Cell, Vol. 98, 229–238, July 23, 1999, Copyright ©1999 by Cell Press

Integrators of the Cytoskeleton that Stabilize Microtubules

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Summary

Sensory neurodegeneration occurs in mice defective in *BPAG1*, a gene encoding cytoskeletal linker proteins capable of anchoring neuronal intermediate filaments to actin cytoskeleton. While *BPAG1* null mice fail to anchor neurofilaments (NFs), *BPAG1/NF* null mice still degenerate in the absence of NFs. We report a novel neural splice form that lacks the actin-binding domain and instead binds and stabilizes microtubules. This interaction is functionally important; in mice and in vitro, neurons lacking BPAG1 display short, disorganized, and unstable microtubules defective in axonal transport. Ironically, BPAG1 neural isoforms represent microtubule-associated proteins that when absent lead to devastating consequences. Moreover, BPAG1 can functionally account for the extraordinary stability of axonal microtubules necessary for transport over long distances. Its isoforms interconnect all three cytoskeletal networks, a feature apparently central to neuronal survival.

Introduction

Composed of actin filaments, microtubules, and intermediate filaments (IFs), the cytoskeleton is vital for many cellular processes, including mechanical strength, movement, adhesion, polarity, and intracellular trafficking. Cytoarchitecture is organized by a diverse array of associated proteins, most of which fulfill specialized roles of individual filament systems. Recently, proteins have been discovered that bind to more than one filament network, enabling new insights into how the cytoskeleton integrates and coordinates the functions of its three filament networks. The plakins are such proteins, and they appear to be crucial for the integrity and survival of many eukaryotic cells and tissues.

Plakins are a group of large (>200 kDa) coiled-coil cytoskeletal-associated proteins that were initially thought to associate exclusively with IFs (Ruhrberg and Watt, 1997). Some plakins decorate IF networks, while others anchor IFs to junctions such as desmosomes and hemidesmosomes. A conserved IF-binding domain resides in the carboxy "tail" segment of most plakins, and deciding where to anchor the IFs involves the amino "head" segment (Fuchs and Cleveland, 1998). Plakin genes possess multiple tissue-specific promoters and encode differentially expressed splice forms. An interesting feature of plakins is that the isoforms encoded by a single gene perform different functions tailored to the cytoskeletal needs of each specialized cell (Yang et al., 1996).

Recently, certain plakin isoforms were found to interconnect IF and actin cytoskeletons (Yang et al., 1996; Andra et al., 1998). Plectin has this capacity, and interestingly, cultured plectin null fibroblasts display perturbations in cell motility and actin rearrangements (Andra et al., 1998). In humans, plectin mutations cause a rare form of muscular dystrophy associated with skin blistering, reflective of plectin's expression pattern and a role for IF linker proteins in mechanical integrity (Fuchs and Cleveland, 1998).

In sensory neurons, two plakin splice forms of the bullous pemphigoid antigen 1 gene, BPAG1n1 and BPAG1n2, also possess binding domains for both actin and IFs, perhaps enabling these proteins to anchor axonal IFs to the cortical actin cytoskeleton (Brown et al., 1995; Yang et al., 1996; Leung et al., 1999). Indeed, the *dystonia musculorum* (*dt/dt*) mutant mouse, defective in the *BPAG1* gene, exhibits axonal swellings packed with disorganized IFs, accompanied by sensory neuron degeneration and death by 4–6 weeks of age (Brown et al., 1995; Guo et al., 1995). *BPAG1* null mice also display mild blistering due to the absence of an epidermal splice form, BPAG1e, that lacks an actin-binding domain and instead anchors IFs to the integrin-based hemidesmosomes that adhere epidermis to underlying basement membrane.

While linkage to IFs has been assumed to be a key feature of plakins, recent observations suggest that they may possess functions independent of IF association. *Drosophila melanogaster* are devoid of IF cytoplasmic networks, and yet the *kakapo* mutant, defective in integrin-based adhesion, encodes a plakin that lacks the IF-binding domain (Gregory and Brown, 1998; Prokop et al., 1998; Strumpf and Volk, 1998). Furthermore, in *dt/dt* (*BPAG1* null) mutant mice, neuronal degeneration persists when the axonal neurofilament (NF) network is removed genetically to avoid NF aggregation (Eyer et al., 1998). Even though NF disorganization on its own can cause neuronal degeneration (Cote et al., 1993; Xu et al., 1993), NF null mice are surprisingly healthy (Zhu et al., 1997; Fuchs and Cleveland, 1998). Thus, if neuronal IF anchorage to the cortical actin cytoskeleton were the only function for neuronal BPAG1s, removal of the axonal NF network should enable the double mutant mice to survive. It does not.

What might the additional function(s) of plakin proteins be, and why are they so critical to cell survival? One clue stems from the fact that in flies, integrin-mediated junctions are stabilized by microtubules rather than IFs.

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Correspondingly, *kakapo* mutant embryos display abnormalities in microtubule organization and detachment at sites where muscle cells and neurons adhere to underlying support cells (Gregory and Brown, 1998; Prokop et al., 1998). An association between plakins and microtubules is also implicated when microtubules are depolymerized in fibroblasts, causing disorganization of plectin and IFs (Seifert et al., 1992). Conversely, after extracting actin from fibroblasts, microtubules and IFs are interconnected by molecular cross bridges that label with antibodies against plectin (Svitkina et al., 1996).

Despite these tantalizing relationships, cultured plectin null fibroblasts display a seemingly normal microtubule network (Andra et al., 1998), and BPAG1n1 has no obvious association with the microtubule network when transiently expressed in foreign cells (Yang et al., 1996; Leung et al., 1999). Other questions arise from studies on *dt/dt* (*BPAG1* null) mutant mice; while the microtubule network is perturbed in their sensory axons (Dalpe et al., 1998), these aberrations could easily be a secondary consequence of disrupting the neuronal IF cytoskeleton. Thus, it remains unclear as to whether plakin connections to microtubules exist in vivo and whether they are physiologically relevant.

In this report, we show that perturbations in axonal microtubules are directly due to the *BPAG1* null condition and cannot be attributed to secondary consequences of neuronal IF perturbations. We also identify a novel BPAG1 neural isoform that preferentially associates with microtubules and possesses a bona fide microtubule-binding domain. This domain renders foreign microtubule networks stable, and conversely, without endogenous BPAG1s, neuronal microtubules are less stable than their wild-type counterparts. Taken together, our findings define BPAG1 plakins as novel integrators of all three cytoskeletal networks, a feature that in neurons appears to be responsible for the high degree of microtubule stabilization that allows axonal transport over long distances.

