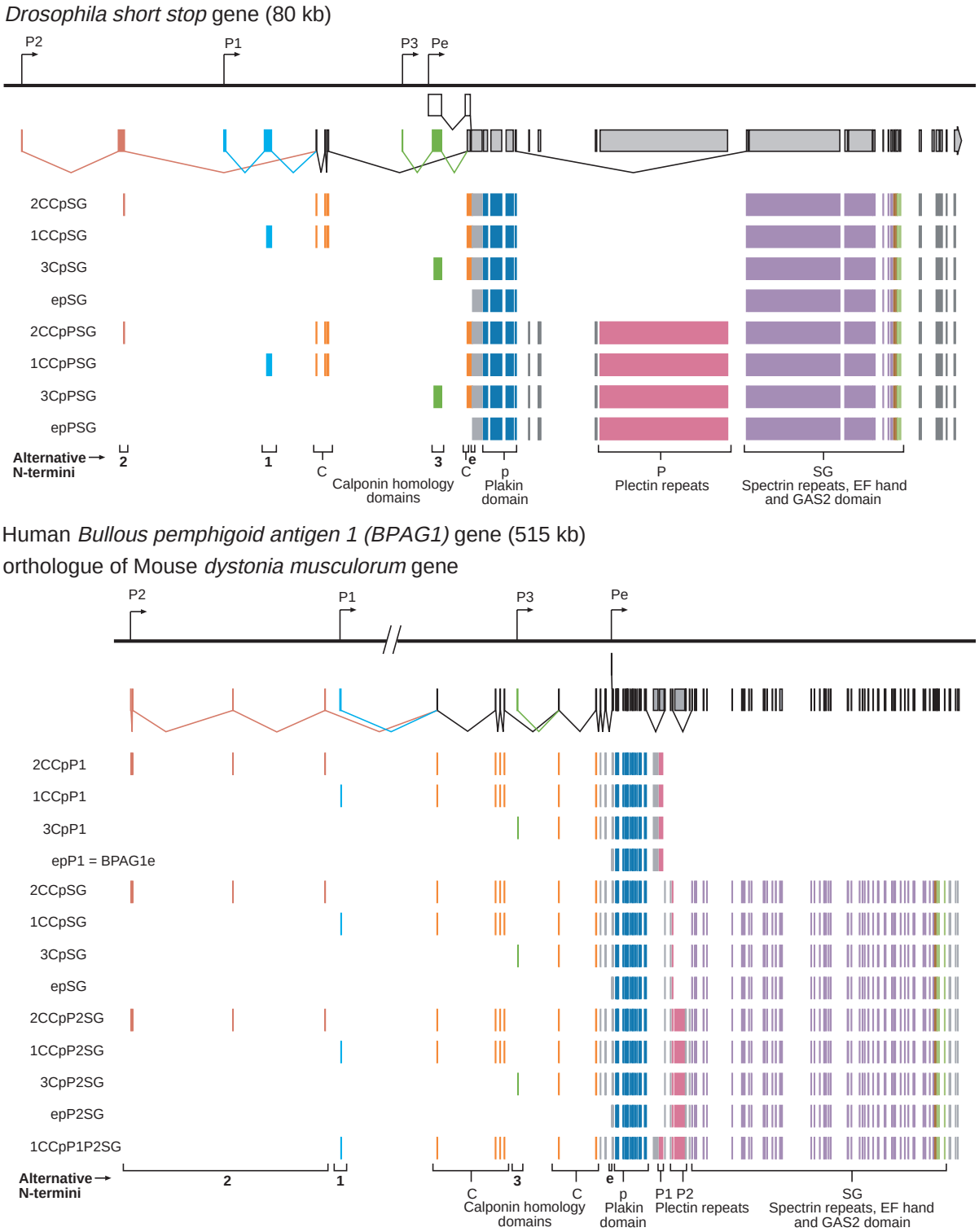

Fig. 3. Spectraplakin gene structure, splicing and protein isoforms. The *Drosophila shot* gene and the human *BPAG1* gene are shown. The arrows above the line show the alternative starts of transcription (P2, P1, P3, Pe). Exons are indicated by boxes, and splicing is only shown for the alternative starts or where exons are bypassed. Different alternative transcripts are then shown below each gene. The colours of the alternative N-termini are random, but the colours of the other exons reflect the protein domains encoded, as used in Figs 1 and 2. Below these is the labelling of the different domains used in the new nomenclature: the different N-termini (2,1,3,e for the very N-termini, C for each of the two calponin homology domains), the common plakin domain (p), the plectin-repeat domain (P in *shot*, P1 or P2 in *BPAG1*), the spectrin-repeat domain, EF hands and the GAS2 domain (SG). On the left is the description of each isoform using the new nomenclature. Additional variation in the GAS2 domain for both genes and in the splicing linking the plakin domain to the spectrin repeats in *shot* are not shown. It is not known whether all these forms exist. For example recent northern analysis could not detect BPAG1 transcripts containing the C domains with P1, or P1 with P2 (Leung et al., 2001b).

activation of the ABD is needed when it is in the context of the large isoform.

The existence of microtubule-binding activity in the spectraplakins was first suggested by the colocalisation of Shot with microtubules and the detachment of microtubule bundles from the plasma membrane in *shot* mutant embryos (Gregory and Brown, 1998; Prokop et al., 1998b). The plakin domain of the isoform BPAG1n3 (3CpP1) has microtubule-binding activity (Yang et al., 1999), as does the isolated plakin domain of MACF1 (Karakesisoglou et al., 2000), but this activity cannot be demonstrated in the context of the surrounding protein