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Isoform-specific functions of the dystonin cytoskeletal organizing proteins

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This thesis is submitted as a partial fulfillment of the Ph.D. program in Cellular and Molecular Medicine.

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Abstract

Dystonin/Bpag1 proteins, whose loss of function results in neuromuscular dysfunction in mice, have been implicated as being essential cytoskeletal organizing proteins in sensory neurons. How exactly they function as such is unclear, however. I have attempted to characterize the subcellular localizations and interactions of different dystonin isoforms in order to help place these proteins into their relevant cellular contexts. By using fusion protein analysis and observing the localization of endogenous dystonin in tissue culture cells, I have determined that the dystonin-a2 isoform can insert into internal membranes around the nucleus via an N-terminal transmembrane domain. Subsequently I demonstrated that a full-length dystonin-a2 fusion protein can reorganize the endoplasmic reticulum (ER) and associate with an outer nuclear membrane protein. The dystonin-a2 isoform is predominantly neuronal, with its strongest expression being in sensory ganglia. Analysis of dystonin in dorsal root ganglia (DRG) and of the DRG phenotype in dystonin mutant mice indicated that loss of the dystonin-a2 isoform, in particular, results in perikaryal abnormalities including disorganization of the rough ER. Analysis of other dystonin isoforms in cell culture indicated that they rely on different domains to dictate their localizations. Each of the dystonin isoforms may reorganize the cytoskeleton via direct interactions with actin filaments and microtubules. Our analysis indicates that a key cause of cell-intrinsic neuronal dysfunction and degeneration in dystonin mutant mice may occur as a result of aberrant cytoskeletal attachment to internal membranes, in particular the ER and nuclear envelope.

Contents

Page

Chapter 1. General Introduction 1

1.1 The cytoskeleton and nucleoskeleton provide an essential framework for normal cell function 2

1.1.1 Actin microfilaments 4

1.1.2 Intermediate filaments 4

1.1.3 Microtubules 5

1.2 Cytoskeletal abnormalities in neuromuscular disease 9

1.2.1 Neurofilament abnormalities and neurodegeneration 9

1.2.2 Tauopathies 10

1.3 Nucleoskeletal abnormalities in neuromuscular disease 11

1.3.1 TorsinA and dystonia 13

1.4 Dystonin deficient mice – the *dystonia musculorum* mice 15

1.4.1 The phenotype of *dt* mice 15

1.4.2 Histopathological analysis of *dt* mice 16

1.5 Dystonin and spectrin superfamily proteins are structural components of the cell 18

1.6 Hypothesis and specific aims 21

Chapter 2. Spectrin repeat proteins in the nucleus **26**

Summary 29

List of abbreviations 30

Introduction 31

Spectrin repeats 32

Spectrin proteins in the nucleus 37

Syne/nesprin, mAKAP, and Bpag1 – spectrin repeat proteins in the nucleus 40

Evolutionary considerations 43

Spectrin repeat proteins and nuclear positioning 46

Spectrin repeat proteins as scaffolds in the nucleus 47

Conclusion and future directions 49

References 52

Supplemental material 63

Chapter 3. Bpag1 localization to actin filaments and to the nucleus is regulated by its N-terminus **64**

Summary 67

Introduction 68

Materials and methods 71

Results 76

Page

Discussion 101
References 109

Chapter 4. A Bpag1 isoform involved in cytoskeletal organization surrounding the nucleus 113
Abstract 116
Introduction 117
Materials and methods 119
Results 124
Discussion 145
References 152
Supplemental material 158

Chapter 5. Dystonin/Bpag1 is a necessary endoplasmic reticulum/nuclear envelope protein in sensory neurons 161
Abstract 164
Introduction 165
Materials and methods 166
Results 171
Discussion 193
References 199
Supplementary materials 203

Chapter 6. General Discussion 208
6.1 Dystonin and human disease 209
6.2 What form of dystonin is the most relevant to understanding neurodegeneration? 210
6.3 Is dystonin in the nucleus important? 214
6.4 What is the relevance of neurofilament aggregates and axonal swelling in *dt* mice? 216
6.5 What is the relevance of perikaryal abnormalities in *dt* mice? 217

References 220

List of tables

Page

Chapter 1

Table I. An overview of mutant structural proteins involved in various pathologies . . 3

Table II. Neuromuscular disorders involving deficiency or defects in nuclear envelope proteins 12

Chapter 2

Table I. A summary of the locations and putative functions of different spectrin repeat containing proteins 33

List of figures

Page

Chapter 1

Figure 1. The cytoskeletal organization of a peripheral neuron and a skeletal muscle fiber 7
Figure 2. Domain architecture of the dystonin proteins, and comparison to other spectrin superfamily members 19
Figure 3. Deciphering the dystonin (*Dst*) gene and transcripts 22

Chapter 2

Figure 1. Structure of the spectrin repeat-containing proteins in the nucleus 35
Figure 2. Spectrin repeat proteins in the nucleus 38
Supplemental Figure 1. Alignment of the putative NuMA spectrin repeat 60

Chapter 3

Figure 1. Bpag1 isoforms expressed in C2C12 myoblasts, and the N-terminal fusion proteins used in this study 77
Figure 2. An antibody specific for Bpag1 isoform 2 detects protein co-aligning with actin stress fibers 80
Figure 3. Endogenous Bpag1a/b2 stress fiber localization does not extend into focal contact sites, and most of the A' Ab staining occurs in and around the nucleus 82
Figure 4. Disruption of actin filaments, but not of microtubules, alters Bpag1a/b2 localization 85
Figure 5. Bpag1e localizes predominantly to the nucleus in C2C12 myoblasts 87
Figure 6. The interaction of Bpag1 N-terminal fusion proteins with actin filaments in C2C12 myoblasts 89
Figure 7. The Bpag1 ABD, but not the isoform 2 N-terminus, localizes to focal contacts 91
Figure 8. The ABD-containing N-terminal regions of Bpag1 can self-associate but require the presence of the unique regions upstream from the ABD to do so 93
Figure 9. The Bpag1 isoform 2 N-terminus, but not the isoform 1 N-terminus, localizes to the nucleus of C2C12 cells when the plakin domain is included in the fusion protein 95
Figure 10. Bpag1 contains a functional NLS in the plakin domain 98

Chapter 4

Figure 1. The Bpag1 isoform 2 N-terminus contains a putative transmembrane domain 125
Figure 2. The Bpag1 isoform 2 N-terminus targets to the region surrounding the nucleus 128
Figure 3. The Bpag1 isoform 2 N-terminus is inserted into membranes surrounding

the nucleus 131
Figure 4. Perinuclear localization of endogenous Bpag1 isoform 2 134
Figure 5. Full-length Bpag1 fusion proteins 136
Figure 6. Bpag1a2 is a cytoskeletal-associated protein that concentrates in the perinuclear region of the cell 138
Figure 7. Bpag1 isoform 1 associates with microtubules and actin filaments throughout the cytoplasm in Cos-1 cells 141
Figure 8. Perinuclear FLAG-Bpag1a2 accumulates actin microfilaments around the Golgi and nucleus in C2C12 myoblast cells 143
Supplemental Figure 1. FLAG-Bpag1a2 nuclear and perinuclear localization in Cos-1 cells 158
Supplemental Figure 2. FLAG-Bpag1a2 nuclear foci and perinuclear FLAG-Bpag1a2 in C2C12 myoblasts 159
Supplemental Figure 3. Microtubule-associated FLAG-Bpag1a1 cross-linking with F-actin 160

Chapter 5

Figure 1. Dystonin isoform 2 is a unique plakin protein, absent in *dystonia musculorum* (*dt*) mice 172
Figure 2. Neuronal defects in *dt* mice include early abnormal F-actin punctae and a widespread loss of Nissl staining 176
Figure 3. Dystonin expression in the dorsal root ganglia 180
Figure 4. Dystonin in C2C12 cells co-localizes with ER and NE markers 183
Figure 5. Full-length dystonin isoform 2 fusion protein expressed in Cos-1 cells reorganizes the endoplasmic reticulum 186
Figure 6. The dystonin isoform 2 N-terminus can associate with nesprin-3α 188
Figure 7. Nesprin-3 and dystonin co-localize at the nuclear envelope in a small subset of DRG neurons 191
Figure 8. Putative model indicating associations of dystonin-a2 at the ER and the contiguous outer NE 195
Supplementary Figure 1. Dystonin isoforms 204
Supplementary Figure 2. Cloning of the dystonin isoform 3 5' end 205
Supplementary Figure 3. Full-length dystonin isoform 2 fusion protein expressed in Cos-1 cells primarily localizes around the nucleus and ER in association with F-actin 206

Chapter 6

Figure 1. Summary of the demonstrated and putative localizations and interactions of dystonin-a proteins 211

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Chapter 1

General Introduction

1.1 The cytoskeleton and nucleoskeleton provide an essential framework for normal cell function

In working towards therapies or treatments for pathological conditions, gaining an understanding of the basic context in which the conditions occur can be of great value. Implicit in this is an understanding of the basic architectural framework of cells and the molecular determinants that are required in establishing a normal framework. Cytoskeletal and nucleoskeletal structure are involved in determining almost all aspects of cell shape, size, function, and death. The structure of the nucleus, cytoplasm, and membranes of the cell depend upon the organization of a few basic types of filaments. These filaments, along with organizational and motor proteins associated with them, mediate a multitude of protein interactions throughout the cell, serving both mechanical and non-mechanical roles (reviewed in Dent and Gertler, 2003; Coulombe and Wong, 2004; Mallik and Gross, 2004). While loss of essential organizing proteins is often incompatible with survival, and redundancy between proteins in other cases allows for normal survival with little or no pathological symptoms, loss of function or the production of deleterious mutants of many basic structural proteins lead to pathologies resulting in shortened lifespan and a multitude of disabilities in humans (Table I) (Chavanas, 1996; Salmikangas et al., 2003; Ilkovski et al., 2004; Yao et al., 2004; reviewed in Cairns et al., 2004; Magin et al., 2004; Ramaekers and Bosman, 2004).

Three basic types of filaments exist in eukaryotic cells, having been described originally from electron microscopy images based on their sizes (reviewed in Janmey, 1991; Alberts et al., 1994; and Strelkov et al., 2003). These are the microfilaments (7 – 10 nm diameter) composed of actin polymers; intermediate filaments (10 – 12 nm

Table I. An overview of mutant structural proteins involved in various pathologies

Disease	Phenotypic traits	Affected protein(s)	Cytoskeletal role*
Epidermolysis bullosa simplex with muscular dystrophy	skin blistering and muscle weakness	plectin	multifunctional crosslinker interacting with IFs, MFs, and adhesion proteins
Limb girdle muscular dystrophy type 1A	weakness and atrophy of shoulder and hip muscles	myotilin	actin MF bundling in muscle sarcomere
Nemaline myopathy	muscle weakness	nebulin	regulation of MF length
		muscle α-skeletal actin	muscle MF component
Duchenne/Becker muscular dystophies	muscle weakness and atrophy; cardiomyopathy	dystrophin	tethering MFs to muscle sarcolemma
Desmin myopathy	muscle weakness; cardiomyopathy	desmin	muscle IF component
Amyotrophic lateral sclerosis	progressive neuropathy affecting motor neurons	neurofilament heavy	neuronal IF component
		tau	neuronal MT stabilization
Charcot-Marie-Tooth disease types 1 and 2	neuropathy affecting motor and sensory neurons with muscle atrophy	neurofilament light	neuronal IF component
Alexander disease	early childhood spasticity and dementia	glial fibrillary acidic protein	glial IF component
Focal segmental glomerulosclerosis	kidney dysfunction	α-actinin-4	MF organization
Spherocytosis	red blood cell fragility leading to anemia	α-spectrin	cortical MF organization
Inflammatory bowel disease	inflammation of the intestines	keratin 8	epithelial IF component
mutliple skin disorders	skin blistering and fragility	various keratin proteins	epidermal IF components

*IF = intermediate filament; MF = actin microfilament; MT = microtubule

diameter) composed of various structurally-related proteins; and microtubules (25 nm diameter) composed of tubulin polymers.

1.1.1 Actin microfilaments

Actin microfilaments, in general, are highly cross-linked filaments that provide rigidity to the cell. They are organized into a multitude of structures involved in a wide variety of functions such as cell division, cell movement, attachment of intracellular organelles, and tissue-specific roles such as muscle contraction. Actin monomers and polymers are in a constant state of flux, and actin filaments can be rapidly assembled and disassembled in conjunction with filament nucleating, assembly, and disassembly proteins (reviewed in Paavilainen et al., 2004). This allows actin filaments to rapidly adapt to the changing needs of a cell and to be a driving force in processes such as cellular motility. More recently, actin monomers and short oligomers have been demonstrated to play a role in various nuclear functions including chromatin remodelling and RNA polymerase activity (Hofmann et al., 2004; McDonald et al., 2006; reviewed in Percipalle and Visa, 2006). Actin microfilaments have also been implicated in contributing to the structure of the nuclear lamina (Holaska et al., 2004). Thus, actin monomers and filaments, expressed ubiquitously throughout all phyla, have come to play important roles throughout multiple regions of all cell types.

1.1.2 Intermediate filaments

Intermediate filaments are commonly thought to be important for adding to the mechanical strength of cells, and exhibit a high tensile strength. They are found in high

abundance in mechanically challenged tissues such as skin and muscle. In various cell types different classes of intermediate filaments play prominent roles in specialized structures, such as desmosomes and hemidesmosomes in keratinocytes, neuronal axons, and the Z-lines of skeletal muscle fibres. In addition, lamin intermediate filaments are essential to nucleoskeletal structure and cell survival throughout all cell types of the animal phylum (Schulze et al., 2005). Aside from lamin intermediate filament proteins, the other intermediate filament proteins are often partly or completely dispensible, with mouse genetic knock-outs often showing strikingly little phenotype (Capetanaki et al., 1997; Zhu et al., 1997; Zhu et al., 1998; Levavasseur et al., 1999; Reichelt, 2001), and the fruit fly being considered to lack all non-lamin intermediate filaments entirely (Gregory and Brown, 1998). This is indicative of the specialized functions of intermediate filaments often being either easily compensated for, or not necessary for normal survival. However, several human diseases have been linked to mutant intermediate filament proteins (Table I), and intermediate filament aggregates are often associated with neuronal dysfunction (see below).

1.1.3 Microtubules

Microtubules are rigid filaments that emanate from the center of the cell (the microtubule organizing center). They are, in general, important for determining the overall organization of a cell by regulating organelle positioning and the trafficking of various subcellular components. Despite their rigidity, microtubules display a highly curved appearance in cells, and are capable of bearing large compressive loads when coupled to the surrounding cytoskeleton (Brangwynne et al., 2006). This enables them to contribute

significantly to the mechanical behaviour of cells. Microtubules, like microfilaments, are also highly dynamic, and their rapid polymerization and depolymerisation contributes to processes such as neurite outgrowth (Buck and Zheng, 2002), and chromosome segregation during cell division (Mitchison et al., 2005).

The cytoskeleton, though composed of only the few types of filaments, is extremely plastic. While actin and tubulin proteins are the only two cytoskeletal proteins that are conserved throughout all phyla, multiple isoforms of these two proteins provide for some diversity (Hoyle and Raff, 1990; Wagner et al., 2002; Hinz et al., 2002). For instance, muscle α-skeletal actin is an isoform unique to muscle that contributes to the specialized mechanical properties of this tissue (Rubenstein, 1990; Crawford et al., 2002). A large variety of intermediate filament proteins, such as vimentin, desmin, neurofilament proteins, or analogous bacterial proteins such as crescentin, also provide for diversity in cytoskeletal structure (McCormick et al., 1991; Ausmees et al., 2003). An enormous diversity of cytoskeletal organizing proteins are ultimately responsible for manipulating the basic structures of actin microfilaments, microtubules, and intermediate filaments, and determining the incredible array of cytoskeletal structures necessary to give different cell types their unique morphologies and functions (Fig. 1).

This thesis discusses the functions of one group of cytoskeletal interacting proteins, whose loss of function in mice results in a movement disorder with characteristics similar to human dystonia. These proteins, products of the *dystonin/Bpag1* (*Dst*) gene, constitute a group of cytoskeletal organizers capable of interactions with microfilaments, microtubules, and intermediate filaments (Brown et al., 1995a;

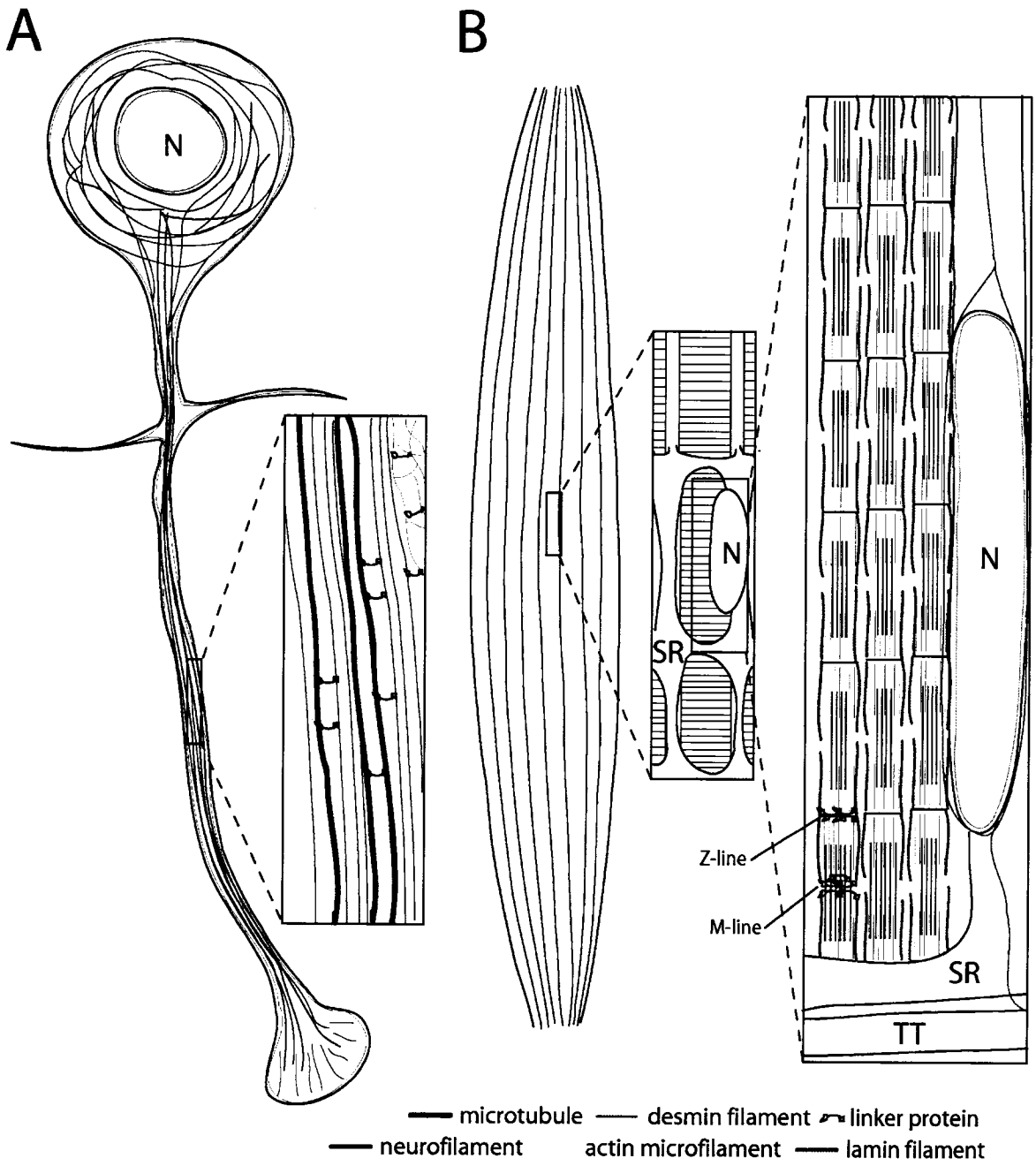

Figure 1. The cytoskeletal organization of a peripheral neuron and a skeletal muscle fiber. **A,** Schematic diagram of a peripheral ganglion neuron, showing a simplified view of the perikaryal (cell body), axonal, and growth cone cytoskeleton. Microtubules and intermediate filaments, including neurofilaments, are prominent in the axon and

perikaryon. Actin microfilaments play a prominent role in cellular protrusions, such as the growth cone of a neuron. Cytoskeletal organizing proteins, including linker proteins, are shown interconnecting axonal filaments along the length of the axon. Underneath the plasma membrane, a meshwork of cortical actin filaments is connected by a different set of proteins, which also link to the membrane. **B,** Schematic diagram of the organization within a muscle, with a section of an individual myofibril showing its sarcomeric organization. An individual sarcomere is bounded by Z-lines, which contain multiple organizational proteins that tether actin and desmin filaments. In the middle of the sarcomere, extending out from the M-line, are the myosin thick filament which interdigitate with the actin thin filaments. Microtubules play a less prominent structural role in mature muscle, with a limited localization surrounding the nuclei and connecting to the sarcolemmal membrane (of which the transverse tubules are an extension). N = nucleus; SR = sarcoplasmic reticulum; TT = transverse tubule.

Leung et al., 2001a). The focus of this work has revolved around placing the various dystonin isoforms into their relevant cellular contexts to help elucidate exactly what cytoskeletal interactions they are involved in, and what necessary function is lost with mutation of the *Dst* gene.

1.2 Cytoskeletal abnormalities in neuromuscular disease

The etiology of neuropathologies and muscle disorders varies widely. Defects and deficiencies in cytoskeletal filament proteins and cytoskeletal interacting proteins play a large role in many of these disorders. While some cytoskeletal filament proteins and organizing proteins are dispensable for normal neuromuscular function, the loss or aberrant function of many have proven to be important in the development of neuromuscular dysfunction.

1.2.1 Neurofilament abnormalities and neurodegeneration

Amyotrophic lateral sclerosis (ALS) and Charcot-Marie-Tooth (CMT) disease are two neuropathies which can be caused by mutations in neurofilament (NF) genes, and consistently involve neurofilament aggregation (reviewed in Cairns et al., 2004). Neurofilaments are specialized neuronal intermediate filaments implicated in the etiology of several neurological disorders also including Alzheimer's disease, diabetic neuropathy, dementia with Lewy bodies, giant axonal neuropathy, neuronal intermediate filament inclusion disease, and Parkinson's disease. Several mutations in the *NF-L* (neurofilament light) gene affecting various regions of the protein are associated with CMT disease (Perez-Olle et al., 2002; Jordanova et al., 2003). Likewise, various mutations in the *NF-H*

(neurofilament heavy) gene can cause ALS (reviewed in Robberecht, 2000). While genetic mouse knock-outs for NF-L and NF-H exhibit no overt phenotype with a loss of neurofilament function (Zhu et al., 1997; Zhu et al., 1998), the involvement of neurofilament disorganization in multiple neurodegenerative diseases and neurofilament mutations in ALS and CMT indicates that aberrant neurofilament organization and function are common features of neurodegeneration.

1.2.2 Taupathies

Tauopathies are a group of neurological disorders which involve the aberrant function, or loss of function, of the microtubule associated protein, tau (reviewed in Cairns et al., 2004). Neuromuscular disorders associated with abnormal accumulations of the tau protein include Alzheimer's disease, ALS, corticobasal degeneration, dementia pugilistica, Down's syndrome, Pick's disease, and progressive supranuclear palsy, among others. The *tau* gene itself is mutated in a neuropathy known as FTPD-17 (Hutton et al., 1998). Tau is a small axonal protein produced as six variable isoforms generated by alternative splicing. It is also present at low levels in astrocytes and oligodendrocytes. Tau isoforms serve to stabilize and promote the polymerization of microtubules. Hyperphosphorylated and polymerized tau is found in neurofibrillary tangles, and the number of tangles correlates with the degree of dementia in Alzheimer's disease (Alonso et al., 2006). However, it is the ability of unpolymerized hyperphosphorylated tau to sequester normal tau that results in neurofibrillary tangle formation and microtubule destabilization. This indicates that the neurofibrillary tangles themselves have nothing to

do with progression of tauopathies, but rather it is the aberrant sequestration of normal tau that causes the resulting neuronal dysfunction.