Results

A Direct Correlation between Microtubule Abnormalities and Loss of BPAG1

Sensory axons of *BPAG1* null (*dt/dt*) mice exhibit axonal swellings packed with NF aggregates, vesicles, and mitochondria (Figures 1A and 1A′; Brown et al., 1995; Guo et al., 1995). In some regions, the axoplasm displays fewer and disorganized neuronal IFs as well as shorter, more disorganized microtubules (Figure 1A; see also Dalpe et al., 1998). In other regions, areas are devoid of microtubules, appearing as a sandwich of IFs flanked by vesicles (Figure 1A′). The abnormalities in microtubule organization, transport, and accompanying neurodegeneration are most striking for the large myelinated sensory axons of dorsal column and dorsal root ganglia (DRG). These features differ from wild-type sensory axons, which align microtubules and IFs along the axon (Figures 1B and 1B′).

To determine whether these microtubule abnormalities arise secondarily as a consequence of neuronal IF aggregates, we bred our *BPAG1* null mice (Guo et al., 1995) with *NF-L* null mice, which despite an absence of NFs are phenotypically healthy and possess largely normal neurons (Zhu et al., 1997). In agreement with Eyer et al. (1998), *BPAG1/NF* double null mice exhibited rapid degeneration of their sensory nervous system, a feature characteristic of *BPAG1* null animals and not seen in *NF* null mice. In fact, our double knockout mice were indistinguishable phenotypically from *BPAG1* knockout animals. These data unmask the existence of an additional function for *BPAG1* gene products that is independent of their ability to bind and anchor neuronal IFs.

The removal of the NF cytoskeleton from dorsal root axons of *BPAG1/NF-L* double knockout mice gave us the opportunity to examine the microtubule network in the absence of NF aggregates. As shown in Figure 1C, microtubules of sensory axons from double null mice appeared short and disorganized, as in *BPAG1* null mice (Figure 1A). Notably, axonal swellings still occurred that were similar to those of *BPAG1* null sensory axons except they lacked NF aggregates (Figure 1C inset; Eyer et al., 1998). Only occasional IFs, perhaps peripherin filaments, were detected in these swellings.

NFs form the bulk of the axoplasm and play a role in determining axonal caliber (Fuchs and Cleveland, 1998). When NFs were absent in either *BPAG1/NF-L* double null or *NF-L* DRGs, the density of axonal microtubules was increased (Figures 1C and 1D, respectively). Most importantly, however, the abnormalities in microtubule organization persisted ± NFs, correlating with loss of *BPAG1* function and not with NF aggregation.

To more carefully examine defects in microtubule length differences, we cultured neurons from DRGs of newborn *BPAG1* null, *BPAG1/NF-L* double null, *NF-L* null, and wild-type mice. Neurite outgrowth was similar for all four cultures. The largely two-dimensional axoplasm enabled us to cut ultrathin sections parallel to microtubules and IFs. Under these conditions, differences in microtubule length and organization between *BPAG1/NF* double null (or *BPAG1* null) and *NF* single null (or wild-type) axons were more striking than in vivo (Figures 1E and 1F, respectively). Taken together, our in vivo and in vitro data argue that defects in microtubule organization within *BPAG1* null DRGs do not arise simply from primary disorganization of NFs but rather appear to be an inherent feature of the *BPAG1* null condition.

A Novel Neural Isoform of BPAG1 Lacking Half of the Actin-Binding Domain

In transiently transfected cells, the known neural isoforms of BPAG1 associate with IF and actin cytoskeletons but not microtubules (Yang et al., 1996). To assess whether there might be an additional neural isoform of BPAG1 capable of interacting with microtubules, we screened a human fetal brain cDNA library with a cDNA to a 5′ coding segment of BPAG1n1. Seven overlapping cDNA clones were identified, the longest of which contained 778 bp of a 5′ novel sequence.

To verify the existence of the mRNA encoding this isoform, we used RT-PCR with high-fidelity long-range polymerase to amplify an ~8 kb fragment encompassing a novel BPAG1n3 sequence and extending through the encoded coiled-coil rod shared among BPAG1 isoforms. This PCR band was not generated from DRG RNAs of