sequences (Leung et al., 1999b). A second microtubule-binding domain has been identified, consisting of a region including the GAS2 domain (Sun et al., 2001; Lee and Kolodziej, 2002a). Segments of MACF1 and Shot containing this domain associate with microtubules and stabilise them both in vitro and when expressed in cell culture (Leung et al., 1999b; Sun et al., 2001). Deletion of this domain from the full-length (1CCpSG) form of Shot causes loss of microtubule binding. In addition, this domain binds directly to α-tubulin in a yeast two-hybrid analysis (Lee and Kolodziej, 2002a). Recently, the equivalent domain of GAS2 itself has also been shown to associate with microtubules in transfected cells (Sun et al., 2001), which suggests that microtubule binding is the conserved function of this domain.

Plakins associate with intermediate filaments (Ruhrberg and Watt, 1997; Leung et al., 2002). The plectin-repeat regions of plectin, desmoplakin and BPAG1e bind to intermediate filaments (Nikolic et al., 1996; Leung et al., 1999a; Choi et al., 2002). This appears to be the only intermediate-filament-binding site, because longer forms of MACF1 lacking the plectin repeats (1/2CCpSG) do not associate with intermediate filaments (Leung et al., 1999b; Karakesisoglou et al., 2000).

From these studies we are confident that the spectraplakins are able to interact with all three cytoskeletal components. One of the most abundantly expressed isoforms, BPAG1e (epP1), binds to intermediate filaments. The BPAG1n1 (1CCpP1) and BPAG1n2 (2CCpP1) isoforms can link actin to intermediate filaments (Yang et al., 1999), whereas the BPAG1n3 (3CpP1) form has been shown to align neurofilaments with microtubules (Yang et al., 1996). The spectraplakin 1/2CCpSG form can link actin to microtubules, as demonstrated for BPAG1 and Shot – as can an engineered mini version of Shot consisting of just the ABD fused to the GAS2 domain [CCG (Leung et al., 1999b; Lee and Kolodziej, 2002a)].