Although neurofilament aggregates and neurofibrillary tangles associated with hyperphosphorylated tau are two common features of neuropathologies, the underlying mechanisms that cause these features are very heterogenic, even within individual disorders. For example, CMT disease and the associated neurofilament aggregates can, in addition to being caused by *NF-L* mutations, be caused by mutations in genes coding for tyrosyl-tRNA synthetase (Jordanova et al., 2006), an enzyme required for protein synthesis, mitofusin 2 (Verhoeven et al., 2006), a mitochondrial membrane protein, and laminA/C proteins (De Sandre-Giovannoli et al., 2002), structural components of the nuclear lamina. In ALS, only rare cases involve mutations in the *NF-H* or *tau* genes. Mutations in the gene coding for superoxide dismutase 1, an antioxidant enzyme with chaperone activity, are more commonly found in this disease (in approximately 20% of patients with familial ALS) (Al-Chalabi et al., 1999; Al-Chalabi and Miller, 2003; Munch et al., 2005; Tummala et al., 2005). Thus, the role of the cytoskeleton in neuropathologies involving cytoskeletal aggregates or disorganization is not clear.

1.3 Nucleoskeletal abnormalities in neuromuscular disease

In addition to defects in the cytoskeleton and cytoskeletal interacting proteins resulting in a wide range of neuromuscular pathologies, defects in the nuclear lamina, a filamentous network underlying the inner nuclear membrane, can cause several neuromuscular disorders (Table II). These are primarily laminopathies, caused by mutations in the lamin

Table II. Neuromuscular disorders involving deficiency or defects in nuclear envelope proteins*

Disease	Inheritance	Phenotypic traits	Affected protein
Emery-Dreyfus muscular dystrophy	X-linked or AD	slowly progressing contractures and muscle weakness; wasting of sk. muscle and cardiomyopathy	emerin or lamin A/C
Limb-girdle muscular dystrophy type 1B	AD	slowly progressing pelvic girdle weakness; development of contractures and cardiac disturbances	lamin A/C
Dilated cardiomyopathy with conduction defect	AD	dilated cardiomyopathy	lamin A/C
Charcot-Marie-Tooth disorder type 2B1	AR	motor and sensory neuropathy; diabetes	lamin A/C
Early onset torsion dystonia (dystonia musculorum deformans)	AD	poor muscle tone and prolonged muscle contraction; results in body twisting and abnormal posture	torsin A

*adapted from Somech et al., 2005

A/C (*LMNA*) gene (reviewed in Ostlund and Worman, 2003; Worman and Courvalin, 2004). Multiple disease causing mutations throughout this gene have been described, and are associated with various types of disorders ranging from Emery-Dreyfus muscular dystrophy to progeria to lipodystrophy. While lamin A and C proteins are clearly important structural filament proteins within the nuclear envelope, they also likely play important roles in gene regulation (reviewed in Hutchison and Worman, 2004). In mechanically challenged cells such as muscle and peripheral neurons, loss of structure may result from a structural compromise of the nuclear lamina, but it is just as likely that aberrant gene regulation is a significant contributing factor to many or all of the laminopathies.

1.3.1 TorsinA and dystonia

Recently, a mutant form of the torsinA protein, produced in patients with early onset torsion dystonia (*dystonia musculorum deformans*) (Ozelius et al., 1997), has been demonstrated to interfere with nuclear envelope structuring (Naismith et al., 2004; Goodchild et al., 2005). TorsinA is an endoplasmic reticulum/nuclear envelope protein belonging to the AAA+ (*A*TPase *a*ssociated with different cellular *a*ctivities) family of chaperone proteins (Ozelius et al., 1997). While torsinA itself is not likely to play a structural role at the nuclear envelope, loss of a normal interaction between torsinA and a structural nuclear envelope protein or proteins is thought to cause the nuclear envelope abnormalities observed in cells expressing the mutant protein. That expression of mutant torsinA results in a loss-of-function phenotype is suggested by the fact that neuronal

nuclear envelopes from torsinA knock-out mice contain defects similar to those seen in cells expressing the mutant torsinA (Goodchild et al., 2005).

In both tissue culture cells expressing mutant torsinA, and in neurons from transgenic mice expressing the mutant protein, an increased localization to the nuclear envelope compared to the wild-type torsinA has been observed (Goodchild and Dauer, 2004; Naismith et al., 2004; Goodchild and Dauer, 2005; Goodchild et al., 2005). Cells expressing mutant torsinA have dramatic alterations in the structuring of the outer nuclear membrane, with membranous inclusions forming within the perinuclear space. One normal interaction between torsinA and a nuclear envelope structural protein that could be affected is an interaction with the lamina associated polypeptide 1 (LAP1) protein (Goodchild and Dauer, 2005). Based on the phenotype of cells expressing the mutant torsinA, and neuronal nuclear envelopes of torsinA knock-out mice, it is likely that loss of normal torsinA function results in the alteration of a link between the nuclear lamina and the outer nuclear membrane and surrounding cytoskeleton (Goodchild et al., 2005; and reviewed in Gerace, 2004). Though the nuclear envelope can be adversely affected in non-neuronal cells expressing mutant torsinA in culture, this effect appears to be limited primarily to neurons in torsinA knock-out or mutant torsinA knock-in mice (Goodchild et al., 2005). These neuronal nuclear envelope defects may help explain the early onset torsion dystonia phenotype, which has been primarily attributed to a neuropathy affecting basal ganglia neurons (Rostasy et al., 2003).

Thus, defects in filament and filament-organizing proteins can cause a variety of pathologies, which may ultimately lead to a mechanical breakdown in cell structure

and/or an alteration in gene expression and subsequent cellular dysfunction. Early onset torsion dystonia, in particular, appears to be caused by a breakdown in structure of the neuronal nuclear envelope due to a break in the link between the nuclear lamina and the cytoskeleton surrounding the nucleus. This is an example where there is a clear structural defect within a central organizing feature of the cell (the nucleus) which may lead to a widespread mechanical breakdown of the cell and which, due to potential deformations within the nucleus, may also involve the aberrant regulation of gene expression. As with the laminopathies, dissecting out the exact cause of the resulting neuronal dysfunction in torsion dystonia should shed light on the importance of specific mechanical roles of nuclear envelope-associated proteins for normal neuronal function, and indicate what is necessary to cause dysfunction.

1.4 Dystonin deficient mice – the *dystonia musculorum* mice

1.4.1 The phenotype of *dt* mice

Dystonia musculorum (dt) mice were originally described in the mid 1960s as being a spontaneously arising mutant with a severe movement disorder (Duchen et al., 1963; Duchen and Strich, 1964). They were much later demonstrated to contain mutant alleles of the *dystonin/Bpag1* gene (Brown et al., 1995a). The condition of the mice resembles human dystonia in that both are generated primarily due to a neuropathy accompanied by poor muscle tone, abnormal posturing, and uncoordinated twisting movements. Human dystonia does not normally result in a shortened lifespan, though this is the case in *dt* mice, with death often occurring by the time of weaning. However, among the originally described mutants, some lived well into sexual maturity (as long as 8 months), and it has

been suggested that factors such as an inability to groom and to feed normally contribute significantly to the early death of the mice (Duchen and Strich, 1964; Messer and Strominger, 1980). This is supported by the observation that affected mice tend to develop a more severe phenotype and die earlier when born into larger litters, where there would be more competition for maternal care and feeding (De Repentigny, unpublished observations). The *dt* mutation in and of itself, therefore, is likely not lethal, though the resulting movement disorder causes an extremely debilitating condition.

1.4.2 Histopathological analysis of *dt* mice

Upon histopathological analysis of the neuromuscular system of *dt* mice, three basic features of sensory neurons were initially described as being abnormal (Duchen and Strich, 1964). The first two were the appearance of focal swellings along the nerve fibres and the gradual loss of nerve fibres in both the central and peripheral regions of the sensory pathways. The third was an abnormality in some of the neuronal cell bodies consisting of chromatolysis (which involves a dispersion of rough endoplasmic reticulum from around the nucleus) with eccentricity (misplacement) of the nucleus. As the genetic defect was unknown at the time, only mice with an observable phenotype were used for histological analysis, and it is therefore possible that some of the observed abnormalities were secondary to the original defect. With this in mind, nerve fibre swellings were the one noted defect that was most prominent in the youngest animals studied.

Based on extensive histological analysis at the electron microscope and light microscope levels of peripheral and central neurons, Sotelo and Guenet (1988) have hypothesized that the *dt* mutation causes a "dying back" of the sensory neurons. That is,

the disorder may initially affect axons, largely of the first order sensory neurons and spinocerebellar neurons, and progressively lead to abnormalities in the cell bodies. That neurofilament accumulation in focal swellings of the neurites is prominent in *dt* mice (Sotelo and Guenet, 1988) suggests a disruption in cytoskeletal organization, and possible problems with axonal transport. More recently, neurofilament and microtubule disorganization within *dt* axons was correlated with axonal transport defects of acetylcholinesterase in the sciatic nerve (De Repentigny et al., 2003). Thus axonal specific defects may be the primary cause of the *dt* phenotype.

Alternatively, however, cell body defects including chromatolysis with eccentric nuclei have been consistently reported for several *dt* mutant lines (Duchen and Strich, 1964; Hanker and Peach, 1976; Messer and Strominger, 1980; Sotelo and Guenet, 1988), and axonal neurofilament aggregates in *dt* axons have been clearly demonstrated to be irrelevant to the progression of the disease (Eyer et al., 1998). In transgenic mice expressing a mutant neurofilament that is restricted to the cell body and which restricts endogenous neurofilaments to the cell body, axonal swellings still develop in mice additionally homozygous for the *dt* mutation (Eyer et al., 1998). Cellular components such as mitochondria now aggregate within the neurite swellings of these mice. Additionally, no intrinsic axonal transport defect has been noted in DRG neurons from *dt* mice which were grown in culture, and microtubules appeared normal in this situation (Pool et al., 2006). Therefore, the *dystonin* gene products are not absolutely necessary for axonal cytoskeleton organization, and neurofilament aggregates, a hallmark of several neurodegenerative disorders, are likely a secondary feature of the disorder. While eccentric nuclei and chromatolysis may also be a secondary consequence of the *dt*

mutation, with these features known to occur following axotomy (McIlwain and Hoke, 2005), they may also be more indicative of the primary defect.

1.5 Dystonin and spectrin superfamily proteins are structural components of the cell

Neuronal and muscle isoforms of dystonin (dystonin-a and dystonin-b isoforms, respectively), generated by alternative splicing of the *dystonin* gene, contain multiple spectrin repeat domains, and belong to the spectrin superfamily of proteins (Fig. 2) (Leung et al., 2001a; reviewed in Leung et al., 2001b; Roper et al., 2002). Spectrin-related proteins have consistently been described as serving primarily structural roles within the various cell types in which they are expressed (reviewed in Roper et al., 2002; Beck, 2005; Worman and Gundersen, 2006). Indeed, every spectrin superfamily member for whom a function has been ascribed has been characterized, at least in part, as being a structural component of the cell. It is therefore reasonable to assume that any newer members of this superfamily of proteins would play at least some kind of structural role.

Early studies examining the functions of non-epithelial dystonin isoforms came to the conclusion that dystonin proteins were essential cytoskeletal cross-linkers capable of linking actin filaments to intermediate filaments (Yang et al., 1996; Leung et al., 1999a) or stabilizing axonal microtubules (Yang et al., 1999). These studies based their conclusions largely on the ability of actin and intermediate filament binding domains in dystonin to associate with microfilaments and neurofilaments or peripherin intermediate filaments, or the ability of a "cryptic microtubule binding domain" to associate with and stabilize microtubules (Yang et al., 1996; Yang et al., 1999; Leung et al., 1999a). Along

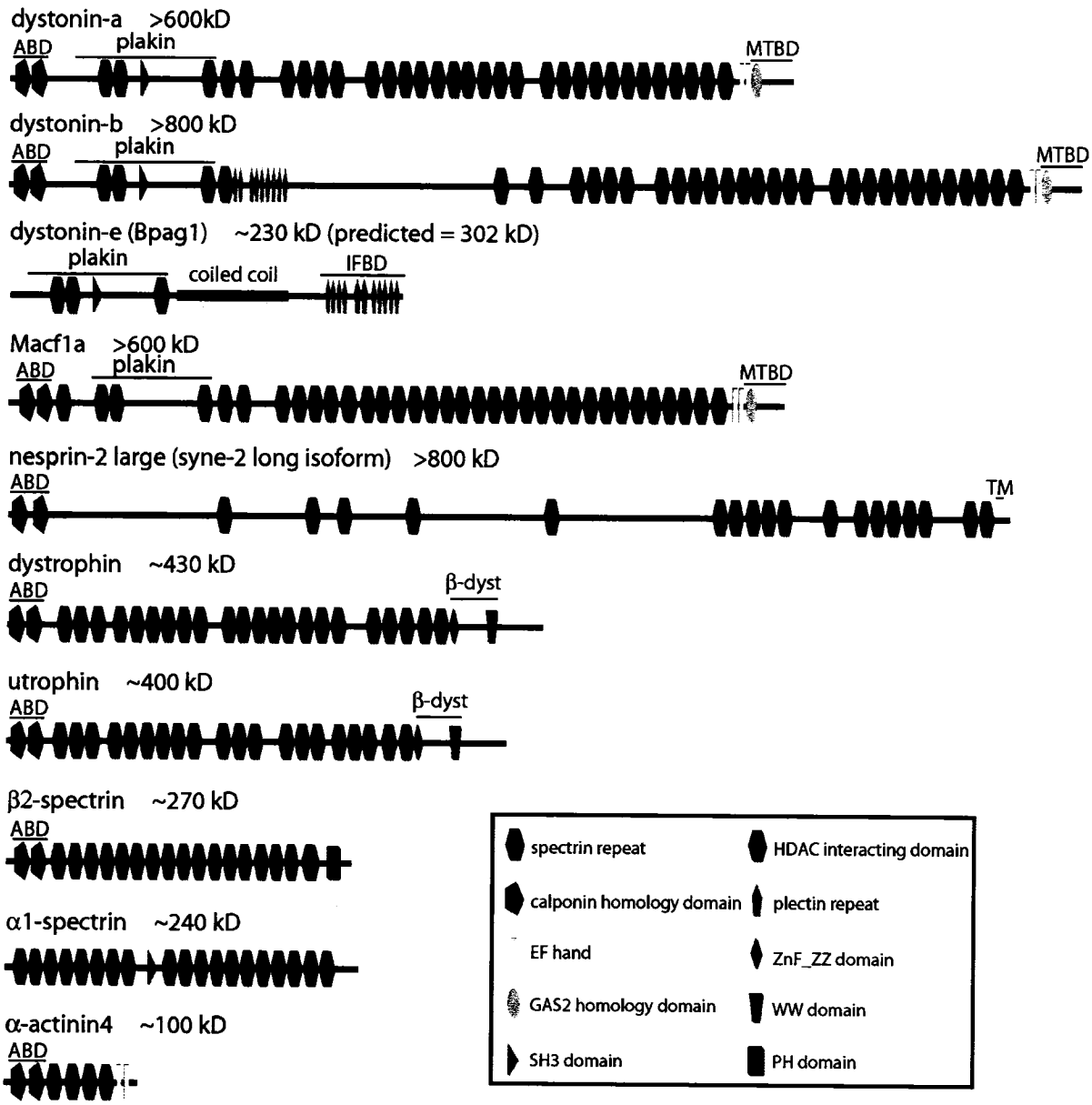

Figure 2. Domain architecture of the dystonin proteins, and comparison to other spectrin superfamily members. Spectrin-related proteins are all relatively large modular proteins. In general, these proteins contain an N-terminal actin binding domain (ABD), composed of tandem calponin homology domains, a central coiled coil region consisting of multiple spectrin repeats, and a C-terminal functional domain. The C-terminal ends of the neuronal and muscle dystonin-a/b isoforms contain EF hand motifs, indicative of calcium binding, and a GAS2 homology domain, which serves as part of a microtubule binding

domain (MTBD) (Leung et al., 2001a). The epithelial dystonin e isoform contains an intermediate filament binding domain (IFBD) within its C-terminus, composed of multiple plectin repeats (Fontao et al., 2003). Additionally, the shared plakin domain region of the dystonin isoforms contains putative histone deacetylase (HDAC) interacting and SH3 domains. Macf1a (shown) and Macf1b (not shown) isoforms are similar in structure to the corresponding dystonin isoforms, and exhibit extensive amino acid identity. Nesprin-2 is expressed as multiple isoforms, the longest of which contains an N-terminal ABD and a C-terminal transmembrane (TM) domain (Zhang et al., 2001; Zhen et al., 2002). Muscle dystrophin and the similar utrophin protein each contain C-terminal β-dystroglycan interacting regions (β-dyst), composed in part by ZnF_ZZ and WW domains (Ishikawa-Sakurai et al., 2004). β2-spectrin contains a C-terminal pleckstrin homology (PH) domain in its long isoform which is possibly involved in regulating membrane associations. α1-spectrin, which would typically dimerize with a β-spectrin isoform (Goodman and Branton, 1978; Woods and Lazarides, 1986), contains a central SH3 domain, involved in mediating protein interactions. α-actinin-4 contains C-terminal EF hand motifs. Domains shown by coloured symbols are those identified by SMART (Simple Modular Architecture Research Tool) (http://smart.embl.de/) analysis of the respective mouse protein sequences, and domain descriptions are provided on the SMART server. All protein lengths are drawn to scale according to the number of amino acid residues in each. The putative dystonin HDAC interacting domain was identified only with the use of a recent version (v. 5.0) of SMART. Predicted and/or approximate empirical molecular weights are given; for the large dystonin, Macf1, and nesprin proteins, empirical determinations of size is difficult.

with the cytoskeletal disorganization in neurons and glia described in dystonin mutant mice by these and other authors (Dalpé et al., 1998; Bernier et al., 1998), a reasonable assumption was that mice lacking functional dystonin isoforms developed a dystonic phenotype as a direct result of the inability to properly maintain cytoskeletal organization in neuronal cells, and most importantly within the axons.

This assumption, however, has to be re-evaluated in light of the fact that early descriptions of the dystonin transcripts were both incomplete and erroneous (an overview of the dystonin gene, and evolution of the proposed transcript structures is given in Fig. 3). Corrected transcripts and the translated proteins (Leung et al., 2001a; Goryunov et al., 2006; Jefferson et al., 2006; Young et al., 2006) vary greatly in their overall structure compared to the originally described neuronal dystonin. Notably, the originally described neuronal dystonin isoforms did not show the extensive overall similarity to spectrin superfamily members that the revised isoforms do (Fig. 3). As well, a lack of reagents such as antibodies for immunodetection of endogenous dystonin proteins has hampered early research into the function of these proteins. And, though it is suggested from studies of dystonin deficient mice that dystonin proteins are important cytoskeletal-interacting proteins required for the maintenance of subcellular architecture, exactly how they function as such is not clear.

1.6 Hypothesis and Specific Aims

While the domain structure of neuronal dystonin-a isoforms has strongly indicated a role for dystonin in cytoskeletal organization, specific functions indicated for endogenous dystonin isoforms are unclear following a correction of the overall transcript structure of

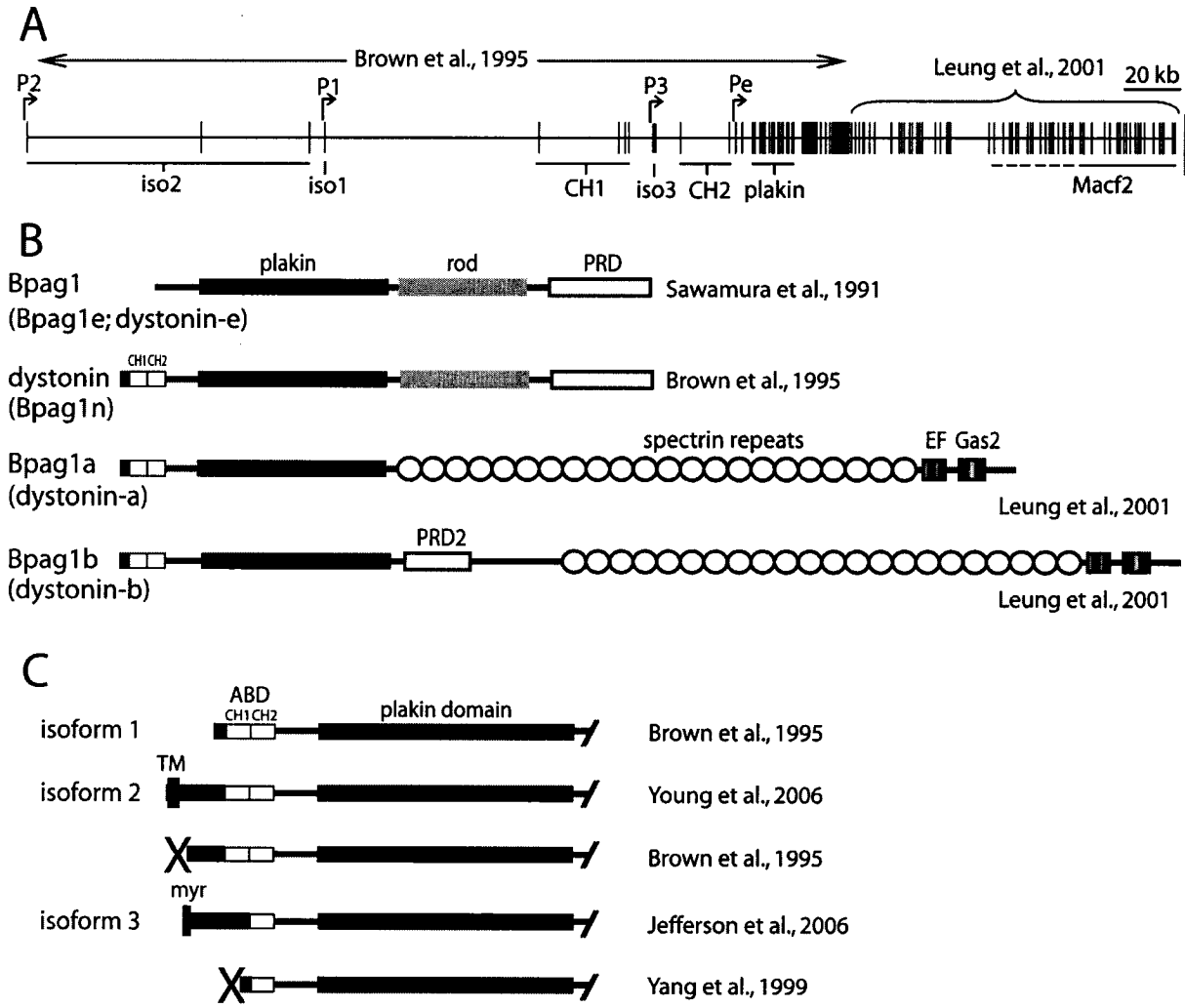

Figure 3. Deciphering the dystonin (*Dst*) gene and transcripts. **A,** Overview of the mouse dystonin gene. The original description of dystonin gene sequence was from Brown et al. (1995a), in which exons coding for alternate 5' mRNA transcript sequence and sequence coding for an actin binding domain (ABD; composed of the CH1 and CH2 regions) were demonstrated to be upstream from a Bpag1 coding region. No analysis of the 3' end of the transcipts was carried out in this study, however, and the 3' end of the gene was not described. The 3' end of the gene, coding for what was originally named Macf2, was later described in Leung et al. (2001a). The whole gene is ~ 400 kb in mice, ~500 kb in humans, and contains >100 exons in both. **B,** The original full-length cDNA sequence for a *Dst* gene product was presented in Sawamura et al. (1991), and codes for the epithelial

Bpag1 protein (dystonin-e). The protein contains sequence conserved in related epithelial proteins, the plakin domain. Downstream from this is a rod domain and plakin repeat domain (PRD), which acts as an intermediate filament binding domain. Transcripts with upstream coding sequence for the ABD were subsequently demonstrated to be produced in neuronal tissue, and were referred to as dystonin (Brown et al., 1995a). It is still unclear whether transcripts identical to the originally described dystonin are produced to any great extent. The predominant neuronal transcripts were later demonstrated to contain coding sequence for a large spectrin repeat region and downstream EF hands and Gas2 homology domain (Leung et al., 2001a), instead of the originally proposed rod domain and PRD. The predominant muscle transcripts are similar to the neuronal transcripts, but additionally contain sequence coding for a novel PRD region (Leung et al., 2001a). The original names are used here, with alternate nomenclature in brackets. Transcript lengths are not to exact relative scale. **C,** The neuronal and muscle transcripts can vary at the 5' end, producing protein isoforms specified by differing N-terminal regions [putative promoter regions for these isoforms and the known promoter for epithelial isoform (P1, P2, P3, and Pe) are shown in A]. The isoform 2 variant contains sequence coding for a highly conserved N-terminal transmembrane (TM) domain, and the isoform 3 variant contains sequence coding for a conserved myristoylation (myr) motif (Young et al., 2006; Jefferson et al., 2006). Most of the isoform 2-specific sequence was originally identified in Brown et al. (1995a), though the start site was not identified (indicated by an X). The original isoform 3-specific sequence was largely incomplete and erroneous due to an apparent sequencing error (Yang et al., 1999; indicated by an X).

dystonin-a (Leung et al., 2001a). As well, the need for neuronal dystonin isoforms defined by alternate N-terminal regions (Brown et al., 1995a) had not been addressed. We had hypothesized that the alternate N-termini would impact on the function of the common N-terminal actin binding domain, and influence the localization and function of the various isoforms. Loss of isoform-specific functions may provide information necessary to determine why the *dystonia musculorum* phenotype develops. To examine the localization and potential functions of dystonin-a isoforms, we had the following specific aims:

1. To determine the impact of alternate N-terminal unique regions on the localization and interaction with actin filaments of dystonin proteins.
2. Following a further refinement of the dystonin isoform 2 transcript sequence, to examine the role of a novel N-terminal transmembrane domain in determining subcellular localization and its impact on the localization and cytoskeletal interactions of full-length dystonin-a fusion proteins.
3. To correlate loss of possible organizing functions of dystonin associated with the nucleus and perinuclear structures with the pathology in *dystonia musculorum* mice.