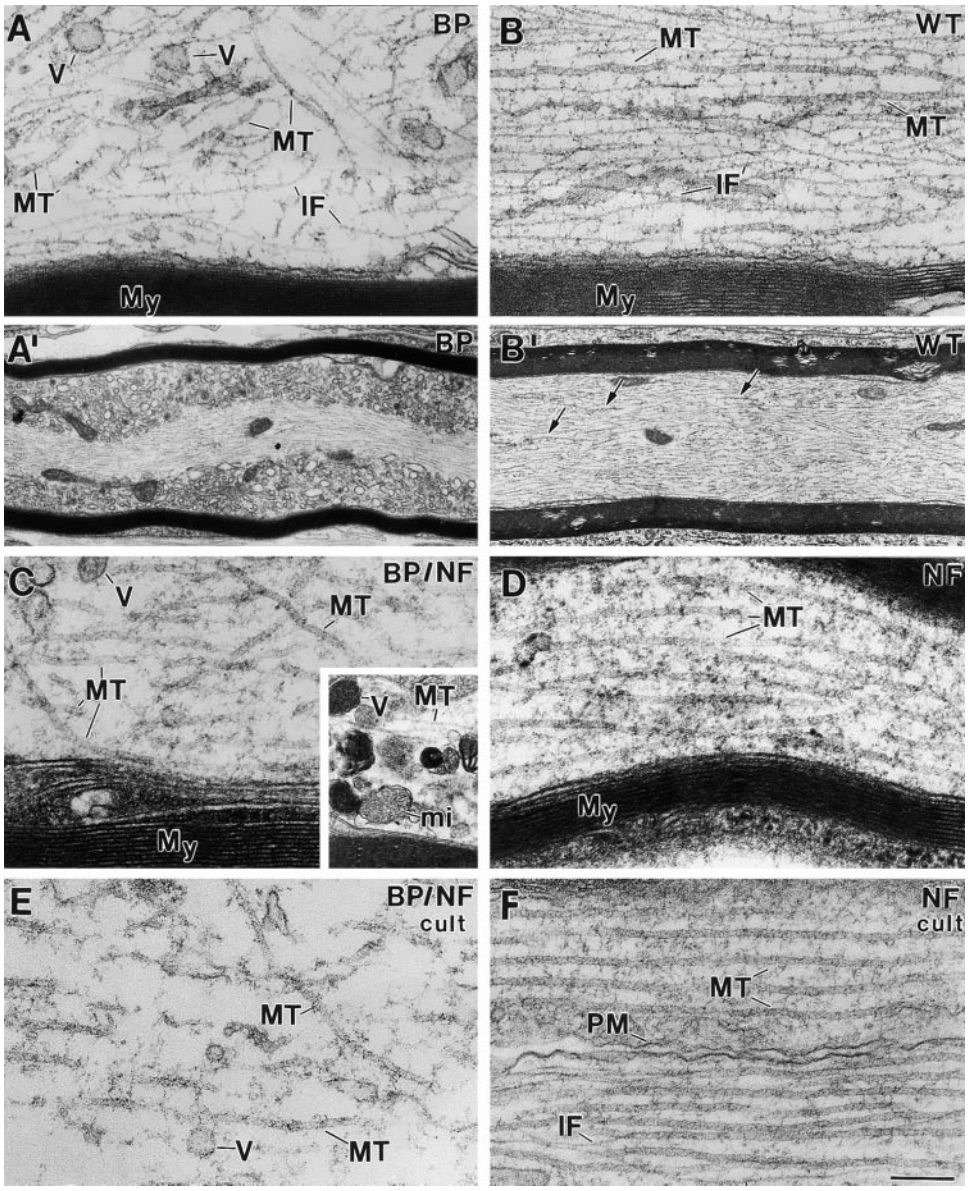

Figure 1. A Defective Microtubule Network in BPAG1 Null Sensory Axons Is Not Rescued by Genetic Removal of the Neurofilament Cytoskeleton

Dorsal root ganglia (DRG) were isolated from *BPAG1* null (BP), wild-type (WT), *BPAG1/NF-L* double null (BP/NF), and *NF-L* null (NF) mice and either processed directly for electron microscopy or cultured to allow neurite outgrowth and then processed.

(A–D) Portions of large myelinated axons from *BP* null (A and A′), WT (B and B′), *BP/NF* double null (C), and *NF* null (D) dorsal roots. Note short and disorganized microtubules when BPAG1, but not NFs, are absent from dorsal roots. Note axonal transport defects in (A′), a region with few or no microtubules; in contrast, MTs are easily seen (arrows) in WT axons, which also display normally dispersed vesicles (B′). Inset in (C) denotes portion of axonal swelling.

(E and F) Neurites cultured from *BP/NF* double null (E) and *NF* null (F) DRGs.

mi, mitochondria; V, vesicles, PM, plasma membrane; My, myelin sheath; MT, microtubule; IF, intermediate filament. Bar in (F) represents 200 nm in (A–F), 1 μm in (A′) and (B′), and 500 nm in (C) inset.

BPAG1 null mice. Northern analysis with a BPAG1n3-specific probe revealed an ~12 kb hybridizing band from DRG, brain, and spinal cord similar to other neural isoforms (Brown et al., 1995; Yang et al., 1996; Dowling et al., 1997). Sequence analysis of cDNAs and PCR products confirmed the identity of the novel BPAG1n3 isoform, and after generating a BPAG1n3 peptide-specific antibody, we verified BPAG1n3's existence and size (~280 kDa) by immunoblot analysis (Figure 2C). The band was not detected in *BPAG1* null DRG tissue, confirming antibody specificity.

Figure 2D depicts the three known BPAG1 neural isoforms. All contain the 908 amino acid coiled-coil rod segment for dimerization and the 633 residue tail harboring the IF-binding domain (Yang et al., 1996). In addition, the majority of the head segment, including the 1184 residue midsegments (M1 and M2), is shared by neural isoforms. BPAG1n3 is distinguished by its initial 32

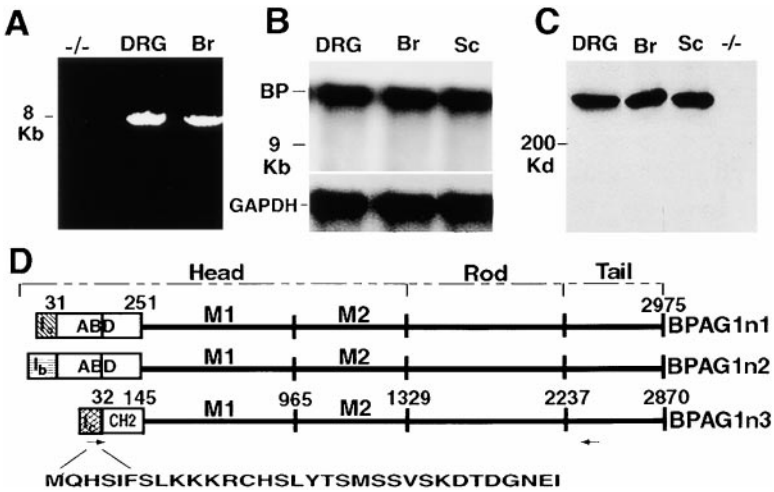

Figure 2. Characterizing BPAG1n3, A Novel Neural Isoform that Lacks Half of the Actin-Binding Domain

(A) PCR. Total RNAs were isolated from DRGs and brain (Br) tissue of 14-day-old wild-type and *BPAG1* null mice (−/−), and RT-PCR was performed using the BPAG1n3-specific oligonucleotide primer pair indicated by arrows in (D). Fragments were analyzed by electrophoresis through agarose gels stained with ethidium bromide.

(B) Northern analysis. RNAs from DRG, brain (Br), and spinal cord (Sc) were poly(A)$^+$ selected, and 2 μg per lane were resolved by formaldehyde gel electrophoresis (Yang et al., 1996). Blots were hybridized with radiolabeled probes against a 500 bp mouse BPAG1n3 cDNA unique to the 5′ noncoding region (BP) or a 1 kb mouse glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA as control. Migration of RNA standard is indicated at left.

(C) Immunoblot of neural proteins. Analysis was carried out with a 1:2000 dilution of anti-BPiso3, and blots were developed by chemiluminescence (ECL method; Amersham Corp., Arlington Hts., IL). Migration of muscle myosin standard is indicated.

(D) Stick diagram of the three neural BPAG1 isoforms. Sequence unique to n3 isoform is shown. Numbers represent relevant residues of n1 and n2 isoforms, with 1 equal to ATG translation start codon. ABD, the actin-binding domain, is split into calponin homology CH1 (grey) and CH2 segments. M1 and M2, midsegments of the neuronal head domains. The ID code for BPAG1n3 is AF165191.

amino acid residues and by the absence of the amino-terminal half of the actin-binding domain (ABD). The ABD half that is missing in BPAG1n3, as well as the novel segment of BPAG1n3, are encoded by separate exons (Y. Y. and E. F., unpublished). We conclude that differential splicing is responsible for this novel neural isoform.

The Half Actin-Binding Domain Abrogates the Ability of the BPAG1n3 Head Segment to Efficiently Associate with Actin Microfilaments

The ABD of BPAG1n1 and BPAG1n2 is similar in sequence and in K_d to that of α-actinin, β-spectrin, and dystrophin (Yang et al., 1996; Andra et al., 1998). This domain consists of two homologous segments called calponin homology (CH) domains. Based upon crystal structure and biochemistry, the major determinant for actin binding is typically in the first of these domains (CH1) rather than the second (CH2) (Gimona and Winder, 1998). The CH1 domain is missing in BPAG1n3.