Spectraplakin function

In addition to our knowledge derived from the phenotypes of mutations in the *shot* and *BPAG1* genes (see below), it is helpful to extrapolate from the functions of other members of the spectrin and plakin superfamilies. The simple picture of the members of both of these superfamilies is of two globular protein interaction domains, often separated by a long α-helical rod. Most attention has focused on two activities of the spectraplakins: crosslinking cytoskeletal filaments, and linking the cytoskeleton to plasma membrane proteins (Fig. 4, 1 and 2). These activities are shared with other members of the

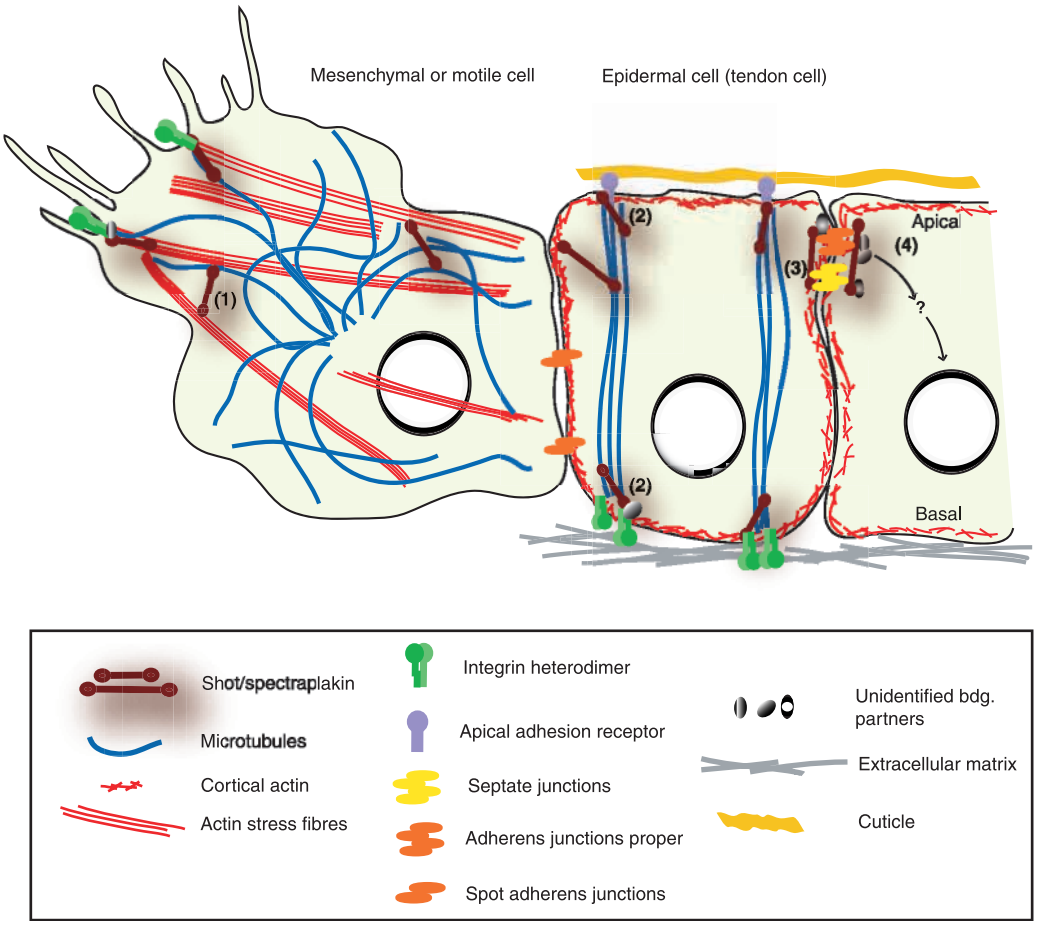

Fig. 4. Functions of spectraplakins. Spectraplakins have at least four different known or proposed modes of function in a cell, depicted here in the *Drosophila* context: (1) crosslinking of cytoskeletal filaments; (2) linking the cytoskeleton to plasma membrane proteins; (3) organising the interaction between the cortical cytoskeleton and plasma membrane proteins to generate membrane subdomains; and (4) acting as a scaffold protein that recruits signalling proteins to sites of cytoskeletal activity.