While dystonin isoforms resemble spectrin-related proteins involved in regulating the organization of cytoplasmic filaments, they also resemble the nuclear/nuclear envelope spectrin-related proteins, the nesprins. This similarity is elaborated on in Chapter 2 where possible roles of a nuclear localized dystonin and related proteins are discussed and in Chapter 4 where the identification of the transmembrane domain in the

dystonin-a2 isoform is detailed. In trying to elucidate a necessary role for neuronal dystonin, we have elaborated on potential structural roles for dystonin in and around the nucleus, and extending out to the endoplasmic reticulum, and have tried to define how loss of these structural functions may contribute to the phenotype of dystonin deficient mice.

Elucidation of the exact roles that dystonin proteins play in neurons, and the ultimate reason for neuronal dysfunction and degeneration caused by the loss of neuronal dystonin, should help provide fundamental insight into the etiology of neuropathologies. As with several other neurodegenerative disorders, murine *dystonia musculorum* was early on associated with neurofilament abnormalities and aggregation. Yet, it remains unclear what role neurofilaments play in neurodegeneration, as diseases such as CMT and ALS that can be caused by neurofilament mutations are more commonly caused by gene mutations that have little obvious connection to cytoskeletal organization. As well, though cytoskeletal organization is clearly important to all tissues and cell types, to what extent cytoskeletal disorganization may be causative of various neuropathies, and for what exact reasons it may be causative, are key questions.

Chapter 2

Spectrin repeat proteins in the nucleus

Contributions of the authors

I had written the original manuscript for this article independently. Input from Rashmi Kothary and others was provided during the editing of the paper.

Spectrin repeat proteins in the nucleus

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Summary

Spectrin repeat sequences are among the more common repeat elements identified in proteins, typically occurring in large structural proteins. Examples of spectrin repeat-containing proteins include dystrophin, α-actinin, and spectrin itself – all proteins with well-demonstrated roles of establishing and maintaining cell structure. Over the past decade, it has become clear that although these proteins display a cytoplasmic and plasma membrane distribution, several are also found both at the nuclear envelope, and within the intranuclear space. In this review, we provide an overview of recent work regarding various spectrin repeat-containing structural proteins in the nucleus. As well, we hypothesize about the regulation of their nuclear localization and possible nuclear functions based on domain architecture, known interacting proteins, and evolutionary relationships. Given their large size, and their potential for interacting with multiple proteins and with chromatin, spectrin repeat-containing proteins represent strong candidates for important organizational proteins within the nucleus.

List of abbreviations

BAF - barrier-to-autointegration factor
Bpag1 - bullous pemphigoid antigen 1
CH - calponin homology
ELF – embryonic liver fodrin
FANCA/C - Fanconi anemia complementation A/C proteins
MACF - microtubule actin cross-linking factor
mAKAP - muscle A kinase-anchoring protein
NLS - nuclear localization signal
NuMA - nuclear mitotic apparatus protein
PML - promyelocytic leukemia
SpR - spectrin repeat

Introduction

Eukaryotic cell structure and function depends upon the presence of cytoskeletal filaments – actin microfilaments, intermediate filaments, and microtubules – as well as their interaction with proteins that bundle these filaments or connect them to organelles, transmembrane proteins, and to each other. Microfilaments and microtubules represent ancient cellular structures, and are akin to those present in prokaryotes.[1-3] In eukaryotes, filament forming proteins are organized into a diverse array of cytoskeletal structures such as thickly bundled actin stress fibers, cortical actin meshwork, acto-myosin sarcomeres, microtubular axonemes and centrioles, and intermediate filaments. As well, numerous types of proteins interact with the cytoskeletal filaments to mediate cell motility, shape, size, function, and death.

The functions of filament proteins, and of the vast majority of their interacting proteins, have been characterized predominantly based on their roles in the cytoplasm, since a filamentous network similar to the cytoplasmic network has not been clearly identified in the nuclei of cells (reviewed in reference 4). Whether cytoskeletal filament components are present in the intact (interphase) nucleus has been controversial at times. However, it is now clear that filament proteins are indeed present in the nucleus. For example, actin is an important component of the nucleus,[5-7] and lamin intermediate filaments underlie the nuclear membrane.[8,9] Less clear is an understanding of the actual functions of actin and lamin in the nucleus, and of the importance of various protein interactions with nuclear actin and lamins that have been described recently.[7,10] Possible roles for nuclear actin include contributing to (1) the organization of chromatin remodeling complexes, (2) RNA processing, (3) a nucleoskeleton structure, or (4)

regulating DNaseI function (reviewed in references 5,7). While lamin filaments play a structural role in the nucleus, the importance of their interactions with proteins such as syne/nesprin, emerin, barrier-to-autointegration factor (BAF), histones, and protein kinase C remains to be determined.[10,11] The possibility that lamins participate in the regulation of gene expression also needs to be further explored. Thus, although cytoskeletal elements are present in the nucleus, how they contribute to nuclear organization and function remains a key question.

Another group of cytoskeletal-associated proteins that is present in the nucleus is the spectrin protein superfamily, which is better known for establishing and maintaining cell structure[12-16]. In the last few years, several members of this family of large molecular weight proteins have been identified at the nuclear envelope and within the intranuclear space (summarized in Table 1). While some of these proteins may anchor actin or lamin filaments to the outer or inner nuclear membranes, respectively, the localization and known binding-partners of others suggests that they are involved in mediating processes, such as DNA repair, within the internal regions of the nucleus. Here, we review the work of several groups that have provided evidence of spectrin repeat proteins in the nucleus. Furthermore, we speculate on how these proteins make it into the nucleus, what they may be doing there, and who their potential binding partners are.

Spectrin repeats

A hallmark of spectrin-related proteins is the presence of spectrin repeats (SpRs), which are large modular repeat elements. Originally identified in human erythrocyte

Table I. A summary of the locations and putative functions of different spectrin repeat containing proteins.

Name	Localization*	Putative nuclear function(s)	Reference
αSpectrinII	DNA repair foci or diffuse nuclear in lymphoblastoid cells	Scaffolding for DNA repair proteins	41-43
βSpectrinII	Cytoplasmic or nuclear in HepG2 hepatocytes	Regulation of Smad localization in the TGF-β signaling pathway	45
βSpectrinIVΣ5	Cytoplasmic or nuclear; PML body-associated in various cultured cells	Scaffolding for PML body proteins	34
Syne/nesprin	Nuclear envelope or intranuclear domains in various tissues and cultured cells	Nuclear positioning; scaffolding protein associated with lamin filaments; intranuclear scaffolding?	26, 46-48, 50, 51
Bpag1	Cytoskeletal-associated or nuclear in C2C12 myoblasts	Intranuclear scaffolding?	35
mAKAP	Nuclear envelope in heart and cultured myocytes	Regulation of nuclear ryanodine receptors	33, 53

* The localization is as described in the cited reference(s). Variant isoforms in the same or different tissues and cells may localize differently.

spectrin,[17,18] SpRs consist of three α helices connected by reverse turns and coil regions (Fig. 1A).[19,20] Analysis of α-actinin, which is thought to most closely resemble the ancestral form of the spectrin superfamily, has revealed that the multiple SpRs originated via duplication events of the exons coding for the original progenitor SpR sequence.[21,22]

Traditionally, SpRs have been viewed as modular domains that separate functional regions within large structural proteins.[20] The composition of the approximately 100 amino acid length of the SpR can vary considerably, with homologous SpR domains identifiable in different proteins. The uniqueness in structure of many SpRs is one indication that some have evolved specialized functions. As well, the inclusion or exclusion of individual SpRs in different spectrin isoforms suggests that these repeat elements may contribute to isoform-specific functions.[23] In this regard, SpRs of α-actinin and micro-dystrophin are not functionally interchangeable.[24] Functions attributed to SpRs include an involvement in self-association,[25,26] interaction with various structural or regulatory proteins,[20,27-32] targeting to the nuclear membrane,[33] targeting to promyelocytic leukemia (PML) nuclear bodies,[34] and mediating active transport into the nucleus via a classical nuclear localization signal (NLS) motif located within an SpR domain (Table 1).[35]

A common feature of SpR-containing proteins is the presence of an N-terminal actin binding domain, composed of two calponin homology (CH) domains (Fig. 1). Typically, the SpRs will separate the N-terminal CH domain region from a C-terminal region containing a distinct functional domain, such as a membrane localization domain (for example, the pleckstrin homology domain in spectrin or the klarsicht domain in syne/nesprin), a binding domain for β-dystroglycan (in dystrophin and utrophin), an

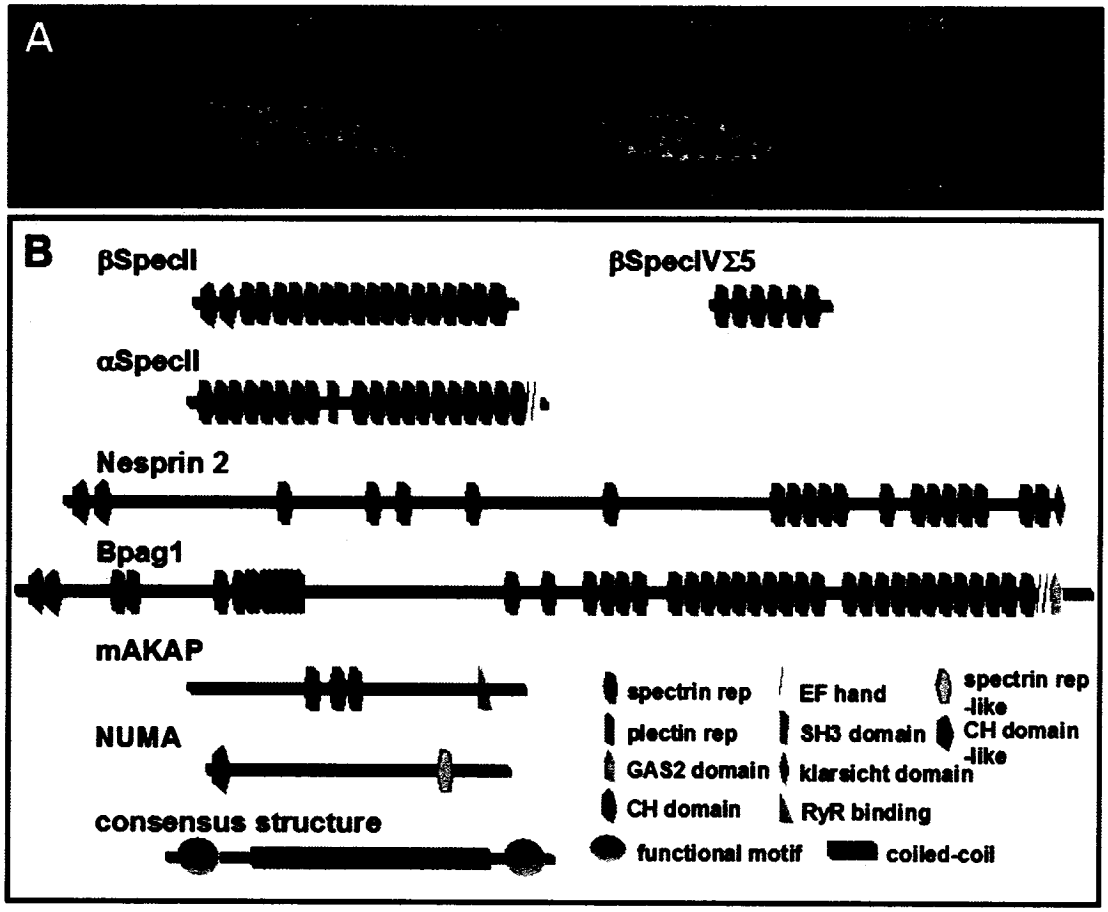

Figure 1. Structure of the spectrin repeat-containing proteins in the nucleus. **A**, Rod domain of α-actinin shown as an anti-parallel dimer. The four individual spectrin repeats (SpR1 – 4) are labeled. This graphic was generated with MOLMOL[91] using the AAC2_HUMAN SwissProt code. **B**, Domains identified by SMART and InterProScan analysis are shown, and protein lengths are drawn according to the relative number of amino acids in each. The klarsicht domain (KASH domain) in syne/nesprin is not identified by SMART or InterProScan, but is included because it has been described by several groups.[26,46-48] The ryanodine receptor (RyR) binding region in mAKAP is also shown, as this has been mapped.[33] The CH-like domain of NuMA was determined by computer modeling.[74] The spectrin-like domain in NuMA is identified as a spectrin

repeat by InterProScan (score = 0.0029), though not by SMART analysis. Alignment of this domain indicates a high degree of similarity compared to other spectrin repeats, with several key residues differing, however (see Supplemental Figure 1). Other functional motifs that have been demonstrated previously are not shown. These motifs do not have a clearly defined consensus or have not been precisely mapped and include microtubule binding regions at the C-terminus of NuMA and Bpag1. The consensus structure indicates that all of these proteins contain a coiled-coil region in the middle, which can vary substantially in length. This region often serves to separate functional domains at either end. Sequences used for this figure are NP_033286 (βSpecII); AAG42474 (βSpecIVΣ5); Q13813 (αSpecII); AAL33548 (Nesprin 2; NUANCE); NP_604443 (Bpag1b); NP_004265 (mAKAP); NP_006176 (NuMA) from GenBank.

intermediate filament-binding domain (in plectin, Bpag1e, and desmoplakin), or a microtubule-binding domain (in Bpag1a/b and MACF). Thus, the overall structure of SpR-containing proteins is consistent with their role as important regulators of cytoskeletal organization and as linker proteins at membrane structures in the cell.

Spectrin proteins in the nucleus

Spectrin was initially characterized as a cytoskeletal protein underlying the plasma membrane of the mature erythrocyte, and was thought to be absent in other cell types.[36] In the decade following the description of non-erythrocyte isoforms of spectrin,[37,38] reports of spectrin isoforms in the nucleus of cells began to appear.[39,40] These latter studies used newly generated spectrin antibodies to demonstrate that, as well as being present at the plasma membrane, spectrin was also detected in nuclei. α-spectrin was identified at the nuclear envelope as well as in intranuclear granules within liver cells.[39] Similar localization of α-spectrin in brain cortex sections was also reported.[40]

More recently, Lambert and colleagues demonstrated that an isoform of α-spectrin (αSpecII, see Figs. 1 and 2) is involved in DNA repair in the nucleus.[41-43] Indeed, αSpecII was initially described as a component of a chromatin-associated complex that is involved in the repair of DNA interstrand crosslinks and it was identified as being a direct binding partner of the DNA repair proteins, FANCA and FANCC.[41] Subsequent studies have shown that αSpecII binds directly to DNA containing psoralen interstrand cross-links.[42,43] As well, in lymphoblastoid cells treated to induce DNA interstrand cross-links, αSpecII localized to multiple nuclear foci along with FANCA and XPF, another DNA repair protein to which it binds directly. This combined work provides

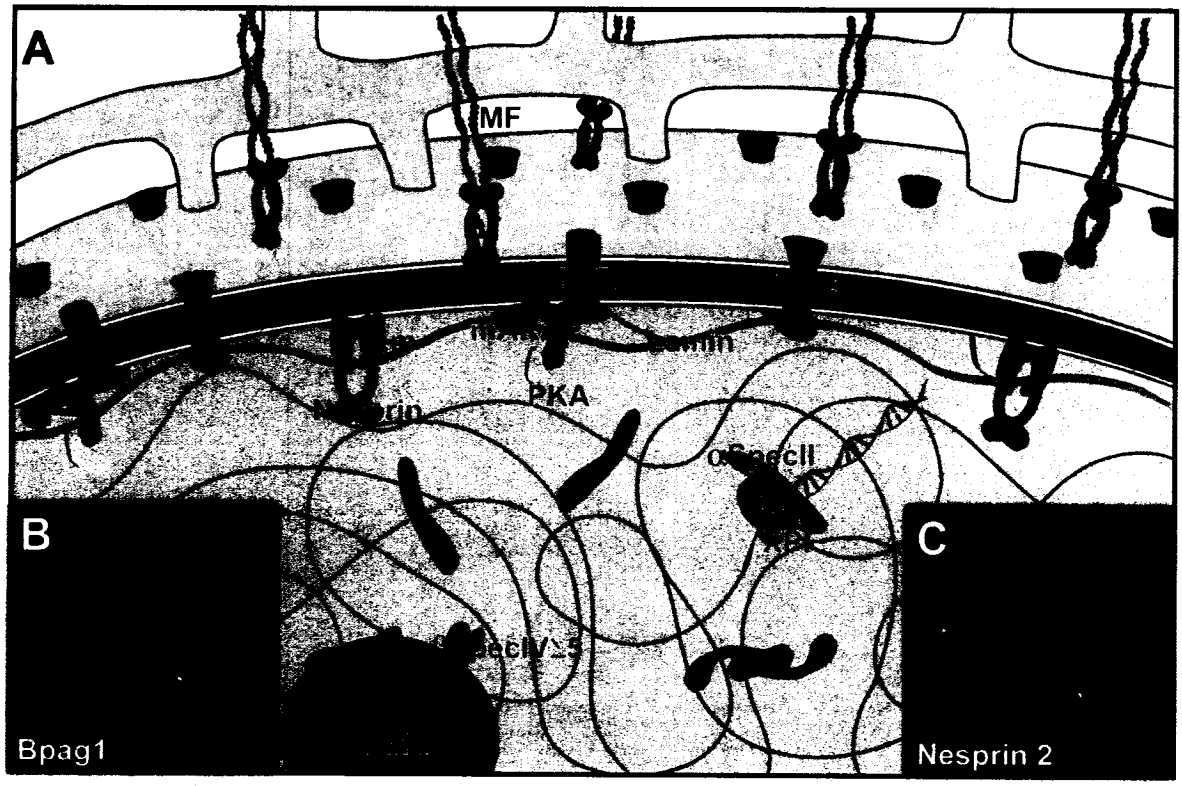

Figure 2. Spectrin repeat proteins in the nucleus. SpR-containing proteins present in the nucleus of various cell types include mAKAP, βspectrinIVΣ5, αspectrinII, Bpag1, and various isoforms of nesprin (syne/NUANCE/myne). **A**, SpR-containing proteins which interact with nuclear proteins or structures. Muscle A-kinase anchoring protein (mAKAP; dark green) anchors nuclear ryanodine receptors (RyR; olive), and mediates the regulation of RyR by protein kinase A (PKA; yellow).[33,53] βspectrinIVΣ5 (βspecIVΣ5; dark blue) associates with PML bodies (light red) in the nucleus.[34] αspectrinII (αspecII; light blue) binds directly to damaged DNA, and associates with DNA repair proteins, FANCA (FA; dark red) and XPF (orange).[41-43] Observations of αspectrinII in nuclei with damaged DNA indicate that it acts as a scaffold for these proteins during DNA repair. Isoforms of nesprin (light green) are present at the outer and inner nuclear membranes, as well as at internal nuclear sites.[26,46-50] At the outer nuclear membrane,

nesprin anchors the nucleus to actin microfilaments (MF).[48] At the inner nuclear membrane, nesprin may associate with lamin filaments, and with emerin (Em; purple).[26] In some cases, the SpR-containing proteins are illustrated as dimers, as these proteins are known to dimerize or multimerize via various regions.[25,26,35] **B**, C2C12 myoblast cells stained with an anti-Bpag1 isoform 2 antibody detected protein throughout the nuclei, as well as in the cytoplasm in association with actin microfilaments.[35] **C**, While some studies of nesprin have focused on this protein at the nuclear membrane,[26,46,47,49] immunofluorescence analysis has also indicated that it is present at internal nuclear structures.[48] Shown is a micrograph of C2C12 cells stained with an anti-nesprin 2 (NUANCE) antibody (a kind gift from Dr. Noegel[48]). Arrow in B points to nuclear staining and in C to nucleolar staining.

strong evidence that the αSpecII isoform of spectrin plays a role in mediating DNA repair, possibly acting as a scaffolding protein for DNA repair proteins.

Two other isoforms of spectrin, βSpecIVΣ5 and βSpecII (also known as embryonic liver fodrin – ELF), are present in nuclei and interact with nuclear proteins. βSpecIVΣ5 is a truncated, 72 kDa isoform, and associates with PML bodies in various cell types in culture (Figs. 1 and 2).[34] PML bodies are thought to be involved in transcriptional regulation, DNA repair, DNA synthesis, and/or RNA transport, though their primary function(s) has yet to be established.[44] Interestingly, βSpecIVΣ5 consists of little more than SpR sequences, and removal of the SpR sequence located at either the N-terminal or the C-terminal end of the protein results in its mislocalization, with the mutant protein diffusely localized in the nucleus.[34] βSpecII associates with Smad proteins in the cytoplasm and once associated, these proteins translocate into the nucleus of liver cells.[45] Disruption of βSpecII blocks the transforming growth factor (TGF)-β induced nuclear translocation of Smad proteins.[45] Thus, not only have isoforms of spectrin been identified in the nuclei of cells, they also have specific functions in these nuclei.