To test directly whether the BPAG1n3 isoform is compromised in its ability to associate with actin, we transfected NIH 3T3 mouse fibroblasts, COS and MCF7 epithelial cells, and the SW13 cell line (lacking cytoplasmic IFs) with BPAG1 expression vectors encoding either CH2 alone or full ABD. Immunoblot analyses confirmed that the proteins were stably expressed and of expected size (not shown). Immunofluorescence revealed that CH2 on its own aggregated, displaying sometimes weak and other times no association with the actin cytoskeleton (Figures 3A and 3B). In contrast, the full ABD, found only in BPAG1n1 and BPAG1n2 (Yang et al., 1996), colocalized with actin (Figures 3C and 3D). Greater than 80% of transfected cells gave the phenotypes shown, irrespective of cell line. Our data imply that the ability to effectively associate with the actin cytoskeleton is a property characteristic of BPAG1n1 and BPAG1n2 but not BPAG1n3.

The BPAG1n3 Isoform-Specific Antibody Decorates Microtubules in Sensory Neurons

To understand how BPAG1n3 might function in sensory neurons, we conducted immunoelectron microscopy using our BPAG1n3-specific antibody, anti-BPiso3 (Figure 4). Greater than 85% of the gold particles identifying anti-BPAG1n3 labeling localized to the axonal cytoskeleton of sensory neurons cultured from wild-type DRGs. Interestingly, BPAG1n3 labeling most heavily decorated

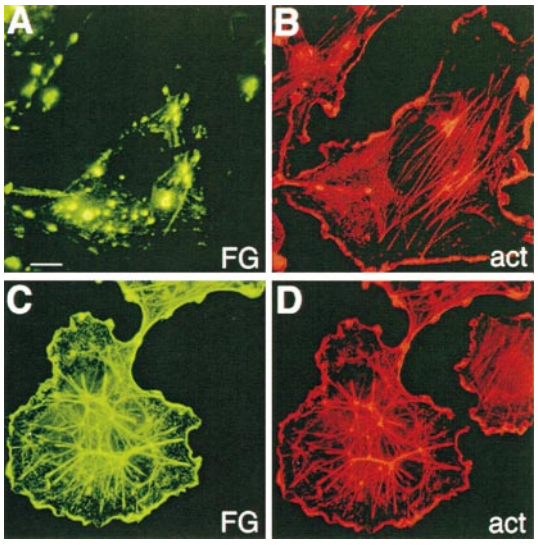

Figure 3. The Half Actin-Binding Domain of BPAG1n3 Does Not Efficiently Associate with F-Actin

IF-less SW13 cells were transfected with pBPch2-FG (A and B) characteristic of BPAG1n3 or pBPabd-FG (C and D) found only in BPAG1n1 and BPAG1n2. After 28 hr, cells were fixed and subjected to immunofluorescence (Yang et al., 1996). Transfected cells were labeled with antibodies indicated in each panel: mouse anti-FLAG (FG), green; rabbit anti-β-actin (act), red. Note that under the conditions used, most cells are transfected. Bar represents 12 μm.

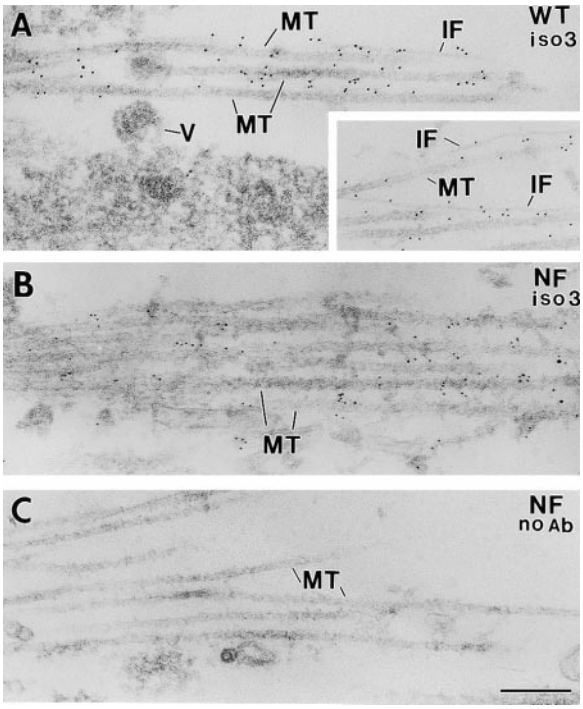

Figure 4. Immunoelectron Microscopy Reveals Association of BPAG1n3-Specific Antibodies with Microtubules and Neurofilaments in DRG Cultures

Wild-type (WT) and *NF-L* null (NF) DRGs were cultured, detergent extracted, and processed for immunoelectron microscopy with anti-BPiso3 (iso3), followed by gold-conjugated secondary antibodies.
(A) Wild-type axons, depicting axonal cytoskeletal labeling. Note strong labeling of microtubules but also IF labeling.
(B and C) *NF-L* null axoplasm labeled with anti-BPiso3 antibody (B) or secondary antibodies alone (C). Note labeling of microtubules even in the absence of NF. Abbreviations are the same as in Figure 1. Bar represents 200 nm.

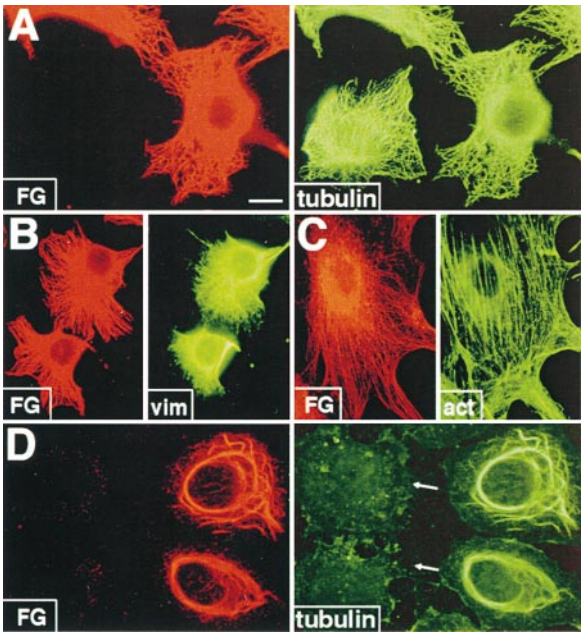

Figure 5. The BPAG1n3 Head Domain Has the Capacity to Bind to and Stabilize Microtubule Networks