spectrin and plakin superfamilies, such as α-actinin, plectin and desmoplakin, and there is good evidence to support these functions. However we also wish to highlight two other potential functions of the spectraplakins. The first is similar to that of spectrins and dystrophin in organising the interaction between the cortical cytoskeleton and plasma membrane proteins to generate cortical subdomains (Fig. 4, 3). This could involve clustering of a subset of membrane receptors and cytoskeleton-binding proteins at sites of cell-cell contact or other specialised membrane domains – this would be similar for instance to spectrin clustering sodium channels and ankyrin-G in axonal membranes at the nodes of Ranvier (Komada and Soriano, 2002). The second function is as a scaffold protein that recruits signalling proteins to sites of cytoskeletal activity (Fig. 4, 4). The giant size of spectraplakins offers a large number of potential binding sites for other proteins.

Before discussing these functions in detail, we need to introduce the mutant phenotypes of spectraplakins. Because *shot* is the only spectraplakin/plakin gene in *Drosophila*, it is not surprising that mutations in *shot* have a stronger phenotype than mutations in *BPAG1*, since there is likely to be redundancy between BPAG1, MACF1, plectin and the other plakins. *shot* is a very large gene; any mutagenesis is therefore likely to generate several alleles, and so several different aspects of its function have been uncovered in screens by different groups looking at different processes (Van Vactor et al., 1993; Prout et al., 1997; Prokop et al., 1998b; Strumpf and Volk, 1998; Walsh and Brown, 1998; Gao et al., 1999). Every allele isolated so far is embryonic lethal when homozygous, although in many cases there are no gross morphological defects. Closer examination has shown that *shot* homozygotes have defects in epidermal integrity, epidermal muscle attachments, axon outgrowth, dendritic branching, neuromuscular junctions, sense organ development and anastomosis of the tracheal branches (Gregory and Brown, 1998; Prokop et al., 1998b; Strumpf and Volk, 1998; Gao et al., 1999; Lee et al., 2000; Lee and Kolodziej, 2002a; Lee and Kolodziej, 2002b). When clones of cells in the adult are made homozygous for mutant *shot* alleles, the most obvious defect is a dramatic loss of adhesion between the two cell layers forming the wing, resulting in wing blisters (Prout et al., 1997; Walsh and Brown, 1998).

Some aspects of these phenotypes occur in mice possessing mutations in the *BPAG1* locus, *dystonia musculorum* (*dt*). These mutations arose spontaneously in mouse populations (Duchen et al., 1963), presumably as a consequence of the large size of the gene. Homozygous *dt/dt* mice suffer progressive degeneration of the sensory neurons, which start about 10 days after birth. Only a subset of the neurons that express BPAG1 degenerates (Bernier et al., 1995; Dowling et al., 1997b), possibly because they are the ones with little expression of MACF1. Defects are first seen during embryogenesis, but loss of motor activity does not occur until after birth. In addition, defects in the functions of Schwann cells and the muscles have been reported (Dowling et al., 1997a; Bernier et al., 1998; Dalpe et al., 1999). The BPAG1e (epP1) isoform was independently discovered as an essential component of hemidesmosomes in epithelial cells. There, it mediates a direct link between intermediate filaments and the hemidesmosomal transmembrane proteins BPAG2 and the β4 integrin subunit (Hopkinson and Jones, 2000). A knockout mutation in *BPAG1*, which eliminates the common plakin-domain-encoding exons and so may be a null allele, has a skin-blistering phenotype that is similar to that evident in the human hereditary disease epidermolysis bullosa (Guo et al., 1995). A different form of this disease, epidermolysis bullosa simplex associated with muscular dystrophy, is caused by mutations in the human *plectin* gene (McLean et al., 1996; Smith et al., 1996), and the *plectin*-knockout mouse has a similar defect (Andra et al., 1997). Characterisation of the *dt* gene and the phenotype of a *BPAG1* knockout revealed that *BPAG1* and *dt* are the same gene (Guo et al., 1995). Not all alleles of *dystonin* disrupt the skin isoform BPAG1e (epP1), and one allele deletes exons encoding part of the ABD, eliminating all forms generated from the transcription start sites 1 and 2 (Brown et al., 1995a; Guo et al., 1995).