Syne/nesprin, mAKAP, and Bpag1 – spectrin repeat proteins in the nucleus

In addition to spectrin, other SpR-containing proteins are found in the nucleus, including syne/nesprin, mAKAP, and Bpag1 (Table 1; Figs. 1 and 2).[33,35,46-50] As well, these proteins contain domains that target the protein to the nuclear membrane, interact with the nuclear proteins lamin and emerin, or transport the protein into the nucleus. Thus, it is

likely that these other SpR proteins have distinct nuclear functions, as is observed for βSpecIVΣ5 and βSpecII.

The syne/nesprin proteins include multiple isoforms generated by two related genes.[26,46-48,50] Syne/nesprin was identified originally as a protein enriched in sub-synaptic nuclei.[46] A klarsicht domain (also referred to as a KASH domain) is present within the C-terminus of all syne/nesprin proteins,[51] and functions to target the protein to the nuclear membrane.[47] The endogenous syne/nesprin is concentrated at the nuclear envelope, where it interacts with lamin and emerin. However, syne/nesprin also appears diffuse or localized to nucleoli,[26,46-50] or to the spindle apparatus during mitotic division.[52] In heart sections or cultured cardiomyocytes, the mAKAP protein is predominantly nuclear and concentrated at the nuclear envelope.[33,53] Fusion protein analysis indicated that two of the three SpR domains within mAKAP target the protein to the nuclear envelope where it associates with ryanodine receptors located in the nuclear membrane.[53] Finally, Bpag1, another SpR protein, is present in the nucleus of C2C12 myoblast cells.[35] Thus, nuclear localization is commonly observed for many SpR-containing proteins.

Given the generally large sizes of the SpR-containing proteins found in the nucleus (72 kDa to >1 MDa), each protein must have a mechanism for being actively transported into the nucleus. This mechanism would involve either encoding an NLS motif within the protein that would be recognized by an import protein such as karyopherin (importin), or through interaction of the SpR-containing protein with another protein which contains an NLS, and that could act to shuttle it into the nucleus. Multiple Bpag1 isoforms contain a conserved NLS within an SpR domain.[35] Anti-Bpag1

antibodies consistently detect the protein within nuclei (Fig. 2), and Bpag1a/b isoform 2 N-terminal fusion proteins containing the NLS motif localize primarily to the nucleus.[35] Recently, we have identified karyopherin β as a candidate interacting protein of Bpag1 by yeast two-hybrid analysis (Young and Kothary, unpublished data). While karyopherin α typically binds to the NLS-containing cargo, and then associates with karyopherin β for transport into the nucleus, several cargo proteins interact directly with karyopherin β.[54-58] This direct interaction with karyopherin β may allow Bpag1 to bypass karyopherin α, and reduce the overall size of the complex entering the nucleus. Thus, we envision that this direct interaction with nuclear shuttle proteins would facilitate the nuclear entry of larger protein cargos.

Syne/nesprin isoforms also contain putative NLS motifs, though none have been verified experimentally.[47,48,50] At least one of the motifs present within a C-terminal spectrin repeat in βSpecIVΣ5, fits the consensus for an NLS which is similar to that found in Bpag1. In βSpecIVΣ5, this motif is KRRRL, and in Bpag1, the identified motif is PVKRRRI, though KRRRI alone may be sufficient, as this fits an experimentally identified classical NLS pattern of KRRRL/V/I.[59] The second β-spectrin isoform, ELF, known to be nuclear, also contains an amino acid motif similar to a known NLS (ERKRRP). Thus, not only are SpR proteins located in nuclei, several have a bona fide NLS as part of the protein.

To get a fundamental understanding of the function of SpR proteins in the nucleus, it is important to know their precise structure. This task is complicated by the presence of multiple isoforms of each of the spectrin-related proteins. It will be important to determine whether, for instance, truncated isoforms which lack functional domains

found in cytoplasmic versions of the proteins are the predominant type in the nucleus. We also do not know whether post-translational processing causes a truncation of the SpR proteins prior to nuclear translocation, as is the case with proteins such as filamin and focal adhesion kinase.[60-62] In the case of syne/nesprin and Bpag1, the large size of the genes, the corresponding large mRNA transcripts, and the high molecular weight protein products make an accurate determination of the overall structure difficult. However, although we may not completely understand the exact structure of nuclear SpR proteins, we do know that the spectrin repeats themselves are important components of the final proteins given their known targeting functions, and the fact that spectrin repeats make up much, if not most, of the predicted structure of these proteins.

Evolutionary considerations

Spectrin repeat-containing proteins are found primarily in animals, but also occur in fungi, plants, and protists.[22,63-65] As well, the Archaea contain at least one SpR-containing protein, the SMC1-family ATPase.[66] Our analysis shows that this protein contains a single spectrin repeat as identified by SMART (smart.embl-heidelberg.de/) or InterProScan (www.ebi.ac.uk/InterProScan/). Similar to spectrin superfamily members, the Archaea SpR-containing protein has an extensive coiled-coil region (predicted at www.ch.embnet.org/software/COILS_form.html). Interestingly, this one known prokaryotic SpR-containing protein is a homolog of the eukaryotic SMC1 protein which belongs to a well-defined family of DNA repair proteins.

As mentioned above, α-actinin best resembles the ancestral proteins of the spectrin superfamily. One recently described isoform of α-actinin (actinin-4) also translocates into the nucleus.[67] The evolution of α-actinin has been examined extensively, and relatives of this protein have been identified in the fungi *Schizosaccharomyces pombe* and *Neurospora crassa*, in the microsporidian *Encephalitozoo cuniculi*, and in the more primitive protist *Entamoeba histolytica*.[22] This evolutionary analysis supports the conclusion that α-actinin originated as an actin binding protein with a single SpR domain which subsequently underwent duplication events to produce the four SpR domains in animal α-actinin. However, the single SpR domain found in primitive α-actinin should be incapable of dimerization, thus excluding an actin cross-linking role for this early ancestor.[22] This suggests that the function of the precursor to the spectrin superfamily proteins may have not involved cytoskeletal organization.

In plants, immunofluorescence and immunoblot analysis using several anti-spectrin antibodies indicated that a spectrin-related protein is present primarily in the nucleus.[63,64] Though no spectrin-related proteins have yet been cloned from plants, genetic evidence for the existence of plant spectrin-related proteins is found in the *Arabidopsis thaliana* genome, which contains at least three regions predicted to code for proteins with spectrin repeats identifiable by SMART and InterProScan analysis (GenBank accession numbers AL161580, AY059745, and ATF5E19). Expressed sequence tags corresponding to each of these predicted proteins also exist, suggesting the transcript for each is produced. The patterns of spectrin-like immuno-staining in plants showed discrete localization to the nuclear envelope and to intranuclear speckles.[63,64]

This distribution is reminiscent of earlier descriptions of spectrin in liver and brain cells,[39,40] and suggests a conserved role for this protein in the nucleus. The speckle or granular-like staining in the nuclei is consistent with localization to PML bodies or DNA repair foci, as described for βSpecIVΣ5 and αSpecII.[34,43] This staining is also consistent with localization to spliceosomal complexes, such as SC35-containing complexes, which typically occur in nuclear speckles organized in part by structural proteins such as protein 4.1.[68,69] Thus, it appears that SpR-containing proteins in both plants and animals may have similar nuclear distribution and function.

One well-known nuclear protein from animals which is not regarded as a spectrin-related protein, but which bears strong structural similarities, is the nuclear mitotic apparatus protein (NuMA). NuMA is primarily a nuclear protein that is implicated in regulating mitotic spindle formation, maintaining nuclear structure in interphase nuclei, and in mediating RNA splicing.[70-72] The overall structure of NuMA is consistent with the spectrin superfamily of proteins, in that it has globular N- and C-termini separated by an elongated coiled-coil region.[73,74] Furthermore, using InterProScan analysis, we observed that NuMA contains a single SpR in the C-terminal half of the coiled-coil region, though SMART analysis scores this region as subthreshold for classification as a SpR. We therefore refer to this domain as a SpR-like domain (Fig. 1; for alignment, see supplemental Fig. 1). In other studies, computer modeling indicates that similar to other spectrin superfamily proteins, the N-terminal region of NuMA contains a CH domain.[74] However, this domain exists as a single CH domain, as opposed to the tandem CH domains in spectrin repeat-containing proteins, and it clearly does not contain the α-actinin actin binding motif commonly present in the first of the two tandem CH domains.

Therefore, we refer to this domain as a CH-like domain (Fig. 1). Finally, NuMA contains a functional microtubule binding domain at the C-terminal end.[75] These data together indicate that the overall structure of NuMA bears strong similarity to the spectrin superfamily, though we have not determined whether this is due to distant homology or coincident similarity, possibly from convergent evolution.

Considering the above evidence on the evolution of spectrin-related proteins, and their structural similarity to a well-known nuclear organizing protein, NuMA, it is tempting to speculate that the original function of SpR-containing proteins may have involved a role in nuclear organization.

Spectrin repeat proteins and nuclear positioning

Spectrin-related proteins associate with both the inner and outer nuclear membranes,[47,51] suggesting that these proteins affect nuclear function from both outside and from within. An example of an SpR protein that is present at the outer nuclear membrane is the *Caenorhabditis elegans* homolog of syne/nesprin, ANC-1, which appears to play a role in nuclear positioning.[76] *C. elegans* mutant for ANC-1 display aberrant positioning of nuclei in their multinucleated syncytial hypodermis. Since this protein associates with F-actin and the outer nuclear envelope, a role in tethering the nucleus to the actin cytoskeleton seems likely (as with syne/nesprin; see Fig. 2). This view of the protein's function is akin to the structurally related dystrophin protein, since both involve tethering actin filaments to either the nuclear membrane (ANC-1 or syne/nesprin) or the plasma membrane (dystrophin) (reviewed in reference 51). Thus, nuclear positioning or

anchoring fits well with the traditional view of the role for a spectrin superfamily member.

Spectrin repeat proteins as scaffolds in the nucleus

Determining the primary functions of SpR proteins in the nucleus is a very active area of research. Given that SpR-containing proteins are present in the nucleus of the cell, and that proteins of this family have very diverse structures, it is likely that they mediate a variety of functions within the nucleus. Furthermore, actin and protein 4.1, both of which interact with various spectrin superfamily members, are present and functionally important in the nucleus. Protein 4.1, long recognized as a major partner for cortical actin and spectrin in erythrocytes, localizes to the nucleus, and is essential for proper nucleus formation in vitro.[77-80] As such, one could speculate that SpR proteins, through their interactions with proteins like actin and protein 4.1, ensure the maintenance of nuclear structure.

Although many proteins have been identified that interact with spectrin superfamily proteins in the cytoplasm, it is not clear if these interactions also occur in the nucleus. For example, one study reported that the nuclear isoform of protein 4.1 (non-erythrocyte isoform 4.1R) may not interact with either spectrin or actin.[78] However, by contrast in a more recent study, protein 4.1 interaction with actin was shown to be necessary for proper nuclear formation.[81] In addition, nuclear protein 4.1 interacts with NuMA. As NuMA appears to be structurally related to the spectrin superfamily (Fig. 1; see above), the interaction of protein 4.1 and NuMA in the nucleus remains consistent with the structural role that protein 4.1 plays with spectrin at the plasma membrane.

Multiple spectrin superfamily proteins contain CH domains that constitute a classical actin binding domain that interacts with filamentous actin in the cytoplasm. However, actin may not form extensive filaments in the nucleus, or it may adopt a different conformation and/or filament structure in the nucleus.[7,82] The interaction of the CH domain of dystrophin with actin is modulated with changes in F-actin structure, such as would occur in different local environments.[83] Therefore, the well conserved actin binding domain of the spectrin superfamily may not be able to interact with much of the actin in the nucleus. On the other hand, the CH-like domain in NuMA was argued to interact with actin or actin-related proteins (ARPs) in the nucleus.[74] Thus, whether SpR proteins actually network with actin in the nucleus remains a controversial topic.

The possibility that SpR-containing proteins serve as a scaffolding network throughout the various regions of the nucleus is suggested by their size, potential for self-association, and interaction with other types of proteins. As well, each of the spectrin superfamily members in the nucleus exhibits discrete intranuclear localizations (e.g. mAKAP at the inner membrane, βSpecIVΣ5 in PML bodies; see Fig. 2), and interacts with a specific subset of nuclear proteins (and potentially with chromatin and RNA). Thus, it is possible that this distinct localization pattern of SpR proteins could serve as a mechanism for compartmentalization within the nucleus.

A scaffolding function has been suggested for spectrin isoforms in the nucleus[43,45] and for syne/nesprin.[26] Spectrin superfamily proteins in the nucleus interact with filamentous networks and with several more proteins that are involved in specific functions. For instance, the association of syne/nesprin with lamin filaments and with emerin may bring it into close association with heterochromatin.[26] Lamin filaments

interact with multiple other structural, gene-regulatory, and signaling proteins.[10] Thus, large regulatory complexes may be established in association with nuclear lamins and their associated scaffolding proteins. The observations that αSpecII interacts with different proteins involved in DNA repair and localizes to focal spots following DNA damage, suggest that this particular spectrin may specialize as scaffolding for DNA repair proteins.[41-43] The association of βSpecIVΣ5 with PML bodies may help to provide a structural framework for the multiple proteins that are part of PML body structure.[34] However, to determine if this is the case, the interactions of βSpecIVΣ5 with other proteins remain to be demonstrated. Spectrin may also help to form the structure of nucleoli,[84] though again, a demonstration of interactions with specific proteins is needed to help confirm this hypothesis. To what extent the nuclear SpR-containing proteins serve as scaffolding molecules remains to be determined with an emphasis on what proteins each interacts with.

Conclusions and future directions

Although traditionally viewed as cytoplasmic structural proteins, it is now apparent that spectrin and spectrin-related proteins are present and functional in various regions of the nucleus of multiple cell types. Given their ability to self-associate, interact with filament-forming proteins, and serve as scaffolding proteins, SpR-containing proteins likely play a variety of organizing roles within the nucleus. The net effect of these roles may be critical for the formation of a highly dynamic and structured organelle, compatible with the current view of compartmentalization and dynamic reorganization in the nucleus.[85-87]

The functions of the various SpR-containing proteins in the nucleus are quite varied. While structurally diverse, all of these proteins contain an extensive coiled-coil pattern throughout the central region, commonly consisting of SpR domains that separate functional motifs at either end. One consistent role for the spectrin repeats within these proteins is nuclear targeting. Specific examples include SpR sequences targeting βSpecIVΣ5 to PML bodies, mAKAP to the nuclear envelope, and Bpag1, and potentially others, to the inside of the nucleus via NLS motifs (Fig. 2). However, the SpR sequences can be quite unique between different proteins, and even within the same protein, and each SpR domain likely has evolved to perform a specialized function. In addition to having specialized unique functions, SpR-containing regions throughout the central part of these proteins may be necessary to provide a common flexible spacer.

Whether the spectrin superfamily proteins serve as critical organizing proteins within the nucleus remains to be determined. While the loss of function of spectrin and Bpag1 causes pathology, these abnormalities have been attributed primarily to an inability to organize the cytoskeleton and associated proteins and, consequently, an inability to maintain proper cell structure.[14,16,88-90] Whether nuclear disorganization also plays a role in these pathologies has yet to be demonstrated. Furthermore, since our knowledge of the proteins which interact with nuclear SpR-containing proteins is limited, we can not yet evaluate whether loss of these interactions also contributes to the disease state. It is clear that we have much to learn about this fascinating family of proteins. Future research should focus on an examination of nuclear structure in the absence of the various nuclear SpR-containing proteins. A demonstration of interactions with various

nuclear components should also help to establish the roles of the spectrin superfamily proteins within the nucleus.

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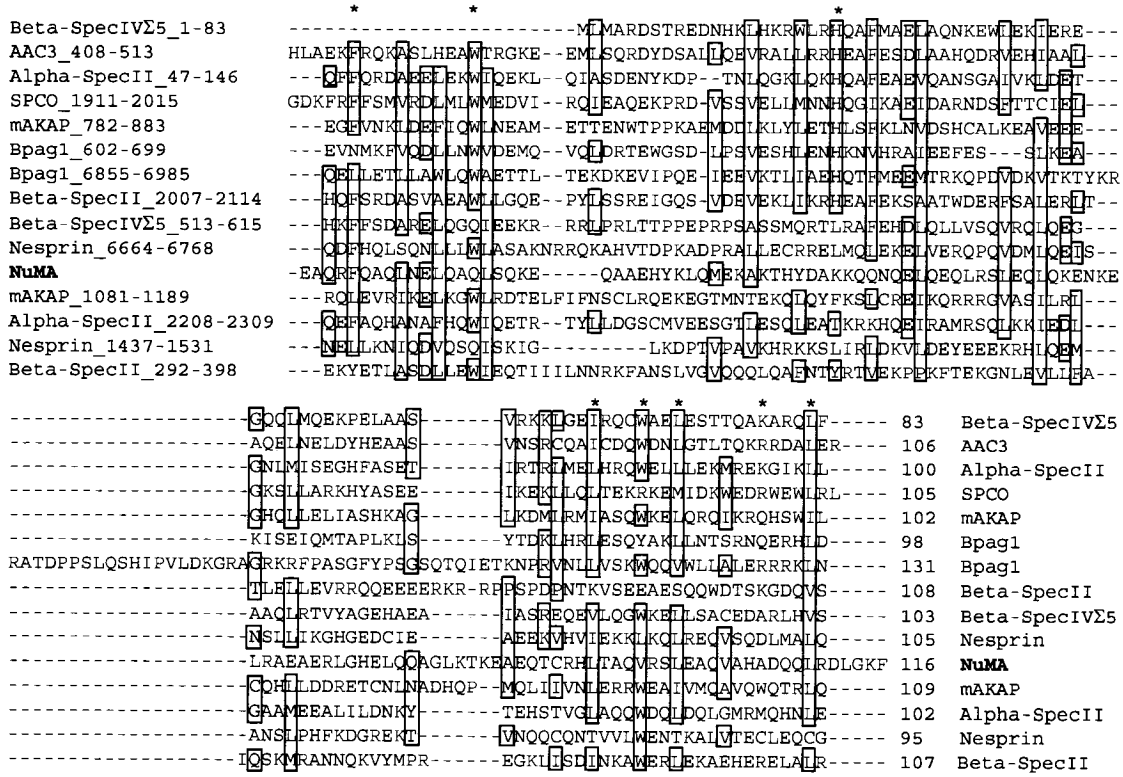
Supplemental Material



Supplemental Figure 1. Alignment of the putative NuMA spectrin repeat with the first and last SpR sequences from each of the proteins depicted in Figure 1, as well as additional human βSpecII (SPCO_1911-2015) and α-actinin (AAC3_408-513) SpR sequences for comparison. The numbering at the left indicates the region of the protein being analyzed and the figures at the right provides the number of residues analyzed per region. Alignment and coloring of similar amino acid residues was performed using ClustalW. Similar residues conserved in the majority of the sequences are boxed. The (*) denotes well-conserved residues identified in the HMM logo. Secondary structure prediction also indicates a predominantly helical structure for the NuMA sequence, corresponding with the SpR pattern (not shown).

Chapter 3

Bpag1 localization to actin filaments and to the nucleus is regulated by its N-terminus

Contributions of additional authors

Madeline Pool contributed to this paper by performing RT-PCR experiments shown in Figure 1, and was involved in the editing of the paper. Writing of the original version of the manuscript was done by myself, with general suggestions from Rashmi Kothary (primarily for the Introduction section). Madeline and Rashmi each helped with editing during the revision process.

Bpag1 localization to actin filaments and to the nucleus is regulated by its N-terminus

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Summary

Plakins are a family of giant cytoskeleton binding proteins. One member of this group is bullous pemphigoid antigen 1 (Bpag1)/dystonin, which has neuronal and muscle isoforms that consist of actin-binding and microtubule-binding domains at either end separated by a plakin domain and several spectrin repeats. The better-characterized epithelial isoform has only the plakin domain in common with the neuronal and muscle isoforms. Here, we have analyzed the localization of muscle/neuronal (Bpag1a/b) isoforms and the epithelial (Bpag1e) isoform within C2C12 myoblast cells. Although an antibody specific to Bpag1a/b isoform 2 detected protein co-aligning actin stress fibers, this same antibody and two Bpag1e antibodies predominantly detected protein in the nuclei. A Bpag1a/b isoform 2 N-terminal fusion protein containing the plakin domain also localized to actin stress fibers and to nuclei. Within the plakin domain, we characterized a functional nuclear localization signal, which was responsible for localization of the fusion protein to the nucleus. Bpag1a/b isoform 1 N-terminal fusion proteins differed in their interaction with the actin cytoskeleton and with their ability to localize to the nucleus, suggesting that Bpag1 isoforms with different N-termini have differing roles. These results show the importance of N-terminal domains in dictating the localization and function of Bpag1 isoforms. We provide the first indication that Bpag1 is not strictly a cytoplasmic/membrane protein but that it can also localize to the nucleus.

Introduction

Bullous pemphigoid antigen 1 (Bpag1) was originally identified as an epithelial protein involved in linking or anchoring keratin intermediate filaments to the cytoplasmic side of hemidesmosomes (Jones et al., 1994; Mueller et al.,1989; Mutasim et al., 1985; Stanley et al., 1988). This epithelial isoform, now termed Bpag1e, is the most important autoantigen in the human skin blistering disease bullous pemphigoid (Mueller et al., 1989). Correspondingly, inactivation of murine *Bpag1* by gene targeting resulted in mice with microscopic skin lesions (Guo et al., 1995). More surprising, however, is that the predominant phenotype in the *Bpag1* null mice was neurological, identical to that seen with *dystonia musculorum* (*dt*) mice, a naturally occurring mutant (Duchen et al., 1963; Duchen and Strich, 1964). We have reported that *dt* mice have mutations in the *Bpag1* gene and further showed that the *dt* locus encodes neuronal and muscle isoforms of *Bpag1*, which we initially called 'dystonins' (Bernier et al., 1995; Brown et al., 1995). For ease of presentation, we will refer to the gene as *Bpag1* and to the gene products as Bpag1.

Analysis of the cytoskeleton in *dt* mice has shown that intermediate filaments and microtubules are disorganized in the axons of sensory neurons (Bernier and Kothary, 1998; Dalpé et al., 1998; Yang et al., 1999). An abnormal cytoarchitecture is also observed in the muscle of *dt* mice, having a reduction in sarcomere length, a thickened z-disk and an abnormal clustering of the mitochondria (Dalpé et al., 1999). These studies led to the conclusion that the neuronal and muscle isoforms of Bpag1 play a role in maintaining cytoskeletal organization.

The family of proteins in which Bpag1 is included, termed plakins, comprises cytoskeleton binding proteins that are expressed as alternative splice forms (Fuchs and Karakesisoglou, 2001; Fuchs and Yang, 1999; Klymkowsky, 1999). To date, there have been at least five predominant isoforms of Bpag1 identified. One of these is the epithelial isoform, Bpag1e, which consists of a globular N-terminal end (referred to as the plakin domain because it is conserved among the various plakins), a central coiled-coil domain and a C-terminal intermediate filament binding domain (IFBD). Bpag1e (also known as BP230) is encoded by a 9 kb transcript. Nonepithelial Bpag1 has also been identified in the form of two major neuronal (Bpag1a1 and Bpag1a2) and two major muscle (Bpag1b1 and Bpag1b2) isoforms (Leung et al., 2001). All Bpag1 isoforms share one region in common – the plakin domain. Apart from the presence of the plakin domain, the nonepithelial forms of Bpag1 are distinct from Bpag1e.