NIH 3T3 fibroblasts, MCF7, COS, and SW13 cells were transfected with pBPM1-FG, expressing epitope-tagged M1 domain from BPAG1n3. Cells were fixed in methanol (−20°C, 10 min) and subjected to double immunofluorescence using antibodies indicated on each frame: mouse anti-FLAG (FG), red; rabbit anti-tubulin (tub), green; anti-β-actin (act), green; or vimentin (vim), green. Each pair of frames represents the same field of cells stained with different antibodies. (A–C), transfected 3T3 cells; (D), transfected IF-negative SW13 cells treated at −20°C for 10 min, a process that depolymerizes the unusually sensitive microtubules of SW13 and MCF7 cells (white arrows denote untransfected cells, displaying depolymerized MTs). Note that in the two cell lines with sensitive MT networks, microtubule bundling occurred upon association with BPAG1n3, a process also seen upon transient expression of other microtubule-associated proteins, including MAP1b (Togel et al., 1998). Bar represents 15 μm in (A), 25 μm in (B) and (C), and 12 μm in (D).

axonal microtubules, although it also localized along neuronal IFs (Figure 4A). 73% ± 10% of the cytoskeletal-associated gold particles were within 15 nm of a microtubule, that is, the limits of resolution of the labeling; 14% ± 6% were within 15 nm of an IF. When microtubules and IFs were in close proximity, labeling was often between the two filamentous structures (Figure 4A inset). No significant BPAG1n3 labeling was detected in other neurite areas, including vesicles, other organelles, and membranes.

To assess whether the interaction between BPAG1n3 and microtubules could take place in the absence of NFs, we repeated the immunolabeling, this time using *NF* null DRG cultures. As shown in Figure 4B, anti-BPiso3 labeling remained prominent on microtubules (80% ± 5%) even when the bulk of the neuronal IF cytoskeleton had been removed genetically. Labeling remained highly specific and was not seen when primary antibody was omitted from the process (Figure 4C). Thus, BPAG1n3's association with microtubules was not dependent upon its interaction with NFs.

A BPAG1n3 M1 Domain that Binds and Stabilizes Microtubule Networks in Foreign Cells

To examine whether a domain within BPAG1n3's head segment might have the capacity to localize specifically to microtubules, we first constructed expression vectors that encoded various portions of BPAG1n3, each epitope tagged with FLAG. Immunoblot analysis of transiently transfected cell protein confirmed that bands of the expected sizes were produced (not shown).

As judged by immunofluorescence microscopy of transfected cells, the M2 domain of BPAG1n3 was diffuse in the cytoplasm (not shown). However, the M1 domain of BPAG1n3 gave a striking pattern of anti-FLAG immunofluorescence: in NIH 3T3 fibroblasts, >90% of the microtubule network was decorated (Figure 5A). In contrast, anti-FLAG colabeling was not seen with either IF or actin cytoskeletons (Figures 5B and 5C, respectively).

An extraordinary feature of cells transfected with either the M1 domain or the BPAG1n3 head domain was that it rendered the endogenous microtubule network resistant to a variety of microtubule-destabilizing agents. In either IF-negative SW13 cells or keratin-positive MCF7 cells transfected with the M1 domain, microtubules were bundled and resistant to cold treatment, while those in untransfected cells (white arrows) were not (Figure 5D; shown are data for IF-negative SW13

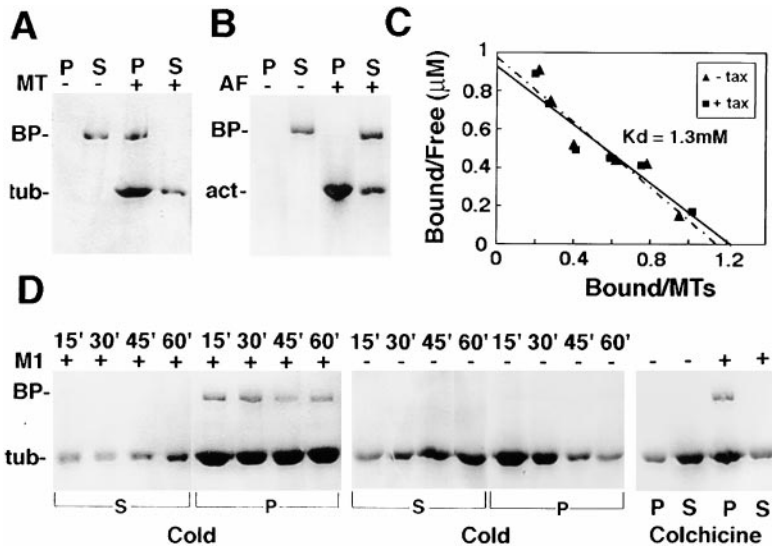

Figure 6. The M1 Domain Binds Directly to Microtubules In Vitro

(A) Microtubule binding assays. Assays were carried out using purified M1 domain of BPAG1n3 and microtubules. Supernatant (S) and pellet (P) fractions were separated by electrophoresis through 10% SDS-polyacrylamide gels, which were then stained with Coomassie blue to visualize protein. Each assay was performed with (+) or without (−) polymerized microtubules (MT).

(B) F-actin binding assay as negative control. Actin was polymerized (AF) and binding assays (Yang et al., 1996) carried out with the M1 domain of BPAG1n3. Note that M1 remained in the supernatant.

(C) K_d of microtubule binding. Microtubule binding assays were carried out as in (A) ± taxol (tax) to provide additional MT stability and with BPAG1 M1 fusion protein at 0.5–10 μM. Protein was quantitated by densitometric scanning. Scatchard analyses for taxol-treated and untreated MTs were conducted on three data sets each, which were averaged (values differed by less than 10%). Plot shown: vertical axis, BPAG1-M1 bound/free BPAG1-M1 μM^{-1}; horizontal axis, bound BPAG1-M1/microtubules. Note that a nonlinear Scatchard plot has also been seen for MAP2 (Wallis et al., 1993; Coffey and Purich, 1995).

(D) BPAG1n3's M1 domain increases resistance of microtubules to cold and colchicine. Microtubules ± M1 were either placed on ice or treated with 2.5 μg/ml colchicine at 37°C for times indicated. Following incubation, samples were centrifuged and supernatant (S) and pellets (P) analyzed by SDS-PAGE. Note persistence of tubulin in (P) fractions after cold or colchicine treatments when BPAG1n3 M1 was present.

cells). Similarly, in all four cell lines, microtubules were more resistant to colchicine and nocadazole when the M1 domain was expressed (data not shown). These findings unveil a microtubule recognition domain within the M1 domain of BPAG1n3 and demonstrate that it can increase the stability of a foreign microtubule network irrespective of the presence of IFs.