The skin blistering observed in *BPAG1*- or *plectin*-mutant animals appears to be analogous to the wing blistering in *Drosophila shot* mutant animals but also to the separation of the epidermis from the muscles in the mutant embryos. The phenotypes show further similarities at the ultrastructural level. There is loss of electron-dense material from the inner surface of the basal cell junction, and the cytoskeleton detaches from the junction, resulting in the epidermal cell ripping in half in response to mechanical stress (Guo et al., 1995; Prokop et al., 1998b). In both mammals and flies, the adhesion of the basal junction to the extracellular matrix is integrin dependent, and the spectraplakin is found associated with the integrin junctions (Gregory and Brown, 1998). However, there are some important differences. The integrin of mammalian hemidesmosomes α6β4 contains a uniquely long cytoplasmic tail, which interacts directly with the ABD of plectin, and the plakin domain of both plectin and BPAG1e (Rezniczek et al., 1998; Geerts et al., 1999; Hopkinson and Jones, 2000). Both plectin and BPAG1e in turn bind directly to the keratin intermediate filament network (Nikolic et al., 1996; Leung et al., 1999a). *Drosophila* lacks orthologues of β4 integrin and BPAG2, and it lacks intermediate filaments (Adams et al., 2000). Instead, it uses a β1-like integrin to mediate basal adhesion (Prokop et al., 1998a), and this junction is connected to microtubules, which in *Drosophila* appear to have adopted the role of intermediate filaments in stabilising cells under shear stress (Mogensen and Tucker, 1988). This difference between the systems increases the number of possible models for how spectraplakins mediate structural connections to the plasma membrane.

The straightforward interpretation of the role of spectraplakins in cell adhesion is that they directly link particular plasma membrane proteins to the cytoskeleton. This is consistent with the idea that BPAG1e binds to BPAG2 through its N-terminus and to intermediate filaments through its C-terminus. It is also consistent with the roles of other members of the spectrin and plakin superfamilies. The plakins plectin and desmoplakin share this function and do so in a similar orientation, the N-terminus making the connection with the plasma membrane and the C-terminus binding to intermediate filaments. Members of the spectrin family also have similar functions, but in most cases it is the C-terminus that interacts with the plasma membrane, whereas the N-terminal ABD interacts with the actin cytoskeleton. Examples of this include α-actinin linking β1-integrin to actin (Otey et

al., 1993), dystrophin linking dystroglycan to actin (Ervasti and Campbell, 1993), and spectrin linking the ankyrin/anion exchanger complex to actin (Anstee et al., 1995). However, in the latter case one usually considers spectrin as linking to very short actin filaments that are themselves performing a bivalent linker role on the basis of models of the red cell cortical cytoskeleton (Bennett, 1990).

So how could Shot function in the above model? Since it is the microtubules that are linked to the plasma membrane, we infer that a microtubule-binding domain is essential, which seems most likely to be the C-terminal GAS2 domain of the spectraplakins. The N-terminus in one of its isoforms would bind to the integrin cytoplasmic domain either directly, as is the case for BPAG1e, or indirectly, as observed for desmoplakin. However, another simple solution presents itself: the integrins are linked to the actin cytoskeleton while Shot links actin to microtubules.

The ability of spectraplakins to link together different elements of the cytoskeleton is a key feature of this family and is not commonly found in cytoskeletal proteins. As mentioned above, individual isoforms have the ability to make all possible linkages between actin, microtubules and intermediate filaments. Plectin also has the ability to bind all three (Wiche, 1998). Given that these proteins also have the potential to form homodimers, both parallel and antiparallel, they may also be able to bundle or crosslink any single filament, which would be akin to the activity of α-actinin (Meyer and Aebi, 1990). Spectrin heterodimers have been shown to form antiparallel homodimers (a tetramer overall), and evidence gathered in yeast also suggests that plectin and BPAG1 form parallel homo- and hetero-dimers (Fontao et al., 2001).