The neuronal and muscle Bpag1 isoforms share a functional actin binding domain (ABD) upstream of the conserved plakin domain (Brown et al., 1995; Yang et al., 1996). This ABD is composed of two classical calponin homology domains, CH1 and CH2, which are similar to those described in a-actinin, β-spectrin and dystrophin (Dubreuil et al., 1990; Koenig et al., 1987; Noegel et al., 1987; Witke et al., 1986). In addition to the ABD and the plakin domain, the neuronal and muscle Bpag1 isoforms are characterized by a spectrin repeat-containing rod domain, putative calcium-regulated EF-hands and a region that shares homology to the growth arrest specific protein 2 (GAS2) (Collavin et al., 1998; Zucman-Rossi et al., 1996). This GAS2 homology region includes a microtubule binding domain (MTBD) (Sun et al., 2001). The predominantly neuronal-specific isoforms, Bpag1a1 and Bpag1a2, are encoded by 17 kb transcripts and predicted

to be around 600 kDa (Leung et al., 2001). The Bpag1 muscle transcripts are identical to their neuronal counterparts but have, in addition, a large exon harboring a second potential IFBD (referred to as IFBD2). These isoforms, Bpag1b1 and Bpag1b2, are encoded by 22 kb transcripts and predicted to be around 800 kDa (Leung et al., 2001). Diversity is introduced by alternative splicing of exons A and A' at the 5' end of the neuronal and muscle transcripts (Brown et al., 1995), resulting in unique Nterminal regions upstream of the ABD. In addition to these isoforms, a neuronal isoform lacking a complete ABD has been described (Bpag1n3) (Yang et al., 1999), although more recent information on the structure of Bpag1 isoforms calls into question the overall structure of Bpag1n3 (Leung et al., 2001).

The ability of the IFBD1 to associate with keratin, neurofilaments, desmin and peripherin (Dalpé et al., 1999; Leung et al., 1999a; Yang et al., 1996), together with the microtubule binding capability of the MTBD (Sun et al., 2001) has been shown with fusion proteins in cell transfection assays. Also, the potential of the ABD to bind actin filaments was shown using FLAG and green fluorescent protein (GFP) fusion proteins (Dalpé et al., 1999; Yang et al., 1996). Whether the IFBD2 can interact with various intermediate filaments remains to be determined. Collectively, the above studies suggest that endogenous Bpag1 proteins crosslink actin to microtubules in neurons and muscle, and possibly also to intermediate filaments, in the case of the muscle isoforms. However, one aspect that remains unclear is the role of the isoform-specific N-terminal sequences encoded by the alternatively spliced exons A and A'. Also, the need for multiple Bpag1 isoforms and the subcellular localization of endogenous non-epithelial Bpag1 has not been addressed in detail.

In the present study, we have used an isoform-specific antibody against the A' unique region of Bpag1a/b2 to localize the endogenous protein in cultured C2C12 myoblasts. The localization of the endogenous Bpag1a/b2 along stress fibers, but not at their tips, corresponded well with the localization of Bpag1 isoform 2 N-terminal fusion proteins. Unexpectedly, the results with the A' and two Bpag1e antibodies, and with plakin domain-containing fusion proteins, showed that Bpag1 localizes predominantly to the nucleus in C2C12 cells. Furthermore, fusion protein analysis in C2C12 cells showed that the nature of the isoform-specific unique region upstream from the ABD has a major impact both on the way the ABD interacts with the actin cytoskeleton and with the role of the plakin domain region in localizing the protein to the nucleus. These results, in combination with recent results with various plectin isoforms in keratinocytes (Andra et al., 2003), indicate that unique N-terminal regions are necessary for regulating the localization and function of plakin proteins.

Materials and Methods

Fusion protein constructs

GFP- and FLAG-tagged fusion proteins were expressed from plasmids constructed from pEGFP (Clontech) and pCMV-Tag2 vectors (Stratagene), respectively. The inserts for the N-terminal regions of Bpag1 isoform 1 and Bpag1 isoform 2 were subcloned from RT-PCR-derived fragments that were fully sequenced in various vectors, and the sizes of the translation products were verified by western blotting (Fig. 1C). Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Stratagene). A 1.6 kb insert from the plakin domain was used for mutagenesis of the

nuclear localization signal (NLS) and was subsequently subcloned into an isoform 2 N-terminus expression plasmid. In a separate experiment, the NLS was introduced between *Bam*HI and *Eco*RI sites at the 5' end of the ABD expression plasmid insert by using annealed oligonucleotides coding for the NLS and flanked by *Bam*HI and *Eco*RI sites. The plasmids were transfected into Cos-1 cells (see below) using the Lipofectamine 2000 transfection reagent (Qiagen) and into C2C12 cells using Lipofectamine Plus (Qiagen), following the manufacturer's protocol.

RT-PCR

For cDNA production, equal amounts of total C2C12 and mouse skin RNA was reverse-transcribed in a standard reaction with MuLV reverse transcriptase (Invitrogen). Semiquantitative PCR was performed using 25 cycles in a thermocycler, with the following primer pairs being used: CTACATGTACGTGGAGGAGCA and CATCGTTTGCACCAATGCC for A-ABD; GAGGGCTGTGCTTCGGATAG and CATCGTTTGCACCAATGCC for A'-ABD; GGGTATTTATCTCGAACTCTC and CCCTACTGGGAAGATAGT for IFBD1; GCCAGAGGCTTCTGCTCTATG and GTGACAAAGTAGAGGCTTCGC for IFBD2; CTATCTCTTCGAAGACTTGTC and CAGTTTGGAGACTCCCAGCAG for MTBD; and CCGTCAGGCAGCTCATAGCTCTTC and CTGAACCCTAAGGCCAACCGT for actin. Either a water control for the PCR, or an RT reaction using C2C12 RNA without the MuLV reverse transcriptase (to check for genomic DNA contamination), were performed as controls. All reactions were run on the same gel and exposures shown

are all identical, except for the actin bands, which are shown with a reduced exposure time.

Cell culture

Cos-1 and C2C12 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Cellgro) + 10% fetal bovine serum (FBS) (Gibco/BRL) in plastic culture dishes, and maintained at 37°C in 5% CO2. The cells were seeded onto round glass coverslips in 12-well plates for microscopic analysis. When C2C12 cells were treated with cytochalasin D or nocodazole, the culture media was replaced with media containing the drug one hour before processing for immunocytochemistry. A control with equivalent dilutions of the vehicle for both (DMSO) was also done for comparison.

Bpag1 antibodies

Antisera were produced using synthetic peptide conjugates injected into New Zealand White Rabbits (Washington Biotechnology). The peptide sequence chosen was from the N-terminal unique region of Bpag1 isoform 2 (Fig. 1A). The antiserum was affinity purified against the immunizing peptide, and characterized by detection of the GFP-fusion proteins containing the N-terminal regions of Bpag1 isoform 1 and Bpag1 isoform 2 in Cos-1 cells by immunofluorescence and on immunoblots. Affinity-purified antibody was pre-absorbed with the immunizing peptide as a control for the immunocytochemistry. Anti-Bpag1e antibodies recognizing the Cterminal half of the human Bpag1e isoform were also used (Santa Cruz Biotechnology, and a kind gift from Takashi Hashimoto, Kurume University School of Medicine).

Immunoblot analysis and immunoprecipitation assays

Cell culture lysates were typically collected in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA and 1% glycerol) containing 1 mM PMSF, 0.01 mg/ml aprotonin, 0.01 mg/ml pepstatin, 0.01 mg/ml leupeptin and 10 mM Na_2VO_4 on ice. Samples were analyzed by SDS-PAGE on standard 10% polyacrylamide gels according to Laemmli (Laemmli, 1970), and transferred semi-dry onto a PVDF membrane (Millipore).

Immunoprecipitations against the FLAG epitope were performed with an anti-FLAG polyclonal antibody (Sigma) using lysates from Cos-1 cells that were co expressing GFP- and FLAG-tagged Nterminal constructs. Lysates were incubated with the polyclonal anti-FLAG antibody for 1 hour at 4°C, following which Protein G-Sepharose beads (Sigma) were added for an overnight incubation at 4°C. The beads were subsequently washed with RIPA buffer repeatedly, resuspended in sample buffer, boiled, and the supernatant was analyzed by SDS-PAGE. A fraction of each lysate collected was set aside and used for input controls.

Anti-GFP polyclonal (Invitrogen), anti-FLAG monoclonal (M2; Sigma) and anti-FLAG polyclonal antibodies were used according to the manufacturer's instructions.

Immunocytochemistry

Cultured cells were rinsed twice in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (J.B. EM Services) for 5-10 minutes, and then rinsed twice for 10 minutes in PBS. The cells were permeabilized and blocked in a solution of 0.4% Triton

X-100 and 10% goat serum in PBS for 30 minutes. Samples were then incubated with the first primary antibody in staining buffer [0.04% Triton X-100 with 3.3 mg/ml of bovine serum albumin (Sigma) in PBS] overnight at 4°C. The cells were washed twice for 10 minutes in PBS, followed by incubation with the secondary antibody diluted in staining buffer for 1 hour. Rhodamine-phalloidin (Molecular Probes) was included with the secondary antibody when counter-staining for the actin cytoskeleton. When double-staining using a second primary antibody, the staining procedure was repeated following three washes in PBS for 10 minutes each. After completion of the antibody incubations, the cells were washed three times for 10 minutes, and then mounted in Slowfade Light (Molecular Probes).

The anti-tubulin monoclonal (E7 supernatant) antibody was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD (University of Iowa). Also used were antipaxillin (clone 349; Transduction Laboratories) and anti-FLAG (M5 or rabbit polyclonal; Sigma). GFP signal was observed without staining. Cells were observed using either a Zeiss Axioplan equipped with epifluorescence illumination, or with a Zeiss LSM 410 invert laser-scanning microscope. Digital images were captured using AxioVision 2.05 (Zeiss) or LSM software (Zeiss), and processed with Adobe Photoshop and Adobe Illustrator.

Where quantifications of the observed staining patterns are given from transient transfections, the percentage was obtained from counting cells on at least three coverslips from two or more separate transfections. A minimum of 100 cells from each coverslip was counted. Quantifications of endogenous Bpag1 staining are from a minimum of two separately stained coverslips, with at least 100 cells being counted from each.

Results

Bpag1 expression in C2C12 myoblasts

Semiquantitative RT-PCR analysis on RNA from C2C12 cells, using a series of primer pairs from different regions of Bpag1, indicated that these cells express what has previously been identified as the predominant transcript in muscle, Bpag1b (Fig. 1) (Leung et al., 2001). This is indicated by the amplification of cDNA corresponding to the IFBD2 region, not found in the Bpag1e or Bpag1a isoforms (Fig. 1B). Conversely, the IFBD1 region, which is unique to Bpag1e, was expressed at relatively low levels in C2C12 cells compared with in skin, where it is the predominant isoform (Fig. 1B). Both isoform 1 (A) and isoform 2 (A') unique N-terminal coding regions were amplified from RNA of C2C12 cells, with comparatively low levels of each being amplified from skin RNA. The MTBD region, present in both Bpag1a and Bpag1b isoforms, was expressed more highly in C2C12 cells than in skin. Therefore, transcripts encoding the Bpag1b isoform are present at higher levels than for the Bpag1e isoform in C2C12 myoblasts. Previous northern blot analysis has shown that undifferentiated C2C12 cells also contain a slightly lower level of the predominant brain transcript (Bpag1a), which then decreases following differentiation (Dalpé et al., 1999). Therefore, we refer to the endogenous Bpag1 isoform 2 localized in this study as Bpag1a/b2.

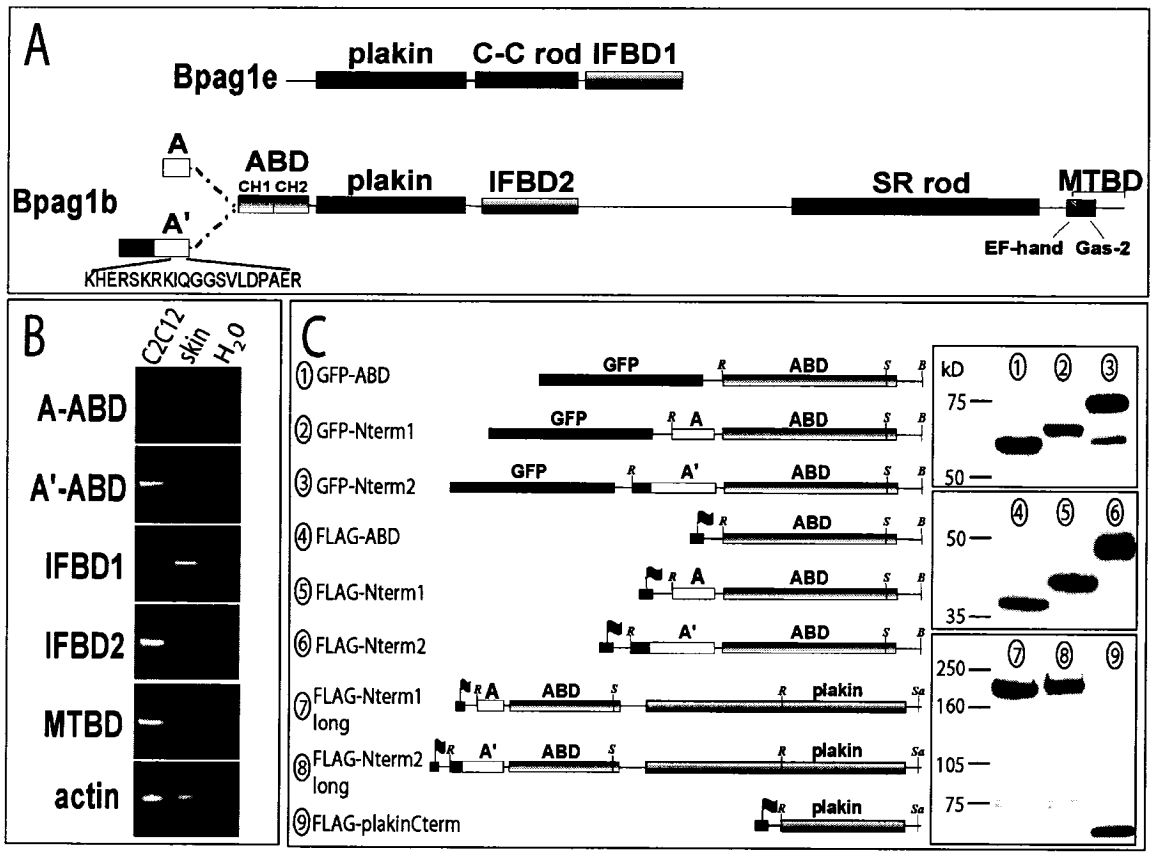

Fig. 1. Bpag1 isoforms expressed in C2C12 myoblasts, and the N-terminal fusion proteins used in this study. (A) The Bpag1e and Bpag1b isoforms. Bpag1b includes the N-terminal unique region encoded by exons A or A' (Brown et al., 1995), an ABD consisting of two calponin homology domains (CH1 and CH2), a plakin domain, an intermediate filament binding domain (IFBD2), a spectrin-repeat region (SR rod), an EF-hand, and a GAS2 homology region which includes part of an MTBD. Only the plakin domain is shared by the Bpag1e isoform, which has a C-terminal half consisting of a coiled-coil rod domain (C-C rod) and an alternate intermediate filament binding domain (IFBD1). The Bpag1a isoform (not shown) differs from the Bpag1b isoform only in that it lacks the IFBD2 and surrounding region. The peptide sequence used for isoform-specific antibody production is also shown. (B) Semiquantitative RT-PCR using primers against various regions of Bpag1. The results indicate that Bpag1b is the predominant

isoform expressed in C2C12 myoblasts, although a band amplified from the IFBD1 region of Bpag1e was also detected at low levels. Conversely, regions from the Bpag1b mRNA were detected at low levels in mouse skin, whereas the IFBD1 region from the Bpag1e mRNA was prominent in skin. Actin mRNA amplification served as a control for RNA amount used. (C) The various fusion proteins used in this study. FLAG and GFP western blots indicated that each fusion protein was expressed at the appropriate size. B, *Bgl*II; R, *Eco*RI; S, *Sal*I; Sa, *Sap*I.

Bpag1a/b2 localizes to the nucleus and actin stress fibers, but not to focal adhesion sites, in C2C12 myoblasts

Polyclonal antisera against a peptide derived from the sequence in the Bpag1 isoform 2 N-terminal unique exon (A') (Brown et al., 1995) were produced in rabbits (sequence shown in Fig. 1A). The antisera and affinity-purified antibody recognized the N-terminal region of Bpag1 isoform 2, but not Bpag1 isoform 1, as determined by immunoblot analysis of GFP-fusion proteins (Fig. 2A). The isoform 2 fusion protein, but not the isoform 1 fusion protein, was also detected by this antibody with immunofluorescence in Cos-1 cells expressing the fusion proteins (data not shown). Preabsorption of the antibody with the immunizing peptide eliminated any specific staining of the endogenous protein observed by immunofluorescence (Fig. 2B).

Undifferentiated C2C12 myoblasts were used to analyze the subcellular localization of endogenous Bpag1a/b2. The most consistent filamentous staining detected with the A' antibody was a pattern localized in the central area of approximately 5 – 10% of cells (Fig. 2C). This pattern was typically observed in all of the cells in small clusters away from the center of the coverslips, suggesting that the filamentous localization may be most prominent in cells that are in the process of proliferation and migration, and/or cells with only limited contact with other myoblasts. Some cells also exhibited very bright perinuclear staining (Fig. 2C), although this was less consistent. The filamentous pattern of the A' antibody co-aligned perfectly with the central actin stress fibers of the myoblasts (Fig. 2C, D and Fig. 3A, B). However, A' staining did not co-align with peripheral stress fibers (Fig. 2C, D). Further analysis by confocal microscopy also

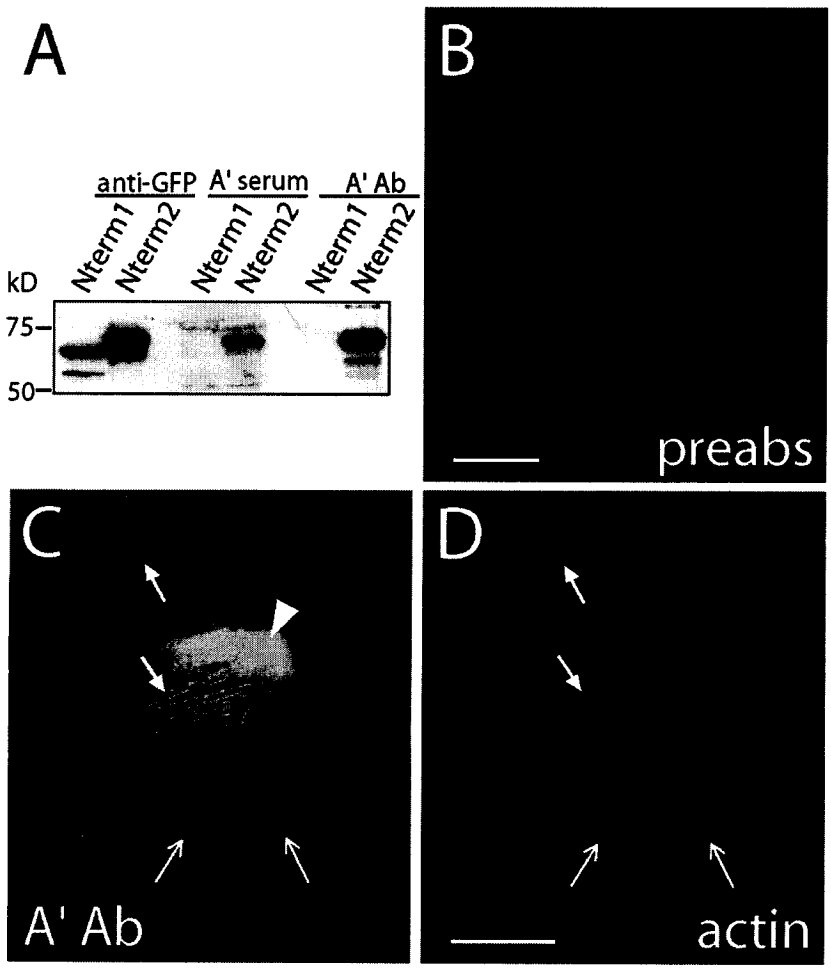

Fig. 2. An antibody specific for Bpag1 isoform 2 detects protein co-aligning with actin stress fibers. (A) Western blot analysis of lysates from Cos-1 cells expressing Bpag1 fusion proteins. GFP-Nterm1 and GFP–Nterm2 were expressed, as shown with an anti-GFP antibody, and used to test anti-Bpag1 sera. Serum containing antibodies against the Bpag1 A' region detected GFP-Nterm2 but not GFPNterm1. GFP-Nterm2 was not detected with the pre-immune serum (data not shown), and was strongly detected with the affinity-purified antibody (A' Ab). (B) Only diffuse background staining was ever observed in cells stained with the pre-absorbed A' antibody. (C, D) In C2C12 cells, the

A' antibody (C) stained in a filamentous pattern co-aligning with centrally located actin stress fibers (stained with rhodamine-phalloidin in D; closed arrows in C and D). The A' staining typically did not occur along more peripheral actin filaments (open arrows in C and D), although colocalization did occur along more peripheral actin filaments in a few cells. Strong peri-nuclear staining was also observed in a few cells (arrowhead in C). Bars, 20 μm.