We do not know whether the M1 domain is operative in BPAG1n1 or BPAG1n2. However, it is worth noting that in transiently transfected cells, the BPAG1n1 head domain interacts with the actin cytoskeleton (Yang et al., 1996), while the BPAG1n3 head domain associates with microtubules (this study). These findings indicate that, at least under some circumstances, the M1 domain behaves differently when in the context of the BPAG1n1 versus the BPAG1n3 head segment.

In Vitro Evidence of Direct Binding and Stabilization of Microtubules by BPAG1n's M1 Domain

To explore further the specificity of the M1 domain of neural BPAG1 isoforms for microtubules, we combined purified bovine brain microtubules with GST-BPAG1n3 fusion protein encompassing the M1 domain. In the absence of microtubules, the BPAG1n3 fusion protein remained in the supernatant after centrifugation at 100,000 × g (Figure 6A). In contrast, after addition of microtubules, most of the BPAG1n3 now pelleted with them. This association was specific: in the analogous experiment with F-actin, the fusion protein remained in the supernatant (Figure 6B).

To estimate the binding constant of this association, we repeated these experiments, this time using a concentration range of BPAG1n3. As expected, as the concentration of BPAG1n3 increased, the association of BPAG1n3 with microtubules became saturating and the level of BPAG1n3 in the soluble fraction increased. A Scatchard plot of the data yielded a K_d of 1.3 μM for BPAG1n3 binding to microtubules (Figure 6C). The data did not vary significantly with addition of taxol, known to stabilize microtubules, and the K_d was comparable to that of MAP2 (1–25 μM; Wallis et al., 1993; Coffey and Purich, 1995), a well-established microtubule-binding protein. Moreover, when in vitro microtubules were exposed to either 0°C or 2.5 μg/ml colchicine, those that had been preincubated with BPAG1n3 M1 were noticeably more stable, shifting from pellet to supernatant more slowly with time than unbound microtubules (Figure 6D).

Axonal BPAG1n3 Is Selectively Lost When Microtubules and Neurofilaments Are Removed from Cultured Sensory Neurons

To more rigorously examine the association between BPAG1n3, microtubules, and IFs in axons, we again turned to DRG cultures from newborn *BPAG1* null, *BPAG1/NF-L* double null, *NF-L* null, and wild-type mice. Abundant neurites containing actin and microtubule cytoskeletons were seen in all cultures, even those that lacked *BPAG1* and NFs (Figure 7A). The BPAG1n3-specific antibody showed strong labeling of neurites from both wild-type and *NF-L* null cultures, even after extracting them with a nonionic detergent to remove water-soluble proteins (Figure 7B; *NF-L* null culture).

We next examined the status of BPAG1 neural isoforms when microtubules were removed from the axonal cytoskeleton. Since neuronal microtubules are resistant to cold and depolymerizing agents (Webb and Wilson, 1980; Morris and Lasek, 1982; Baas et al., 1994), overnight treatment with depolymerizing agents was needed to obliterate all traces of microtubules in cultured DRG neurites. This procedure did not noticeably affect anti-actin immunofluorescence (data not shown), but under these conditions, anti-BPiso3 labeling was quantitatively lost on an *NF-L* null background (Figure 7C) and

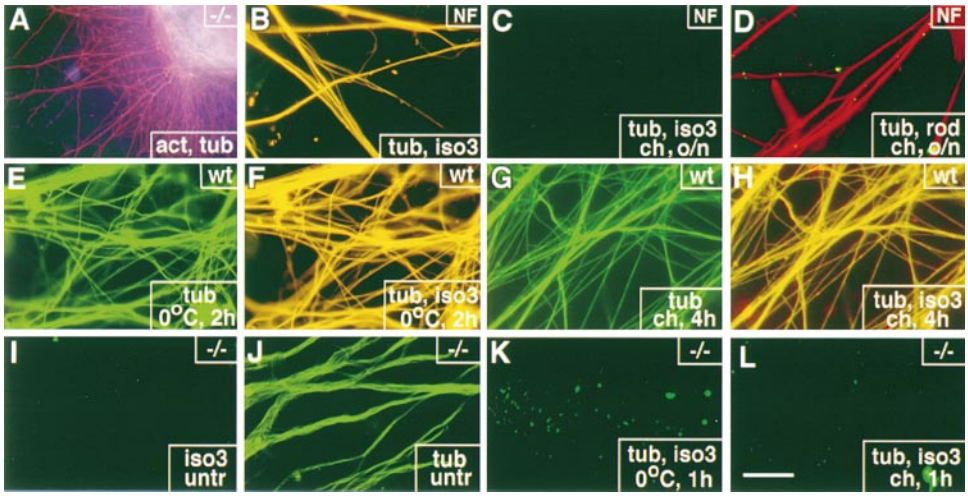

Figure 7. BPAG1n3 Stability Is Dependent upon Microtubules, and Microtubule Stability Is Dependent upon BPAG1n3 in Cultured Sensory Neurons

The dorsal root ganglia (DRG) of wild-type (WT), *NF-L* null (NF), *BPAG1* null (BP), and *BPAG1/NF-L* double null (−/−) (BP/NF) newborn mice were cultured on coverslips. Note that in these assays, *NF* null and WT cultures behaved similarly, as did *BP* null and *BP/NF* double null cultures. The genotype of each specific culture shown is indicated at upper right. Where indicated at lower right, some cultures were treated with 2.5 μg/ml colchicine (ch) at 37°C or incubated at 0°C for the times indicated prior to fixation. After washing two times in 0.1% Triton X-100, 1% BSA, and 0.1% gelatin, DRGs were subjected to immunofluorescence using the antibodies indicated: anti-β-tubulin (tub), red in (A); anti-α-tubulin (tub), green; phalloidin (act), blue; anti-BPiso3 (iso3), red; or anti-BProd (rod), red. Bar in (A) is 50 μm; all others, 10 μm.

reduced on a wild-type background (not shown). In contrast, when cultures were pretreated with recombinant gelsolin to remove the actin cytoskeleton, anti-BPiso3 staining was unchanged (data not shown). These data suggest that the microtubule network is needed for full association of BPAG1n3 with the axonal cytoskeleton, which in addition involves IFs but not actin.

Interestingly, we obtained markedly different results when we repeated these experiments with our anti-BProd antibody, recognizing all three neural isoforms. In this case, after overnight colchicine treatment to depolymerize the microtubule network, anti-BProd antibodies still showed prominent labeling of neurites (Figure 7D). This was true even on an *NF-L* null background (example shown), indicating that neither microtubules nor NFs were essential for maintaining the axonal localization of some neural isoforms. This persistent labeling is likely a reflection of interactions between the actin cytoskeleton and BPAG1n1 or BPAG1n2.