The cytoskeletal crosslinking activity of spectraplakins is thought to be at the root of their role in the nervous system (Yang et al., 1996; Dalpe et al., 1998; Yang et al., 1999; Lee and Kolodziej, 2002a). The name *short stop* comes from the mutant phenotype in sensory and motor neurons, which fail to extend to their correct length and reach their targets (Van Vactor et al., 1993; Lee et al., 2000). The mutant axons have been shown to initiate extension, pathfind and fasciculate normally. In addition, the morphology of the growth cone appears normal. Somehow, lack of Shot prevents full axon extension. The phenotype is not clearly related to the length of the projection, since the sensory axons migrate less far than the motor axons, and in addition Shot is required for the extension and elaboration of dendritic branches (Prokop et al., 1998b), which are much shorter structures. Both the ABD and the GAS2 domain are required for this Shot function, but the central rod domain is not (Lee and Kolodziej, 2002a). Furthermore, they are required in the same molecule, since simultaneous expression of the two protein domains does not rescue the defect (Lee and Kolodziej, 2002a). These results are consistent with a key role of Shot in linking actin to microtubules, because this interaction is essential for structuring the axonal cytoskeleton (Houseweart and Cleveland, 1999), but they do not rule out other interpretations. It is quite possible that these domains bind to other proteins, as does the ABD of plectin (Geerts et al., 1999). The domain requirements are quite different for Shot's function in the development of the tracheae; the rod is necessary, in combination with either the ABD or GAS2 domain (Lee et al., 2002b).

In *dt/dt* mice the neuronal cytoskeleton is also disrupted, but this appears to result from defects in the stability of the axons rather than their initial extension. This disruption is most clearly seen as axonal swellings containing neurofilaments and a variety of vesicles, and these swellings are seen as early as in the embryo (Duchen, 1976; Bernier and Kothary, 1998; Yang et al., 1999). In addition, the microtubules and neurofilaments are disorganised, with abnormal accumulation of neurofilaments in the cell bodies. However, it seems unlikely that disruption of the neurofilament network is the cause of the degeneration, since the knockout of neurofilaments does not have a similar defect (Zhu et al., 1997), and removing neurofilaments from the *dt* mutant mouse does not reduce the defects (Yang et al., 1999). As in the case for *shot*, it seems possible that these phenotypes can be explained by the loss of the link either between actin and microtubules or between the plasma membrane and the cytoskeleton.

Certain aspects of the mutant phenotypes of *shot* and *dt* indicate a role that might be more similar to dystrophin or spectrin. A key function of spectrin is in organising the submembraneous cytoskeleton. This is particularly evident in erythrocytes, in which complexes of spectrin and ankyrin provide essential mechanical support for the membrane bilayer (Discher, 2000). In other cells, spectrin distribution is not uniform around the membrane, and it has an additional role of establishing specialised membrane subdomains. Complexes of ankyrin and spectrin are used in neurons to cluster ion channels and cell adhesion receptors at specialised membrane sites (Komada and Soriano, 2002). In the *Drosophila* embryo, different spectrin isoforms are localised exclusively to the apical or basolateral membrane compartment and are necessary to maintain proper zonula adherens junctions (Dubreuil et al., 1997; Thomas and Williams, 1999; Zarnescu and Thomas, 1999).

Dystrophin appears to have a similar role in the maintenance of membrane-associated complexes. The N-terminus of dystrophin directly binds to actin, and a C-terminal domain can bind to various transmembrane proteins: the laminin receptor dystroglycan and sarcoglycans. This dystrophin protein complex is necessary to protect the muscles from stress-induced damage. Loss of function of dystrophin or other proteins associated with the complex causes loss of proper muscle function and hence various muscular dystrophies through disruption of the essential cytoskeleton-extracellular-matrix link (Bredt, 1999; Burton and Davies, 2002).