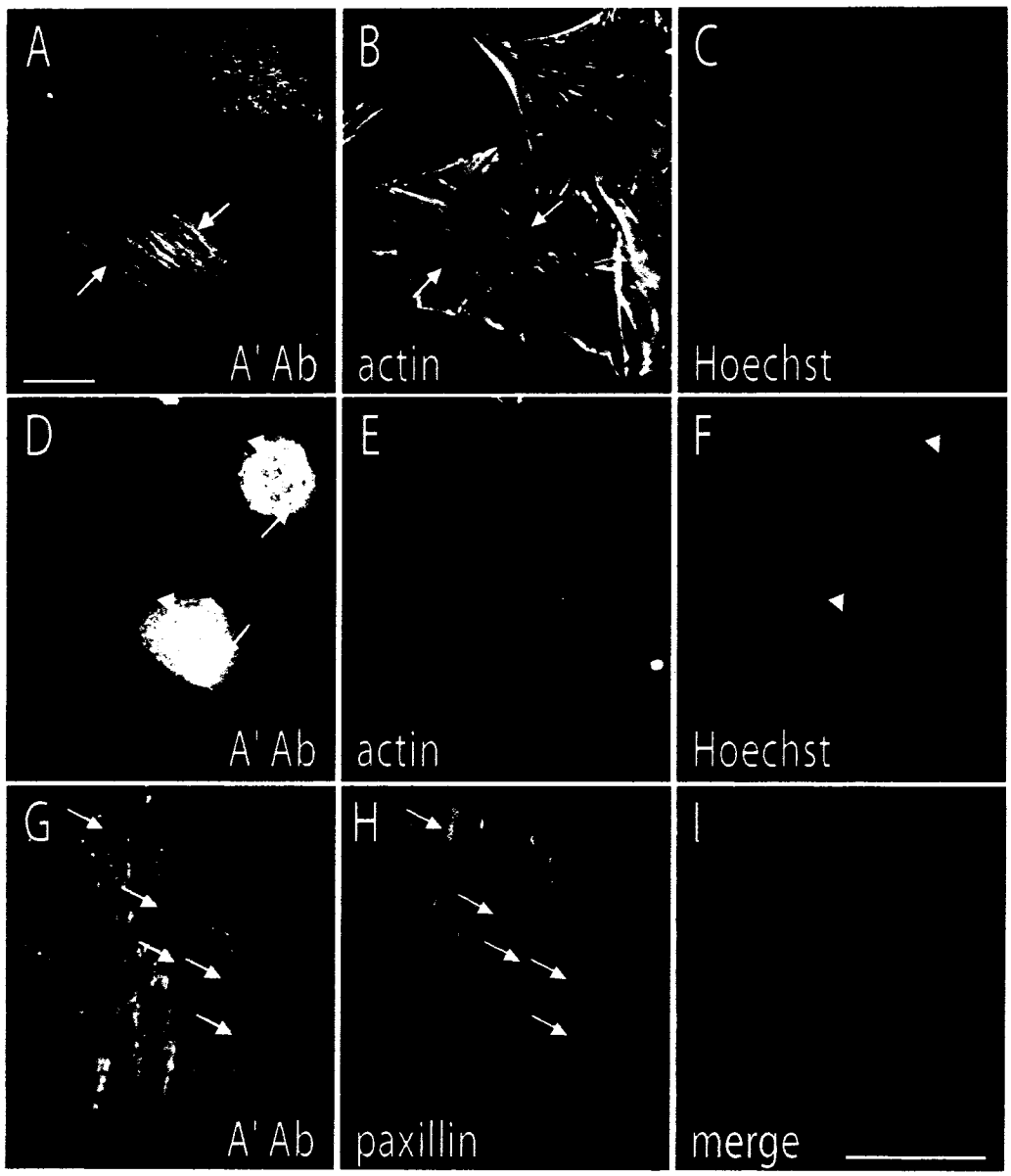

Fig. 3. Endogenous Bpag1a/b2 stress fiber localization does not extend into focal contact sites, and most of the A' Ab staining occurs in and around the nucleus. (A – C) Observed by confocal microscopy, the A' antibody (A) stains in a pattern co-aligning perfectly with centrally-located stress fibers (stained with rhodamine-phalloidin in B; arrows in A and

B). (D – F) In the same cells as in A – C, staining with the A' antibody (D) in an optical plane through the nuclei revealed strong nuclear (arrowheads) and perinuclear (arrow) staining (D). Rhodamine-phalloidin staining was absent from the nuclei (E). Hoechst staining was used to identify the nuclei (C, F). (G – I) When C2C12 cells were stained for both Bpag1a/b2 (G) and paxillin (H), and observed by confocal microscopy, the A' staining and the paxillin staining never overlapped (arrows in G and H). Shown is a region of a C2C12 cell with a large number of focal contact sites (paxillin staining) to illustrate the exclusion of Bpag1a/b2 from these sites (I is the merged image of G and H). Bars, 20 μm. Bar in A applies to A – F; bar in I applies to G – I.

showed that the Bpag1a/b2 protein co-aligned along varying lengths of the actin stress fibers (Fig. 3A – C).

Although filamentous staining was observed only in a small subset of the C2C12 cells, all cells contained varying intensities of staining in the nuclei. In optical sections through the nuclei, staining was noted in all cells imaged in a pattern surrounding the nucleoli (Fig. 3D – F, the same cells as in Fig. 3A – C). This resulted in a 'Swiss cheese' pattern of staining in the nuclei. Perinuclear staining was observed in many of the cells (arrows in Fig. 3D). When double-stained using an antibody against the focal contact protein paxillin and the A' antibody, the two signals were always mutually exclusive (Fig. 3G – I). Thus, Bpag1a/b2 is localized to nuclei in all C2C12 myoblast cells, and to centrally located actin stress fibers in a small subset of the cells; it is excluded from focal adhesion sites.

Bpag1a/b2 staining is not dependent on microtubules

To address whether the Bpag1a/b2 protein functions as a crosslinker between actin stress fibers and microtubules, we assessed the pattern of A' staining after disruption of either microfilaments or microtubules in C2C12 cells. To determine whether microfilament disruption would cause a mislocalization of Bpag1a/b2, we used a concentration of cytochalasin D that would disrupt many, but not all, of the actin stress fibers in all of the C2C12 cells. Thus, clusters of cells with some A' staining could be found, and some of the cells within these clusters lacked intact stress fibers. In cells that contained intact actin stress fibers, some co-aligning A' staining could still be observed (Fig. 4A, B, arrows), but no filamentous pattern was observed with the A' antibody in

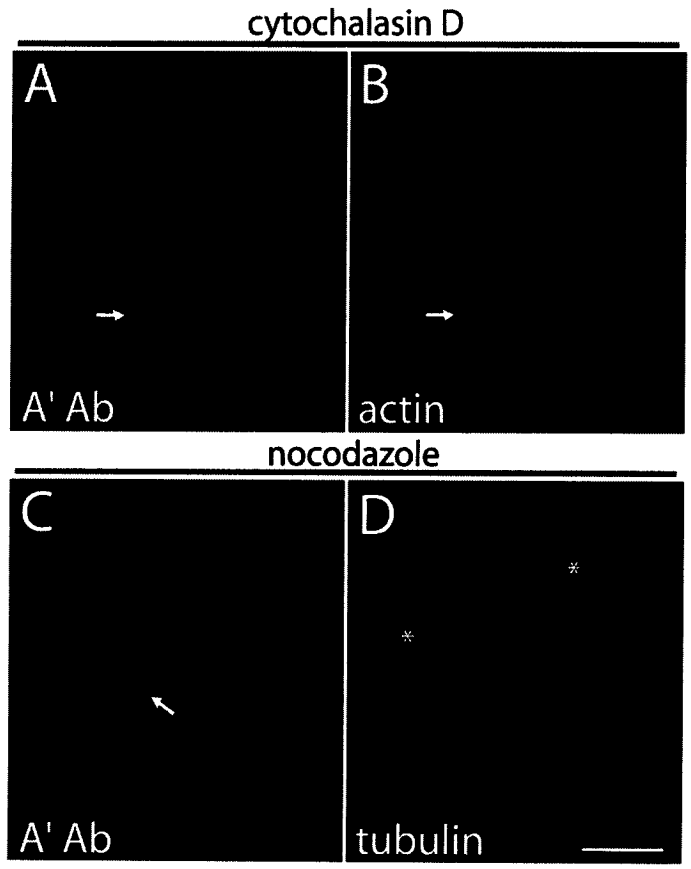

Fig. 4. Disruption of actin filaments, but not of microtubules, alters Bpag1a/b2 localization. (A, B) Treatment with 200 nM cytochalasin D caused the aggregation of stress fibers in many of the C2C12 cells (B). In cells with a few remaining actin stress fibers, some A' staining (A) was still co-aligned with the fibers (arrow in A and B). In cells with no intact central stress fibers, A' staining was diffuse, and no filamentous pattern was ever observed. (C, D) Treatment with 500 nM nocodazole caused almost complete depolymerization of microtubules in the majority of the C2C12 cells (D), with most of the remaining microtubule staining existing at and close to the microtubule organizing centers (asterisks in D). A' staining in these cells (C) was not disrupted, as it was still observed in a filamentous pattern (arrow in C). Bar in D, 20 μm, and applies to all four images.

cells that lacked intact stress fibers.

Following treatment with nocodazole, which causes microtubule depolymerization, the filamentous pattern of A' staining was retained in C2C12 cells that lacked intact microtubules (Fig. 4C, D). Thus, the localization of Bpag1a/b2 to central stress fibers is not dependent on the presence of intact microtubules. We cannot, however, rule out a role for Bpag1 crosslinking actin filaments and microtubules at certain points along the stress fibers.

Bpag1e antibodies detect protein in the nuclei of C2C12 myoblasts

Bpag1e transcripts are present in C2C12 myoblasts, albeit at low levels (Fig. 1B). We therefore expected that Bpag1e protein would also be produced by the myoblasts. However, Bpag1e is normally located at hemidesmosomes in epithelial cell types and, because such structures have not been reported to be present in C2C12 cells, we wondered whether Bpag1e would be present at the other type of adhesive structure – namely, focal contact sites. In a very few myoblasts (<1%), Bpag1e staining was noted in a pattern that corresponded well with focal contact sites, as determined by paxillin double-staining (Fig. 5A – C). The Bpag1e staining, however, localized with only a subset of the focal contact sites, and overlapped only partially with the paxillin staining.

Interestingly, an antibody produced against a peptide from the C-terminal half of human Bpag1e and a human patient serum containing antibody that detects the C-terminal half of human Bpag1e (Ishiko et al., 1993) produced staining in the nuclei of all C2C12 myoblasts (Fig. 5). Notably, the pattern of staining in the nucleus was consistent with that produced by the A' antibody, with staining being weak or absent in the nucleoli,

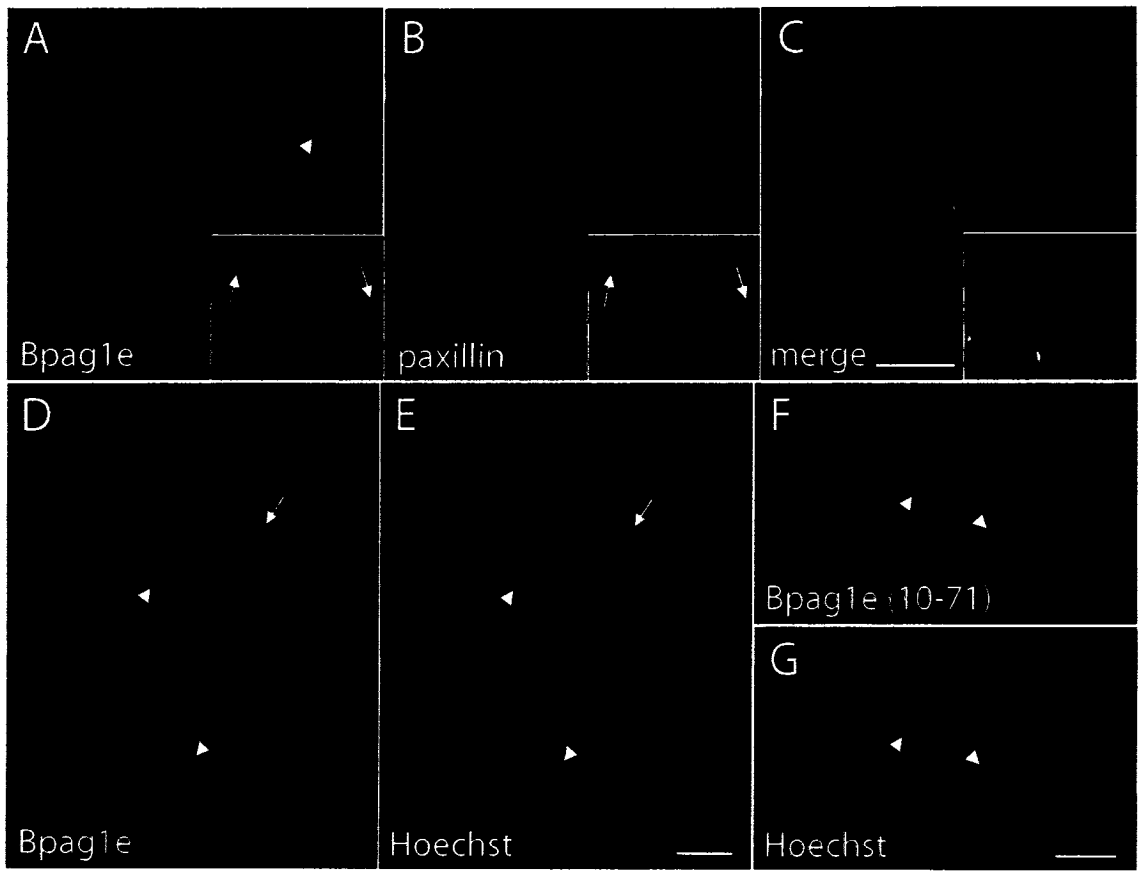

Fig. 5. Bpag1e localizes predominantly to the nucleus in C2C12 myoblasts. (A – C) In cells double-labeled for Bpag1e (A) and paxillin (B), overlap in the patterns of staining was observed in the center of a few cells (arrows in insets; insets are enlargements of the center region from the larger cell). However, the only staining in most cells was light staining in the nuclei (arrowhead in A). (D, E) Bpag1e staining (D) and Hoechst staining (E) showing that most of the Bpag1e staining was limited to the nuclei (arrowheads in D and E). In several mitotic cells noted, Bpag1e staining was absent from condensed chromosomes (arrows in D and E). (F, G) Bpag1e staining (F) was also identified in the nuclei (G; arrowheads in F and G) using bullous pemphigoid patient serum [Bpag1e (10-71)]. Bars, 20 μm.

thus producing a Swiss-cheese pattern of staining in most of the nuclei. Thus, although Bpag1e was observed to localize at adhesion structures as expected, the predominant staining produced with Bpag1e antibodies was nuclear.

Bpag1 N-terminal fusion proteins localize differently and have different effects on microfilament organization

Fusion protein expression plasmids containing the sequence for the N-terminal unique exon of Bpag1 isoform 1 (Nterm1) or Bpag1 isoform 2 (Nterm2) followed by the common ABD, or the ABD alone (Fig. 1C) were assessed in C2C12 cells. These fusion proteins had either GFP or FLAG tagged to the Nterminus (Fig. 1C). N-terminal FLAG tag fusion proteins containing the isoform 1 or 2 unique regions, the ABD, and the plakin domain (Nterm1long and Nterm2long), or the C-terminal half of the plakin domain alone, were also studied (see below). Proteins of the expected sizes for all the expression constructs were detected by immunoblot (Fig. 1C). Because the results for both FLAG and GFP fusion proteins were essentially identical, only the FLAG fusion protein results are shown.

As expected, all of the Bpag1 N-terminal fusion proteins containing the ABD colocalized to the actin cytoskeleton (Figs 6, 9). Expression of the ABD fusion proteins lead to co-alignment with the actin stress fibers, as visualized with rhodamine-phalloidin (Fig. 6A – D). In cells expressing the Nterm1 fusion proteins, the staining of the FLAG tag (Fig. 6E – H) and the GFP signal (not shown) displayed a punctate pattern corresponding to aggregated actin cytoskeleton. The collapse of the actin filaments into aggregates may represent a dominant-negative effect. In addition, some of the Nterm1-

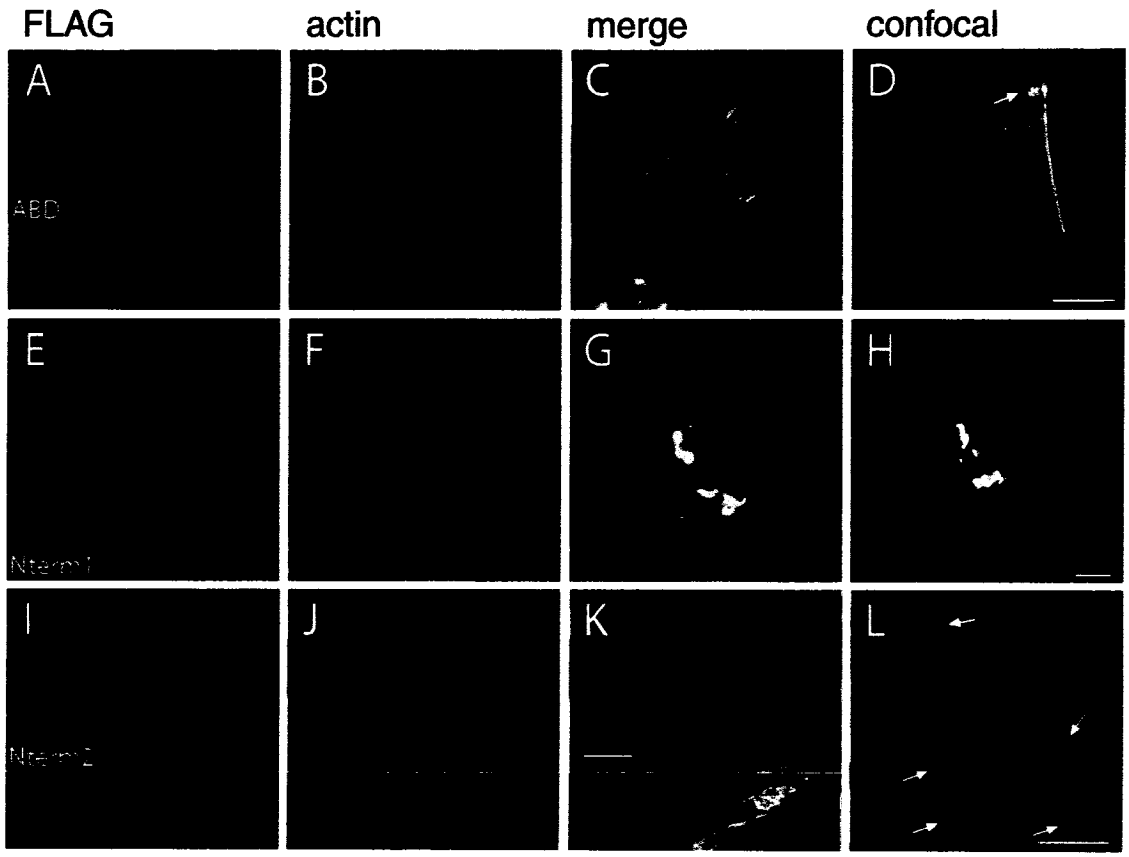

Fig. 6. The interaction of Bpag1 N-terminal fusion proteins with actin filaments in C2C12 myoblasts. (A – D) The FLAG-ABD protein (A) co-aligned with actin stress fibers (B). A relatively stronger signal was also consistently noted at the ends of the fibers (insets in A – C; arrow in D). (E – H) The N-term1 protein (E) colocalized primarily with actin aggregates (F) in all cells. It never colocalized with normal-appearing stress fibers. (I – L) The Nterm2 fusion protein (I) co-aligned with stress fibers (J), but not at the ends (left-hand insets in I – K; arrows in L). In about half of the cells, Nterm2 colocalized with aberrantly bundled actin fibers (right-hand insets in I – K). Bar in K, 20 μm for all conventional fluorescence micrographs. Bars in confocal micrographs (D, H, L), 10 μm.

expressing cells also contained fusion protein colocalizing with thickened filaments. Actin filaments of normal thickness that were observed in these cells did not typically have any co-aligning Nterm1 fusion protein. By contrast, cells expressing the Nterm2 fusion proteins had a normal actin filament network when observed with the FLAG or GFP, and rhodamine-phalloidin labels (Fig. 6I – L and data not shown).

In about half (48%) of the cells expressing the FLAG-Nterm2, many of the actin stress fibers became thickly bundled and no longer appeared as straight stress fibers (insets in Fig. 6I – K). In GFP-Nterm2-expressing cells, a similar result was obtained, with about one-third (35%) of the cells exhibiting thickly bundled stress fibers. In cells that contained fusion protein co-aligning with normal appearing stress fibers, the Nterm2 fusion proteins localized to the stress fibers differently than the fusion proteins containing only the ABD. Although the ABD and Nterm2 fusion proteins both aligned along most of the length of the stress fibers, the Nterm2 proteins never localized to the ends of the stress fibers (lefthand insets in Fig. 6I – K; arrows in Fig. 6L). The ABD fusion proteins consistently gave a strong signal at the end of the fibers (insets in Fig. 6A – C; arrow in Fig. 6D). Thus, the addition of the N-terminal unique sequence from the Bpag1 isoform 2 prevents targeting of the ABD to actin at the tips of stress fibers.

To confirm that the ABD was localizing to focal contact sites, and that the Nterm2 fusion proteins were excluded from these sites, we labeled FLAG fusion proteinexpressing cells with FLAG and paxillin antibodies (Fig. 7). Cells expressing the FLAG-ABD displayed FLAG staining that colocalized with paxillin staining, as assessed by confocal microscopy (Fig. 7A, B). In cells transfected with the FLAG-Nterm2, only fiber-like staining that was excluded from contact sites was observed with the FLAG

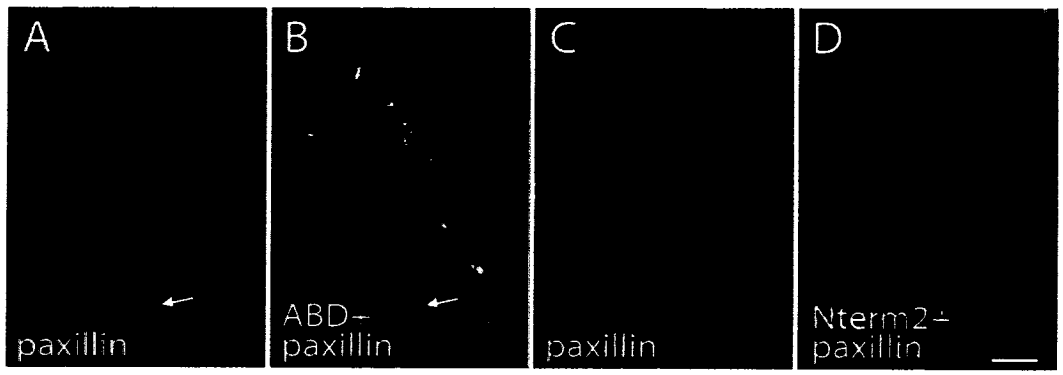

Fig. 7. The Bpag1 ABD, but not the isoform 2 N-terminus, localizes to focal contacts. (A, B) As observed by confocal microscopy, the FLAG-ABD fusion protein staining (green) consistently produced staining that overlapped with paxillin staining (red), indicating its localization to focal contact sites. (C, D) With the FLAG-Nterm2 fusion protein (green), only minor overlap with paxillin staining (red) was ever observed by confocal microscopy. Also, punctate staining consistently observed for paxillin and FLAG-ABD along the periphery of the cells (arrows in A and B) was never observed with the FLAG-Nterm2-expressing cells. Bar in D, 10 μm, and applies to all four images.

antibody (Fig. 7C, D). In addition, although the FLAG-ABD-expressing cells typically displayed extensive paxillin and FLAG staining along the periphery of the cells (arrows in Fig. 7A, B), this type of staining was never seen in FLAG-Nterm2 transfected cells. Thus, it appears that the ABD may localize with focal contact components only if the isoform 2 N-terminus is absent.