BPAG1n Is Directly Responsible for the Stability of the Microtubule Network of Sensory Axons

After 2–4 hr of either incubation on ice or treatment with 2.5 μg/ml colchicine at 37°C, the microtubule network of cultured wild-type or *NF-L* null DRG axons appeared unaffected and still labeled with anti-BPAG1n3 and anti-tubulin antibodies in a fashion indistinguishable from untreated neurites (Figures 7E–7H). To test whether BPAG1 neural isoforms might directly contribute to this in vivo stability of neuronal microtubules, we examined the cold/colchicine sensitivities of *BPAG1/NF-L* double null and *BPAG1* null DRGs. Microtubule networks lacking BPAG1 ± NFs were sensitive to cold or colchicine treatments (Figures 7I–7L). Within 15 min of either treatment, punctate anti-tubulin staining was observed along microtubules; by 30 min, discontinuous antibody staining was seen along ~70% of neurite processes; by 1 hr, staining was no longer detectable in any of the processes (Figure 7K, cold treated; Figure 7L, colchicine treated; compare with Figure 7J, untreated).

These findings reveal a correlation between BPAG1n expression and microtubule stability in cultured DRG neurites. BPAG1n's relatively restricted expression to sensory and sympathetic neurons (Bernier et al., 1995; Dowling et al., 1997), coupled with their preferential association with cold-stable microtubules, provides an explanation for why these proteins were not previously identified among high-molecular-weight MAPs that copurified with isolated brain tubulin.

Finally, as judged by immunoblot analysis of DRG proteins from the four genotypes, we detected no changes in the levels of tubulin, MAP1b (MAP5), or tau (data not shown). Thus, although MAP1b and tau are expressed in sensory axons and influence microtubule stability (Hirokawa, 1994), differences in these proteins are unlikely to contribute to the effects that we ascribe to BPAG1 loss. The lack of concomitant perturbations in tau and MAP1b upon BPAG1 ablation is consistent with the lack of functional equivalence between the severe neural degeneration seen in *BPAG1* knockout mice and the relatively modest alterations in neural integrity observed in mice lacking either tau or MAP1b (Edelmann et al., 1996; Takei et al., 1997).

Discussion

The Missing Link: An Integrator of All Three Cytoskeletal Networks

Plakins have been viewed primarily as IF-associated proteins, or IFAPs (Ruhrberg and Watt, 1997). Through the recent discovery of a bona fide actin-binding domain in neuronal isoforms of BPAG1 and plectin, plakin functions have broadened to include integrating actin and IF cytoskeletons and influencing actin dynamics (Yang

et al., 1996; Andra et al., 1998). These functions alone, however, could not explain why *BPAG1/NF-L* double null sensory neurons displayed fewer and disorganized microtubules and axonal swellings packed with mitochondria and vesicles. This unexplained pathology led us to search for additional plakin functions.

The notion that some plakins might associate with microtubules has been raised previously (Seifert et al., 1992; Svitkina et al., 1996; Dalpe et al., 1998; Gregory and Brown, 1998; Prokop et al., 1998). However, a lack of biochemical evidence coupled with the demonstration of only a partial overlap in anti-plakin and anti-tubulin immunofluorescence has made it difficult to assess the extent to which such an interaction might be real. Prior to our current studies, the major argument for the existence of a connection with microtubules stemmed from the elegant ultrastructural data of Svitkina et al. (1996), demonstrating anti-plectin immunoreactive cross bridges between IFs and microtubules in fibroblasts depleted of their actin cytoskeleton.

Our studies now provide direct biochemical evidence of a microtubule-binding domain that is conserved among some members of the plakin family, including ACF-7, plectin, and kakapo. It is surprising that this domain is also a feature of BPAG1n1, as this protein associated exclusively with actin and IF cytoskeletons in foreign cells (Yang et al., 1996). In this assay, the actin-binding domain dominated over the microtubule association domain in the BPAG1n1 head segment. Further studies will be necessary in order to assess how BPAG1n1's cytoskeletal specificity is determined in vivo and whether it might be influenced by posttranslational modification and/or tissue-specific differences.

Differential expression of isoforms provides a potential mechanism for naturally weakening contacts with the actin cytoskeleton while preserving contacts between microtubules and IFs. It seems likely that previous ambiguities over the localization of plakins in vivo stem at least in part from the selectivity for different cytoskeletons imparted by different plakin isoforms. Indeed, on an NF null background, our BPAG1n3-specific antibody localized predominantly with axonal microtubules, while the broader specificity BProd antibody exhibited colocalization with all three cytoskeletal networks. Given the likely ability of plakins to dimerize and the potential for heterodimerization of isoforms, possibilities exist for bundling and interconnecting cytoskeletal networks through plakin isoforms.

BPAG1ns: MAPs that Are Central to Microtubule Stability, Axonal Transport, and Integrity of Sensory Neurons

Our results here indicate that BPAG1n plakins share considerable similarities to structural microtubule-associated proteins, for example, MAPs and tau, which also bind, stabilize, and bundle microtubules (Drubin and Kirschner, 1986; Lewis et al., 1989; Togel et al., 1998). While sequence homologies are not evident between the microtubule-binding domains of plakins and MAP/tau proteins, this is not surprising given the marked variability already noted among microtubule-binding segments of these proteins (Hirokawa, 1994; Mandelkow and Mandelkow, 1995).

The near perfect colocalization between the M1 domain of BPAG1 and the microtubule network in various transfected cells was even more striking for BPAG1n3 than when bona fide MAP/tau proteins were analyzed in similar fashion (Barlow et al., 1994; Togel et al., 1998). Moreover, in contrast to MAPs and tau proteins, which seem only to render microtubules resistant to depolymerizing drugs and not to cold (Baas et al., 1994), BPAG1n's M1 domain conferred microtubule stability in the face of all depolymerizing agents tested, including cold. The only other protein known to possess this property is STOP, a recently cloned calmodulin-regulated brain protein of as yet undetermined function (Guillaud et al., 1998).

Most importantly, by generating and analyzing DRG cultures from BPAG1 single and double knockout animals, we have provided in vivo functional data that demonstrate that a MAP-like protein is essential for resistance of axonal microtubules to cold and other depolymerizing drugs. Our findings are especially interesting because microtubules in neurons are significantly more stable than in other cell types (Webb and Wilson, 1980; Morris and Lasek, 1982; Baas et al., 1994). It has been postulated that this resistance is key to the remarkable ability of neurons to make axon-dendritic extensions and to transport proteins over long distances (Mitchison and Kirschner, 1988; Hirokawa, 1994; Mandelkow and Mandelkow, 1995). Our findings provide direct evidence in support of this notion.