The swellings produced in the axons of *BPAG1* mutant mice could arise from the loss of a spectrin-like function in providing mechanical support to the plasma membrane. A similar loss of membrane integrity is observed in some alleles of *shot*, in which the epidermis rips during the morphogenetic movements of embryogenesis (Gregory and Brown, 1998). In addition, as observed in *spectrin* and *dystrophin* mutants (Jacobson et al., 2001; Komada and Soriano, 2002), the distribution of transmembrane proteins into discrete domains is impaired by loss of *shot*. Fasciclin 2 is normally restricted to a segment of the axon; in *shot* mutants it is distributed more widely (Prokop et al., 1998b). There is also a failure of epidermal differentiation around the muscle attachment site in *shot* mutant embryos (Strumpf and Volk, 1998). These cells do not receive the correct amount of signal from a neuregulin/EGF-like ligand, which is secreted from the ends of

the muscle. The failure of differentiation could be caused by a loss of specialised submembraneous domains within the receiving cell, or alternatively it could be caused by the loss of a scaffolding function that recruits proteins that assist the signalling process.

Members of the spectrin and plakin superfamilies bind to a variety of potential signalling molecules. Examples include the recruitment of nNOS by dystrophin (Brenman et al., 1996), a PDZ-LIM protein by α-actinin (Vallenius et al., 2000) and the LAP (Leucine rich repeats and PDZ domain) family protein ERBIN by BPAG1 (Favre et al., 2001). As described above, the role for Shot in the morphogenetic events surrounding the anastomosis of the developing tracheal tubules requires the rod domain, but not both ABD and GAS2 domains, which suggests that it involves a function of Shot distinct from crosslinking of the cytoskeleton (Lee and Kolodziej, 2002b). Shot function in the tracheae is required for alterations in cell shape and adhesion, possibly due to recruitment of signalling molecules that regulate RhoA, which also contributes to tracheal morphogenesis. The size and location of spectraplakins might therefore provide a particularly useful scaffold for signalling components.

Conclusions and prospects

The spectraplakins are intriguing proteins with many possible functions related to maintaining the integrity of a cell. An exceptional feature of the spectraplakins is that they are the largest members of superfamilies that are already known for the large size of their members. Fly spectraplakin, with a length of up to 8843 residues, is the third largest protein encoded by the fly genome, after the cuticle receptor dumpy (≥23,000 residues) and the titin-like molecule kettin (17,903 residues). Proteins of such length constitute a challenge for the protein synthesis machinery of a cell; so it seems likely that there is an important reason for their being so big. The role of titin as a molecular ruler that dictates the assembly of the muscle sarcomeres is an attractive model, but we have yet to identify a cellular structure that requires measurement by spectraplakins. The largest spectraplakin isoforms are predicted to have actin- and microtubule-binding sites separated by 400 nm; there might be an intracellular structure that relies on this spacing. In addition, in common with spectrin in red blood cells, spectraplakins may provide a shock-absorbing membrane-cytoskeleton link based on the flexible nature of the spectrin repeats in tissues exposed to high stress, such as skin or tendon cells.

Another general question that arises about spectraplakins is why a single gene is used to make both a spectrin-type protein and a plakin, rather than separate genes? One possibility is that the two isoforms function coordinately and that it is therefore important that they are co-expressed. The more likely explanation lies in the function of the largest possible forms, which only a spectraplakin gene can encode. Therefore an important next step for the field is to determine the function of these largest forms. To our knowledge, no mutants are available that just inactivate these forms, which would reveal their specific function.

The exceptional length, modularity and isoform variability of these proteins make it likely that many more functions than those described above will be assigned to spectraplakins in the near future. In organising protein interactions between cytoskeletal networks as well as linking these to specialised membrane sites, spectraplakins fulfill indispensable functions in invertebrate and vertebrate cells.

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