Bpag1 N-terminal fusion proteins self-associate, but require the upstream unique regions to do so

To explain the actin bundling and aggregation effects observed with Nterm1- and Nterm2-transfected cells, we tested whether these fusion proteins were self-associating. The bundling of actin filaments by a plakin ABD has previously been shown with plectin, and probably occurs via dimerization of the Nterminus (Fontao et al., 2001). The same study also showed by yeast two-hybrid assay that the N-terminal region of non-epithelial Bpag1 self-associates. However, this self-association was shown with only the isoform 2 N-terminal region. We have extended this observation using our FLAG and GFP constructs to determine whether one tagged construct would pull-down the other in an immunoprecipitation assay. Following co-expression of the GFP- and FLAG-Nterm1 or GFP- and FLAG-Nterm2 fusion proteins in Cos-1 cells, the anti-FLAG antibody co immunoprecipitated the co-expressed GFP-tagged fusion protein from the cell lysate (Fig. 8). However, following co-expression of the GFP-Nterm2 with the FLAG-ABD, the GFP-tagged fusion protein was not coimmunoprecipitated (Fig. 8B, lane 6). The FLAG-Nterm1 and GFP-Nterm2 also associated with each other, whereas the GFP- and FLAG-ABD fusion proteins did not associate with each other (data not shown). These results

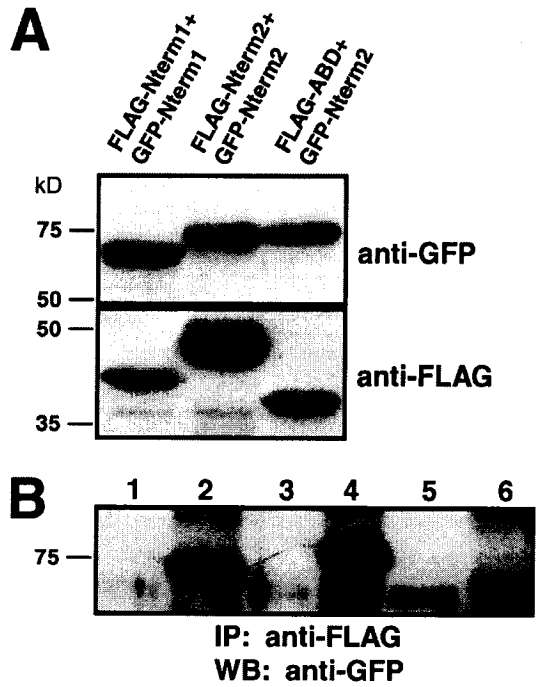

Fig. 8. The ABD-containing N-terminal regions of Bpag1 can self-associate but require the presence of the unique regions upstream from the ABD to do so. (A) Immunoblots showing 1/10 input of the lysates used for performing immunoprecipitations. GFP Nterm1 and FLAG-Nterm1, GFP-Nterm2 and FLAG-Nterm2, or GFP-Nterm2 and FLAG-ABD were co-expressed in Cos-1 cells. All of the GFP- and FLAG-tagged proteins were expressed relatively evenly, with the exception of the FLAG-Nterm2, which was very highly expressed. This was typical of other results looking at the expression of these proteins (data not shown). (B) Immunoprecipitation with an anti-FLAG antibody pulls down GFP-Nterm1 (lane 2) in cells coexpressing FLAG-Nterm1 and GFP-Nterm1, and GFP-Nterm2 (lane 4) in cells co-expressing FLAG-Nterm2 and GFP-Nterm2. However, the GFP-Nterm2 was not co-immunoprecipitated with the FLAG-ABD fusion protein (lane 6). Lanes 1, 3, and 5 are immunoprecipitations corresponding to lanes 2, 4 and 6, respectively, which were performed without the anti-FLAG antibody as controls for the experiment.

indicate that the Nterminal regions of neuronal or muscle Bpag1 can dimerize, or potentially multimerize. This would provide a mechanism for bundling microfilaments, as previously shown for plectin (Fontao et al., 2001), and as observed in this study.

The fusion proteins we used that lacked an N-terminal unique region (GFP- and FLAG-ABD) did not allow for dimerization of the N-terminus, which is consistent with their inability to bundle microfilaments (Fig. 6 and data not shown). Although this may indicate that the ABD region requires an upstream region (provided by either the A or A' unique regions) in order to dimerize, we cannot exclude the possibility that both the GFP and the FLAG tags interfered with the ability of the ABD region to dimerize.

The plakin domain is involved in nuclear localization of Bpag1

Addition of the plakin domain to the Nterm1 fusion protein (Nterm1long) did not alter its localization pattern. The Nterm1long fusion protein localized with actin aggregates, just as the Nterm1 fusion protein did (Fig. 9A – C). By contrast, the presence of the plakin domain at the end of Nterm2 (Nterm2long) did have dramatic consequences. The Nterm2long fusion protein, in a pattern consistent with the A' antibody localization, localized to both actin stress fibers and to the nucleus (Fig. 9D – F). An absence of staining was also noted at the ends of the stress fibers with this fusion protein. The majority of Nterm2long-expressing cells, however, contained no obvious stress fiber localization of the protein, and 44% of the expressing myoblasts contained no cytoplasmic localization of the fusion protein at all. Rather, the predominant localization of this fusion protein, observed in almost all expressing myoblasts (99%), was nuclear (Fig. 9G, H), with 56% of the cells also containing cytoplasmic staining that ranged from

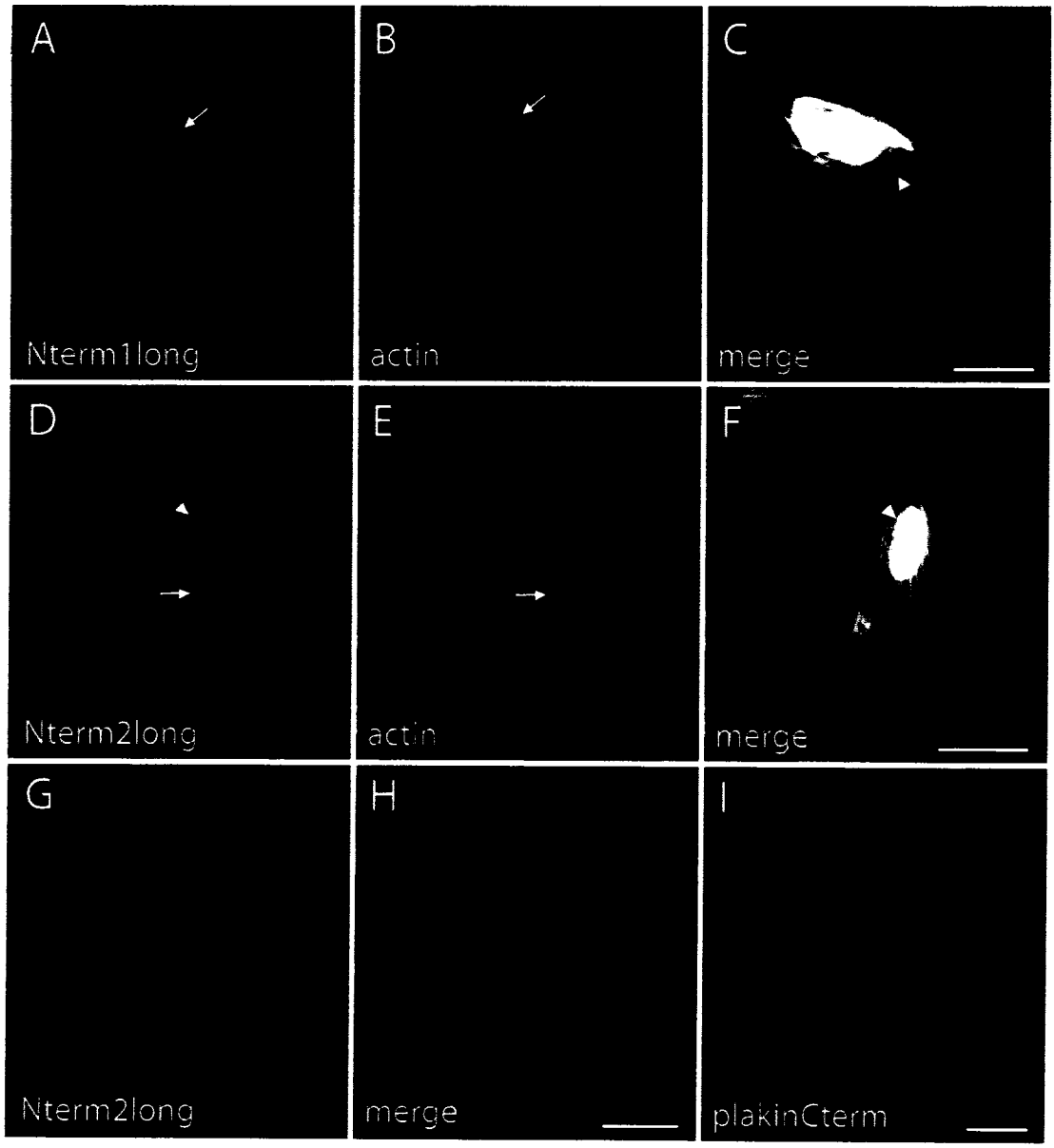

Fig. 9. The Bpag1 isoform 2 N-terminus, but not the isoform 1 N-terminus, localizes to the nucleus of C2C12 cells when the plakin domain is included in the fusion protein. (A – C) Nterm1long (A), similar to the shorter Nterm1 fusion protein (Fig. 6E), localized to actin aggregates (B; arrows in A and B). This protein was never observed in the nuclei (arrowhead in C). (D – F) Nterm2long (D) localized to the nucleus in almost all cells

(arrowhead in D and F), and was also found in the cytoplasm in over half of the cells, including along stress fibers (E; arrows in D and E). (G, H) In many of the cells, Nterm2long staining (G) appeared to be exclusively nuclear, in a Swiss-cheese pattern of staining reminiscent of the endogenous Bpag1 staining (see Figs 3 and 5). (I) The C-terminal half of the plakin domain (plakinCterm) localizes in a similar pattern in the nucleus in all cells expressing this fusion protein. Additional localization to the cytoplasm occurred only in a minority of the cells expressing plakinCterm. Bars, 20 μm.

being filamentous to diffuse. This contrasts sharply with the shorter Nterm2 fusion protein, which did not localize to the nucleus, and always associated with stress fibers or aberrantly bundled actin cables (see Fig. 6I – L). The nuclear pattern of staining was identical to that observed with Bpag1 antibodies in producing a Swisscheese pattern of staining. These results suggested that the addition of the plakin domain caused localization of the protein to the nucleus, and that this localization was influenced by the unique N-terminal sequences.

To examine whether the plakin domain could localize to the nucleus on its own, the C-terminal half of this domain, which contains a putative classical nuclear localization signal (NLS) region [KRRR or PVKRRRI; predicted by PSORTII (http://psort.nibb.ac.jp/)], was expressed as a FLAG-tagged fusion protein (plakinCterm; Fig. 9I). This fusion protein is approximately 69 kDa (see Fig. 1C), and thus is too large to passively localize to the nucleus. Similar to the endogenous Bpag1 proteins and the Nterm2long fusion protein, the plakinCterm protein produced a Swiss-cheese pattern of staining in the nuclei of expressing myoblasts. The plakinCterm protein localized to the nuclei of all expressing myoblasts, and appeared to be exclusively nuclear in 86% of the cells, with additional cytoplasmic staining in the remaining cells.

Within the plakin domain, only the one sequence in the Cterminal half of the domain was predicted to constitute an NLS. If this sequence is of functional significance, it should be conserved between species. Indeed, the PVKRRRI/M motif is conserved between mouse and human Bpag1 (Fig. 10A). This motif is also similar in MACF, but is altered in two other plakins, plectin and periplakin. To demonstrate this motif conclusively as an NLS, we performed two types of experiments. In the first, we used

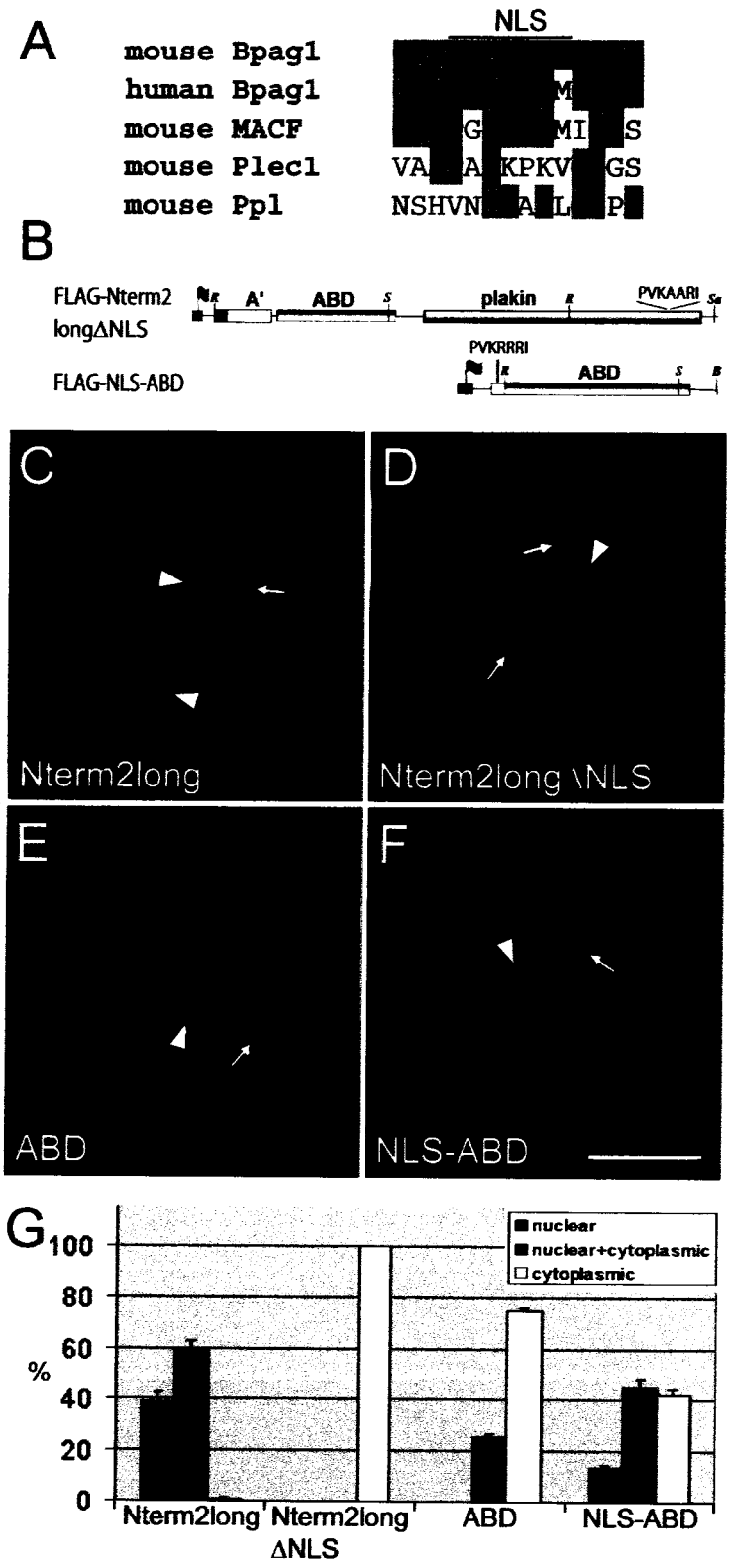
A
NLS
mouse Bpag1
human Bpag1
mouse MACF
mouse Plec1
mouse Ppl
B
FLAG-Nterm2 longΔNLS
A'
ABD
plakin
PVKAARI
PVKRRRI
FLAG-NLS-ABD
C
Nterm2long
D
Nterm2long \NLS
E
ABD
F
NLS-ABD
G
100
80
60
40
20
0
%
nuclear
nuclear+cytoplasmic
cytoplasmic
Nterm2long
Nterm2long ΔNLS
ABD
NLS-ABD

Fig. 10. Bpag1 contains a functional NLS in the plakin domain. (A) In both mouse and human Bpag1 proteins, the PVKRRRI/M motif in the plakin domain is conserved (identical amino acid residues have dark shading; similar amino acid residues have light shading). This motif is similar in the same region of mouse MACF, but differs in plectin (Plec1) and periplakin (Ppl). (B) The FLAG-Nterm2long plasmid was re-made with a mutation within the NLS sequence such that it would no longer resemble a classical NLS (Nterm2longΔNLS). The FLAG-NLS-ABD plasmid had the NLS coding sequence (coding for PVKRRRI) inserted immediately before the ABD insert. The mutant fusion proteins were of similar size to their wild-type counterparts (not shown). Labeling is the same as in Fig. 1. (C) Nterm2long fusion protein produced strong signal in the nucleus of 99% of the cells (arrowheads). The arrow in C points to some of the fusion protein co-aligning with stress fibers. (D) The Nterm2longΔNLS fusion protein staining never produced staining in the nucleus (arrowhead) above the background of untransfected cells. The protein was exclusively cytoplasmic, with much of it co-aligning with stress fibers (arrows). (E) Localization of the ABD fusion protein along stress fibers (arrow), but not in the nucleus (arrowhead). (F) With the NLS sequence placed before the ABD, 57% of the cells had labeling of the fusion protein within the nucleus (arrowhead), which was typically much stronger than the signal in the cytoplasm. The NLS-ABD fusion protein also co-aligned with stress fibers in many cells (arrow), and appeared no different than the ABD fusion protein in many. Bar in F, 20 μm, and also applies to C – E. (G) Summary of the quantification of the localizations of the Nterm2long, Nterm2longΔNLS, ABD and NLS-ABD fusion proteins. Error bars indicate the standard errors from a minimum of three separate transfections.

site-directed mutagenesis to alter the NLS within the context of the Nterm2long fusion protein. In the second, we tagged the PVKRRRI peptide sequence (the putative NLS) onto the N-terminal end of the ABD to assess whether it could relocate the ABD fusion protein into the nucleus.

A mutated Nterm2long fusion protein had the PVKRRRI changed to PVKAARI (Nterm2longΔNLS; Fig. 10B). This change of two amino acids (out of a 1634 amino acid sequence) completely prevented the fusion protein from localizing to the nucleus (Fig. 10D, G). In 100% of transfected cells, staining of the mutant fusion protein appeared to be only cytoplasmic, with obvious stress fiber coalignment in most of the cells (Fig. 10D). This was in sharp contrast to the wild-type Nterm2long fusion protein (Fig. 10C, G). The addition of the putative NLS to the ABD fusion protein (referred to as FLAG-NLS-ABD; Fig. 10B) also had a dramatic effect. Although only a minority (25%) of the FLAG-ABD-transfected cells contained a light staining in the nucleus (probably due to passive diffusion of this small fusion protein), 57% of the FLAG-NLS-ABD-transfected cells contained strong staining in the nucleus (Fig. 10E – G). In all, 12% of the cells had the fusion protein exclusively in the nucleus, whereas the FLAG-ABD fusion protein was never exclusively nuclear. FLAG-NLS-ABD localization in the cytoplasm appeared diffuse in some cells, but still showed nice co-alignment with stress-fibers in most, indicating that the addition of the NLS before the ABD did not have a major impact on the actin binding ability of the fusion protein.

Discussion

In the present study, we have shown that isoform-specific unique regions encoded by alternatively spliced exons upstream from the ABD sequence of Bpag1 alter the interaction of the Bpag1 N-terminus with actin, and alter its ability to localize to the nucleus. We have shown that Bpag1 isoform 2 co-aligned with actin stress fibers in C2C12 cells, excluding the ends, where focal contact sites form. This mimicked the localization observed for its corresponding N-terminal fusion proteins. However, surprisingly, the predominant localization of this isoform in C2C12 cells, and of the Bpag1e isoform, is in the nucleus. These isoforms have only the plakin domain in common, and addition of the plakin domain was required for fusion protein localization to the nucleus. The C-terminal half of the plakin domain alone efficiently targets to the nucleus. Within this region, we identified a functional NLS, which, when mutated, inhibited the fusion protein from localizing to the nucleus. Conversely, when this NLS was placed in front of an ABD fusion, it induced localization of this protein to the nucleus. Our work highlights the importance of N-terminal regions in determining the localization of Bpag1, and presents initial observations that Bpag1 can actively locate to the nucleus.

The N-terminal organization of other plakin proteins, including plectin and MACF (Acf7), is strikingly similar to that of muscle and neuronal Bpag1 in that they all contain unique sequences just upstream of an ABD (Bernier et al., 1996; Fuchs et al., 1999; Karakesisoglou et al., 2000; Leung et al., 1999b). The N-terminal unique regions in plectin were hypothesized to optimize its function in a cell-dependent manner. The possibility of differential subcellular localization and function of plectin isoforms

containing different N-terminal sequences has recently been addressed within a single cell type, keratinocytes (Andra et al., 2003). It was shown that alternate N-terminal sequences could determine the localization of plectin isoforms to either hemidesmosomes or to microtubules. Other work has shown that the N-terminus of plectin is able to bundle microfilaments (Fontao et al., 2001). That plectin may be an important regulator of actin filament dynamics has been shown with cells cultured from plectin null mice, which contain normal microtubule and intermediate filament networks but have aberrant actin stress fiber organization (Andra et al., 1998). Thus, regulation of plakin protein binding to actin filament, microtubules and adhesion structures via unique N-termini may be an important general feature in determining the function of plakin proteins.

A Bpag1 isoform that co-aligns with actin stress fibers

In C2C12 myoblasts, endogenous Bpag1a/b2 aligned along stretches of actin filaments (Figs 2, 3). The only filamentous pattern of staining observed was along the actin stress fibers. Although microtubules crossed through the area of A' staining in some cells, and strong perinuclear staining in some cells colocalizes with at least some tubulin and intermediate filament staining (not shown), the A' staining was not dependent on the presence of microtubules, as assessed in nocodazole-treated cells (Fig. 4). Also, it is clear that although the A' staining aligned the entire length of some stress fibers, microtubules and intermediate filaments do not normally run in parallel to actin stress fibers. Therefore, although the endogenous Bpag1a/b2 may cross-link microfilaments to microtubules and/or intermediate filaments at restricted points, or in selective

situations, its main function may be to help bundle and maintain the integrity of the actin stress fibers. Alternatively, it may interact with other actin filament associated proteins involved in structural or signaling roles.

At the ends of actin stress fibers, it is unlikely that Bpag1a/b2 helps attach the fibers to focal adhesion proteins, given that the isoform 2 unique region excludes Bpag1 from focal contact sites (Figs 6, 7). This makes Bpag1a/b2 distinct from Bpag1e, which has been shown to play a role in anchoring intermediate filaments to membrane adhesion plaques or hemidesmosomes. Other plakins with a similar ABD may localize to focal adhesion sites if using a region upstream from the ABD that does not exclude them from the focal adhesion sites. In this regard, different splice variants of another Nterminal ABD-containing protein, filamin-B, are localized along actin stress fibers either at the focal adhesion sites, or along the length of the fiber excluding the tips (van der Flier et al., 2002). In contrast to Bpag1, the localization of filamin-B either towards or away from the focal adhesion sites was determined by a hinge region in the C-terminal half of the protein.

Bpag1 localizes to the nucleus

Although we initially expected that Bpag1 antibody staining would produce a staining pattern that was consistent with localization predominantly to cytoskeletal structures, the most prevalent staining with the isoform 2 (A') antibody and with Bpag1e antibodies was nuclear (Fig. 3D – F; Fig. 5). The pattern of nuclear staining was consistent between the antibodies, with the staining being weak or absent in nucleoli. Because the Bpag1e isoform and the longer Bpag1a and Bpag1b isoforms have only the plakin domain in

common to their structures (Fig. 1A), we hypothesized that this domain might be involved in the nuclear localization of Bpag1. Indeed, this seems to be true based on the localization of plakin domain-containing fusion proteins (see below).

In epithelial cells, Bpag1e localizes to hemidesmosomes via its plakin domain. This is dependent on binding of N-terminal regions of the plakin domain to hemidesmosomal proteins, BP180 or β4 integrin (Hopkinson and Jones, 2000; Koster et al., 2003). Other than its involvement in targeting to hemidesmosomes, little else is known about the function of the plakin domain. Binding of the N-terminal half of Bpag1e to ERBIN has been described (Favre et al., 2001), suggesting a link to Erb-B2 receptor signaling, although little has been shown regarding this.

The plakin domain is highly conserved among plakin family members, and if this domain targets proteins to the nucleus, other plakin proteins may also localize to the nucleus. In the case of plectin, the most extensively studied plakin, nuclear localization appears to be limited to an association with the nuclear lamina (Wiche, 1998). Consistent with this, the NLS in the plakin domain identified in this paper is not conserved in plectin (Fig. 10A), although nuclear localization of plectin may occur via a C-terminal NLS (Nikolic et al., 1996). At least one other plakin protein, periplakin, has recently been reported to be present within the nucleus (van den Heuvel et al., 2002). This was observed both with a periplakin antibody and a full-length periplakin fusion protein. The authors predicted that the nuclear localization may be dependent on a putative NLS outside of the plakin domain, in a region similar to plectin's putative C-terminal NLS, but the region of periplakin responsible for nuclear localization was not examined. Like plectin, however, the NLS in the Bpag1 plakin domain is not conserved in periplakin

(Fig. 10A), making it likely that a periplakin NLS would indeed be outside of the plakin domain.