When visualized on an NF null background to alleviate the complication incurred by NF aggregation, BPAG1 loss still elicited structural abnormalities in microtubules and their properties. We posit that the loss of BPAG1 directly impairs microtubule stability, which in turn compromises axonal transport and leads to neuron degeneration. This severe pathology is in striking contrast to the surprisingly subtle phenotypes seen in the nervous system of mice lacking MAPs or tau proteins (Harada et al., 1994; Edelmann et al., 1996; Takei et al., 1997).

In summary, our results suggest that a key function of BPAG1 is to orchestrate the organization and stabilization of the microtubule network of sensory neurons to allow axonal transport over large distances. We have (1) identified a direct microtubule-binding and stabilization domain in BPAG1n3, (2) shown that this isoform preferentially associates with microtubule and neuronal IF cytoskeletons in vivo, (3) demonstrated increased sensitivity of microtubules in cultured neurites lacking BPAG1, irrespective of whether they also lack NFs, (4) revealed ultrastructural aberrations in microtubules of sensory axons lacking BPAG1 $\pm$ NFs, and (5) shown that without BPAG1 $\pm$ NFs, sensory axons display swellings packed with vesicles and mitochondria, indicative of transport defects. When coupled with our previous studies, BPAG1-mediated stabilization of microtubules is likely to be achieved in multiple ways that include, but may not necessarily be limited to, integration of the microtubule network with actin and neuronal IF cytoskeletons and stabilization imparted uniquely by BPAG1n's M1 domain. Given the traditional roles ascribed to MAPs and those ascribed to plakins over the past 10 years, our results are both surprising and extraordinary and now pave the way for studies probing

more deeply into regulation and function of the fascinating plakin proteins and their many isoforms.

Experimental Procedures

Screening of Knockout Mice

The isolation and characterization of *BPAG1*$^{-/-}$ and *NF-L*$^{-/-}$ single knockout mice has been described (Guo et al., 1995; Zhu et al., 1997). Double null were engineered by mating and identified by genotyping.

Generation of BPAG1n Antibodies and Immunofluorescence Microscopy

GST fusion proteins were engineered to contain in frame either (1) a peptide encompassing amino acids 18–31 of BPAG1n3 (BPiso3) or (2) 670 residues of the coiled-coil rod segment (BProd) inserted into the EcoRI site of pGEX-2TK (Pharmacia). Proteins were purified, anti-BP antibodies were raised in rabbits, and sera was affinity purified prior to use (Yang et al., 1996). For immunofluorescence, cells were fixed with methanol (−20°C) for 10 min or 1% glutaraldehyde for 15 min at room temperature, washed three times with PBS, preblocked, and then incubated with antibodies in fresh solution at room temperature for 1–2 hr. Antibodies used were anti-FLAG M2 (1:100; IBI-Kodak, New Haven, CT), anti-BProd and anti-BPiso3 (1:50), anti-tubulin (1:100), anti-β-actin (1:100), and blue phalloidin (1:20; Sigma Chemicals, St. Louis, MO). Secondary antibodies were 1:1000 Alexa green and red.

Construction of pBPM1-FG, pBProd-FG, pBPM2-FG, pBPch2-FG, and Transfections

Human BPAG1 sequences encoding (1) 670 amino acids of the rod (amino acids 1208 to 1878 of BPAG1e), (2) 824 amino acids of the M1 segment (amino acids 141 to 965 of BPAG1n3), (3) the first 161 amino acids of BPAG1n3 encompassing the CH2 segment, or (4) 538 amino acids encompassing the M2 segment (amino acids 864 to 1402 of BPAG1n3) were subcloned in frame at the NotI/BamHI site in pCB6, a mammalian expression vector containing the CMV major early promoter and enhancer. Sequences encoding a 9 amino acid FLAG epitope tag were engineered at the C terminus of each segment, followed by a 3′ UTR and polyadenylation site in the vector. These four constructs, pBProd-FG, pBPM1-FG, pBPch2-FG, and pBPM2-FG, respectively, were then used along with pBPabd-FG (Yang et al., 1996) for transfections.

Culturing Sensory Neurons from Mouse Dorsal Root Ganglia

DRGs were cultured on coverslips in MEM medium supplemented with 15% FCS and NGF for 5 days. Coverslips were rinsed with PBS and neurite outgrowths lysed and extracted with 1% Triton X-100 in a cytoskeleton-stabilizing solution (Serva, NY, NY). After 5 min at room temperature, cells were briefly washed with fresh solution minus Triton X-100 and PEG. Samples were fixed in 1% glutaraldehyde in sodium-cacodylate buffer and quenched with sodium borohydride.

Ultrastructural Analyses and Immunoelectron Microscopy

Animals were sacrificed by intravenous perfusion with 2% glutaraldehyde, 4% formaldehyde and tissues processed for electron microscopy (Guo et al., 1995). Immunoelectron microscopy was performed using a preembedding procedure (Nagele et al., 1998) with incorporation of an additional first permeabilization step with digitonin (40 mg/ml) for 1 min. Cultures were treated with primary and secondary gold-conjugated (5 nm) antibodies, postfixed, and embedded in Epon.

In Vitro Microtubule Binding Assays

The BPAG1n M1 domain was fused in frame to GST, and bacterially expressed protein was purified (Yang et al., 1996). Prior to each binding assay, purified M1 domain was centrifuged at 100,000 × g for 15 min. Microtubule binding assays were performed essentially as described (Wallis et al., 1993; Coffey and Purich, 1995). Purified bovine brain microtubule protein (Sigma, St. Louis, MO) was reconstituted and polymerized according to the manufacturer's instructions. Polymerization ± taxol (20 μg/ml) was monitored spectrophotometrically at 350 nm in a cuvette thermostated to 37°C. Purified M1 domain (0.5 to 10 μM) was then added to polymerized microtubules for 20–30 min, followed by centrifugation at 100,000 × g for 30 min at 23°C in a TL-100 Beckman ultracentrifuge, a process that pelleted microtubules and bound protein.

Acknowledgments

We thank Don Cleveland for his critical reading of the manuscript and Mei Yin for her assistance in electron microscopy. We thank Drs. Tatyana Svitkina and Gary Borisy (University of Wisconsin) for their suggestions and recombinant gelsolin. We thank Dr. Robert Evans (University of Colorado Health Science Center, Denver) for SW13 cells. Finally, we thank Ms. Linda Degenstein for her assistance with mice. This work was supported by a grant from the National Institutes of Health (R01-AR27883). E. F. is an Investigator of the Howard Hughes Medical Institute.

Received March 11, 1999; revised June 14, 1999.

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Protein Data Bank ID Code

BPAG1n3 has been deposited in the Protein Data Bank under ID code AF165191.