There are several possibilities to explain why a large structural protein would localize to the nucleus. First, it may be sequestered there, and translocate into the cytoplasm to associate with cytoskeletal elements (e.g. actin filaments or focal contacts) when needed. Second, as has been hinted at for periplakin (van den Heuvel et al., 2002), Bpag1 may play a functional role in the nucleus by associating with other proteins that reside in the nucleus either transiently or over the long-term. Third, Bpag1 may play a structural role in the nucleus. We are currently examining the possibility that Bpag1 interacts with nuclear proteins to address its nuclear localization.

Modulation of Bpag1 ABD localization via unique N-termini

Bpag1 N-terminal fusion proteins differing only in the unique sequence upstream from the ABD have drastically different effects on the actin cytoskeleton when expressed in C2C12 cells. Although overexpression of the Bpag1 isoform 2 Nterminus resulted in a bundling of the microfilaments, overexpression of the Bpag1 isoform 1 N-terminus resulted in an aggregation of the microfilaments (Fig. 6). There is no doubt that the aggregation effect of the isoform 1 fusion proteins was an artifact of overexpressing only the N-terminal region of this protein and may not reflect the function of the endogenous protein. However, the bundling effect and localization of the isoform 2 N-terminal fusion protein was very consistent with the localization of the corresponding endogenous protein. The endogenous isoform 1 protein may associate primarily with nonactin

cytoskeletal structures, or it may interact with actin differently in the context of the rest of the protein. This aspect remains to be studied as specific antibodies become available.

The unique N-terminal regions of Bpag1 isoforms 1 and 2 also had differential effects on the localization of fusion proteins harboring the ABD and the plakin domain. Although the Nterm1long fusion protein still localized with actin aggregates, the Nterm2long fusion protein localized predominantly to the nucleus, as well as to actin stress fibers (Fig. 9). The lack of localization of the Nterm1long to the nucleus may be due to a strong affinity of this fusion protein to actin, which would, in turn, keep it associated with actin aggregates, thus preventing it from translocating into the nucleus.

Within the plakin domain, we identified a functional NLS (PVKRRRI), which may be largely responsible for the localization of Bpag1a/b2 and Bpag1e to the nucleus. Mutation of this NLS in the Bpag1a/b2 N-terminus prevented its localization to the nucleus. The addition of the NLS sequence before the ABD in the FLAG-ABD fusion protein caused the translocation of the fusion protein to the nucleus in a majority of the cells, although it did not prevent the actin binding ability of this fusion protein (Fig. 10). Outside of the plakin domain, the only putative NLS sequences in Bpag1a or Bpag1b reside either within the ABD region, which does not localize strongly to the nucleus (Figs 6, 7), or at the C-terminus. Although the C-terminal region alone localizes predominantly to microtubules, it can also localize to the nucleus (Sun et al., 2001), suggesting that this region may also contribute to the nuclear localization of the endogenous Bpag1a/b2. Notably, the class of NLS identified in this study may be utilized by other large proteins to localize to the nucleus, as it is nearly identical to a putative NLS (PLKRRRV) in the

N-terminal region of the predominantly nuclear protein Ki-67 (359 kDa) (Schluter et al., 1993).

In summary, the ability of the Bpag1 isoform 2 N-terminus to bundle microfilaments, possibly via dimerization, and the localization of the Nterm2 and the endogenous protein along the length of actin stress fibers, indicates that Bpag1a/b2 plays a role in regulating actin filament dynamics. Furthermore, this function is determined by the N-terminal unique region, as it was required for appropriate localization of the ABD. That the N-terminus can probably determine the role of an endogenous Bpag1 was indicated by the fact that the localization of Bpag1a/b2 correlated with that of the corresponding N-terminal fusion protein (Nterm2long) in myoblast cells. Surprisingly, the predominant localization of the Bpag1 isoforms observed in this study was in the nucleus. The various localizations of plakin proteins to the cytoskeleton, adhesion structures, organelles, and the nucleus suggests that differences in the structures of individual isoforms may play a role in guiding different isoforms to different structures. Along with recent results with plectin in keratinocytes (Andra et al., 2003), our data presented here indicates that regulation of localization and function by Nterminal unique sequences may be a common theme in the plakin protein family.

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Chapter 4

A Bpag1 isoform involved in cytoskeletal organization surrounding the nucleus

Contributions of additional authors

Bruno Pinheiro provided technical assistance, primarily in performing cell fractionation experiments (see Figure 3). I had written the original manuscript independently. Rashmi Kothary helped in the editing of the paper.

A Bpag1 isoform involved in cytoskeletal organization surrounding the nucleus

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Abstract

Bpag1/dystonin proteins are giant cytoskeletal interacting proteins postulated to cross-link cytoskeletal filaments and thereby maintain cellular integrity. Loss of function of non-epithelial Bpag1 isoforms results in neuromuscular dysfunction and early postnatal death in mice. Multiple Bpag1 transcripts have been described, including those encoding protein isoforms that vary at the N-terminal end. Here, we have analyzed the subcellular localizations and cytoskeletal interactions of two isoforms, termed Bpag1a1 and Bpag1a2. We demonstrate that novel sequence at the 5' end of the Bpag1a2 transcript codes for an N-terminal transmembrane domain and targets the protein to the perinuclear region of the cell. Furthermore, we show that the endogenous Bpag1a2 protein is also present in the perinuclear region in myoblast cells. Differences in Bpag1a1 and Bpag1a2 with respect to the extent of their interactions with microtubules and microfilaments are also described, with Bpag1a2 fusion protein serving largely to associate with microfilaments surrounding the nucleus and Golgi apparatus. The overall structure and subcellular localizations of Bpag1a2 indicate possible functions in nuclear envelope structuring, nuclear tethering, and organization of membranous structures surrounding the nucleus.

Introduction

Spectrin superfamily members are large cytoskeletal-interacting proteins noted for their structural roles in the cytoplasm and at the plasma membrane. Loss of function of spectrin superfamily members lead to pathological conditions in humans and in mice. For example, loss of dystrophin function leads to Duchenne-Becker muscular dystrophy [1, 2]. Similarly, loss of spectrin function leads to the red blood cell disorders spherocytosis and elliptocytosis [3-5]. In *dystonia musculorum* mice, loss of function of the spectrin-related bullous pemphigoid antigen 1 (Bpag1)/dystonin protein leads to sensory neuron degeneration and deficiencies in glial and muscle cytoarchitecture [6-11]. Each of these disorders is considered to be due primarily to a loss in structural integrity in the cytoplasm and at the plasma membrane of affected cells.

More recently-identified spectrin superfamily members, the Syne (nesprin/myne/NUANCE/enaptin) proteins, have been primarily implicated in regulating nuclear positioning and in maintaining organization at the nuclear envelope and within the intranuclear space [12-15]; and reviewed in [16]. Syne proteins all contain a C-terminal transmembrane domain responsible for attachment to the inner and outer nuclear membranes, and an N-terminal actin-binding domain in the largest isoforms which may play a role in interacting with actin both inside and outside of the nucleus [15, 17-19]. In addition, various isoforms of spectrin and other related proteins have been localized to the nuclear envelope and intranuclear space, and have been demonstrated to interact with components of the nucleus (reviewed in [16]). Syne-1 and MACF1, the closest relative of Bpag1, have also been implicated in the regulation of Golgi apparatus organization and function [20-23]. This, and other data, indicates that spectrin superfamily members are

involved in many aspects of cellular organization including establishment and maintenance of cytoskeletal organization, mediation of multiple organellar interactions with the cytoskeleton, and mediation of nuclear organization.

Observations of proteins which form structural links between the nucleus and the cytoskeleton have helped to provide possible explanations for the intrinsic muscle dysfunction in disorders related to loss of function of the nuclear proteins lamin and emerin. Mutations in both the emerin and lamin A/C genes can lead to Emery-Dreyfus muscular dystrophy, characterized by abnormalities in myonuclear architecture (reviewed in [24]. Loss of interactions between lamin and emerin proteins with Syne in muscle may contribute to a loss of muscle cell integrity [15, 24-26]. Pathology may arise due to aberrant nuclear function (aberrant gene expression) and/or loss of physical interactions with the extended nucleo/cytoskeletal network, mediated by Syne and related proteins [26]. Such a breakdown in physical interactions would result in unstable architecture throughout the cell, especially in cells undergoing constant mechanical stress such as peripheral neurons and muscle.

Among the characteristic pathological features noted early on in *dystonia musculorum* mice was eccentricity of neuronal nuclei [27-29]. In this study, we suggest the possibility of a direct involvement of Bpag1 with subcellular organization surrounding the nucleus and with nuclear attachment to the cytoskeleton. We demonstrate that Bpag1 isoform 2, previously identified as actively localizing to the nucleus and interacting with actin stress fibres adjacent to the nucleus [30], contains an N-terminal transmembrane domain and associates with membranous structures surrounding the nucleus of the cell. Using full-length and N-terminal fusion protein constructs, we

demonstrate that Bpag1 isoform 2 caused the accumulation of microfilaments surrounding the nucleus and Golgi apparatus. In comparison, Bpag1 isoform 1 fusion protein localized throughout the cytoplasm, co-aligning with microtubules and actin stress fibres. The overall structure and localization of Bpag1 isoform 2 show similarities to the Syne proteins, and indicates the possibility of similar roles in the cell. The different interaction and targeting domains of Bpag1 demonstrate that the variant splice forms of this protein are involved in cellular organization throughout multiple regions of the cell.

Materials and methods

Bpag1 cDNA cloning and fusion protein constructs

The 5' end of the Bpag1 isoform 2 transcript was amplified by 5' RACE using reverse transcribed mRNA from BALB/c mouse brain and C2C12 myoblast cells (cDNA fragment 'd' in Fig. 1A). The SMART RACE Advantage 2 kit (BD Biosciences) was used for this according to the manufacturer's instructions. A primer antisense to sequence in the third exon of isoform 2 (5'-GGTGGAAGCACCTTGAACAGAGTG-3') was used as the gene-specific primer for the 5' RACE. Additionally, RT-PCR analysis was performed to confirm the presence of cDNA corresponding to a Bpag1a transcript containing the isoform 2 5' end. First strand synthesized cDNA from the SMART RACE Advantage 2 kit and Advantage 2 PCR reagents were used for this. The following primers were used to amplify the products (a – c) indicated in Fig. 1A: 5'-GCACCATGATCGCCGCCGCTTT-3' (P1) and 5'-CATCGTTTGCACCAATGCC-3' (P2) for 'a'; 5'-GAGGGCTGTGCTTCGGATAG-3' (P3) and 5'-

TCCGCTCGAGCTTCTCCGGGACCTT-3' (P4) for 'b'; and P3 and 5'-CAGCAACCTGCTGACTTGAT-3' (P5) for 'c'.

Expression plasmids encoding full-length and N-terminal Bpag1 fusion proteins were generated primarily by assembling 710 bp – 1.7 kbp cDNA fragments generated by RT-PCR using MuMLV reverse transcriptase and Platinum High Fidelity Taq DNA polymerase (Invitrogen). cDNA fragments were sequenced to ensure accuracy prior to assembly using restriction site ligations. All fragments obtained in this way, with the exception of the isoform 2 N-terminal coding region, corresponded to the previously described Bpag1a transcript sequence [8]. Additionally, sequence from two EST clones was used (GenBank accession #AA064485 and #AI526522). EST #AI526522 contains small splicing variations that differ from the previously described Bpag1a transcript [8] primarily in the sequence 3' to the Gas2 homology domain encoding region. Bpag1 sequence derived from a human brain cDNA (for KIAA0728; GenBank accession #AB018271) also contains similarity in splicing to the AI526522 EST. The C-terminal region encoded by the AI526522 EST localizes with microtubules, similar to the previously described Bpag1a C-terminal region [31], indicating that the major function of this region is not changed with these splicing variations (Pool and Kothary, unpublished data). All Bpag1-encoding cDNA were cloned into either the pCMV-TAG2 vector (Stratagene) encoding an N-terminal FLAG tag, or the pEF1-myc/his vector (Invitrogen) encoding a C-terminal myc/his tag. The endogenous Kozak consensus sequence for Bpag1 isoform 2 was included in the pEF1-myc/his construct, and was necessary for proper expression. The final assembly for the largest plasmid constructs (up to ~21 kbp) was performed in Stabl2 bacteria (Invitrogen) to prevent rearrangement of the plasmids.

Every plasmid preparation was tested with restriction digests to ensure that no rearrangements had occurred.

Cell culture

Cos-1 monkey kidney and C2C12 mouse myoblast cell lines were cultured in DMEM (Wisent) with 10% FBS (Sigma) and 1% pen/strep/antimycotic (Invitrogen). Cells were cultured in 12 well plates on round coverslips when used for immunofluorescence labelling, or in 6 well plates when used for protein extract collection and cell fractionation. Plasmid transfections were performed with either Lipofectamine 2000 (Cos-1) or Lipofectamine Plus (C2C12 and Cos-1; Invitrogen). Treatment with brefeldin A (Sigma) was performed by adding 10 μg/mL of the drug to the culture wells 30 or 60 minutes prior to fixation (with no noticeable difference between the different incubations).

Immunoblotting

Standard SDS-PAGE and blotting conditions were used to demonstrate the sizes of fusion proteins used in this study. Cell lysates were collected by scrapping cells from 6 well plates straight into SDS loading buffer, boiling for 5 minutes, and cooling on ice. Smaller proteins were separated on 10% gels, and transferred onto PVDF membranes. The larger full-length proteins were separated on 5% resolving with 3% stacking gels, and transferred for at least 6 hours, with methanol omitted from the transfer buffer (50 mM Tris, 40 mM glycine, 0.0375% SDS). Anti-FLAG M2 monoclonal antibody (Sigma) and anti-myc monoclonal antibody (Invitrogen) were used for immunodetection.

Cell fractionation

Cos-1 cells transfected with Bpag1 N-terminal constructs were extracted with either a 0.1 M NaOH solution, or an 8 M urea solution, following the protocol of Lin et al. ([23]). Cells from each well of a 6 well plate were scraped into 1 mL of either the NaOH or urea solutions, and two wells for each transfected plasmid were combined. The cells were triturated 20 – 30 times with a yellow pipet tip, and were centrifuged at 100,000 X g at 4°C (for the NaOH extraction) or 18°C (for the urea extraction) for one hour. Following removal of the supernatant from the membrane pellet, proteins in the supernatant were precipitated with trichloroacetic acid. The membrane pellet and the precipitated supernatant proteins were solubilized in equal amounts of SDS loading buffer, and resolved by SDS-PAGE with 10% gels. Proteins transferred to PVDF membranes were immunoblotted with anti-FLAG and anti-myc antibodies, as above, as well as with antibodies to detect emerin (H-12 monoclonal; Santa Cruz) and p35 (C-19 polyclonal; Santa Cruz). The PVDF membranes were stripped between probing with each antibody by incubating the blots at 50°C in a solution of 2% SDS, 62.5 mM Tris (pH 6.8), and 7% β-mercaptoethanol for 15 – 20 minutes.

Immunocytochemistry

Subcellular localization studies were performed by indirect immunofluorescence labelling. Cells were fixed in 4% paraformaldehyde, and blocked and permeabilized in 10% goat serum with 0.4% Triton X-100 in PBS. Antibodies were incubated in a solution of 3% bovine serum albumin with 0.04% Triton X-100 in PBS. For permeabilization with

digitonin, cells were treated as in Zhen et al. ([19]), with no Triton X-100 used in any of the steps. Antibodies used were anti-tubulin monoclonal antibody (clone AB-1; Oncogene), anti-GM130 monoclonal antibody (clone 35; BD Transduction), anti-58K Golgi marker (clone 58K-9; Novus), anti-FLAG polyclonal or monoclonal (M5) antibodies (Sigma), anti-emerin monoclonal antibody, and anti-myc monoclonal antibody. The anti-Bpag1 isoform 2 polyclonal antibody has been described previously (anti-A' antibody; [30]). Rhodamine-phalloidin (Molecular Probes) was used to label F-actin and Hoechst 33342 (Sigma) was used to label nuclei. Appropriate secondary antibodies conjugated with Alexa Fluor 488 (Molecular Probes), Cy3, or Cy5 (Jackson ImmunoResearch) were used for fluorescent labelling.

Microscopy was performed primarily with conventional epifluorescence illumination on a Zeiss Axioplan with the 63X Plan-Apochromat objective, and images were captured and processed using Axiovision software. Extended focus imaging was used for some images (where noted). Figures were assembled with Adobe Photoshop and Adobe Illustrator. Where quantifications of transfected cells are given, at least 150 cells from a minimum of two separate transfections were counted. For comparisons between Triton X-100 and digitonin permeabilized cells, cells were counted from ten random fields at low magnification (20X), imaged from two separate transfections for each group of cells, with exposure times and all other conditions kept constant. Higher magnification pictures comparing these two treatments were also performed with constant exposure times and conditions for comparison between each group.

Results

The Bpag1 isoform 2 N-terminus contains a transmembrane domain, and targets to the perinuclear region

Initially, two alternate N-termini were described for non-epithelial Bpag1 [6]. The isoform 1 N-terminal unique sequence was shown to be encoded by a single exon that was spliced into downstream exons encoding the actin binding domain (ABD). In contrast, the isoform 2 N-terminal unique sequence was shown to be encoded by four exons 5' to the ABD encoding region. However, the start site for the isoform 2 transcript was not described. With the availability of new mouse genomic sequence information from the NCBI (Map Viewer data for the *Dst* gene), and our 5' RACE analysis to confirm the predicted 5' end (Fig. 1A and B), we are now able to report that the start site for Bpag1 isoform 2 transcript lies only a few nucleotides upstream from the beginning of the originally published sequence. This novel DNA sequence has helped to correct the original view of the initial exon sequence (Fig. 1B; new sequence deposited as GenBank accession #DQ023311). Additional RT-PCR analysis also confirmed that the 5' end of the Bpag1 isoform 2 transcript is an alternate end for the Bpag1a transcript previously described in Leung et al. ([8]) (Fig. 1B).

Alignment of the amino acid sequence translated from the newly identified start site indicated a high degree of conservation with sequences from other vertebrate species, including rat, human, and zebrafish, identified using the TBLASTN program (www.ncbi.nlm.nih.gov/BLAST/) (Fig. 1C). Analysis using hidden Markov model prediction indicated that the transcript encodes a putative transmembrane (TM) domain (Fig. 1D; using the TMHMM Server v. 2.0). Analysis of this sequence by TMpred also

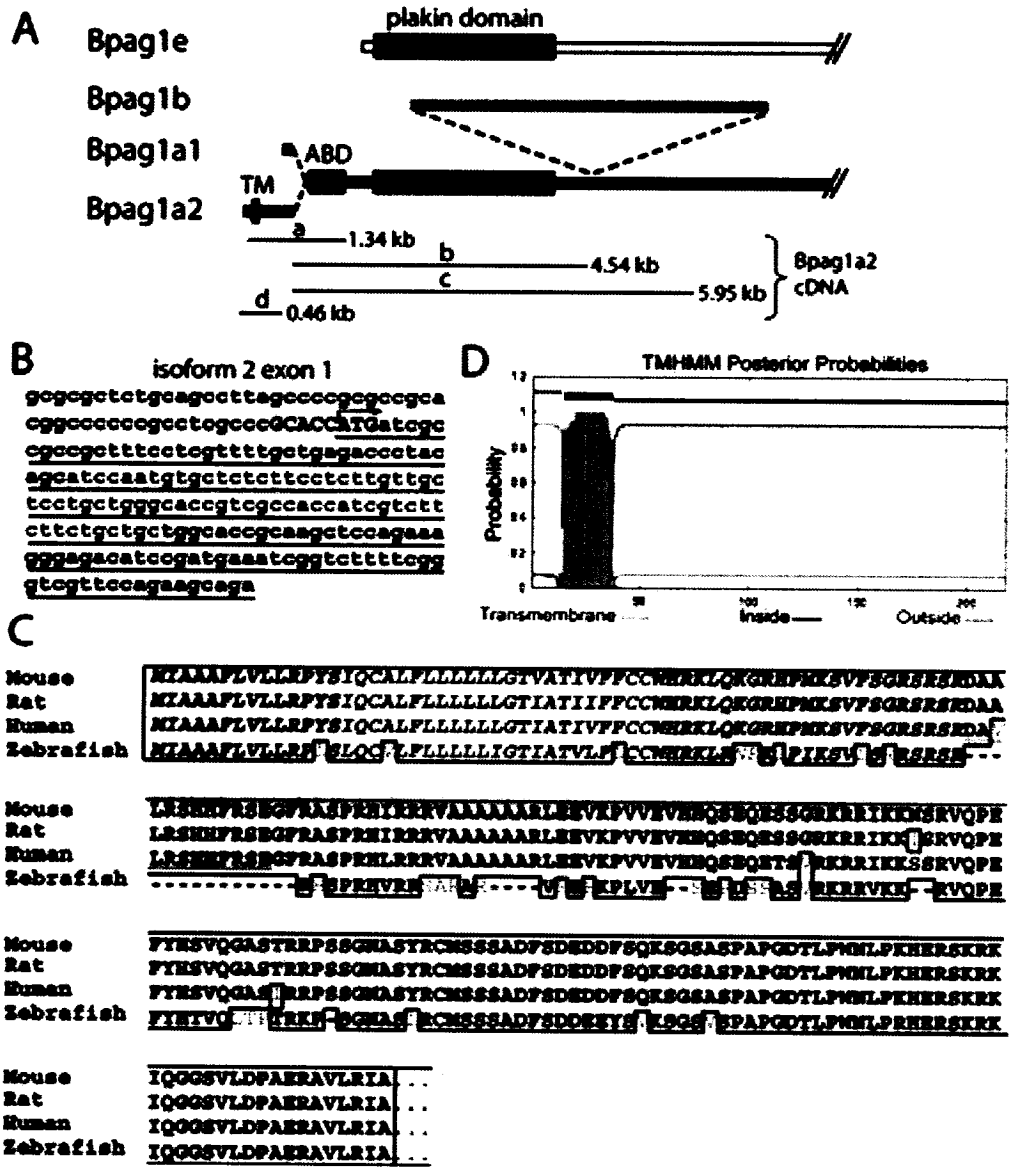

Figure 1. The Bpag1 isoform 2 N-terminus contains a putative transmembrane domain **A,** Schematic representations of the major Bpag1 mRNA transcript variants in neurons and muscle (Bpag1a/b), and skin (Bpag1e). Coding regions for the plakin domain, actin binding domain (ABD), and a transmembrane domain (TM) are indicated. Bpag1b is identical to Bpag1a, with the addition of an ~6 kb region downstream from the plakin

domain coding region. RT-PCR products (labelled a – d) were generated from C2C12 myoblast and mouse brain mRNA corresponding to a Bpag1a variant with the isoform 2 5' end (Bpag1a2). An additional larger expected product from C2C12 myoblasts (~12 kb) using the primer set for fragment 'c', corresponding to a Bpag1b isoform 2, was not observed, possibly due to the difficulty in amplifying such a large fragment. Fragment 'd' was generated using 5' RACE amplification. **B,** Novel exon 1 sequence for Bpag1 isoform 2. The Kozak consensus translation start site is shown in capitalized letters, and the initiating ATG is marked with an arrow. The open reading frame is underlined, and sequence coding for a predicted transmembrane region is indicated in red. Sequence from the longest brain-derived 5' RACE product is shown. Multiple clones derived from C2C12 cell RNA started 7 nucleotides downstream. **C,** Alignment of the translated Bpag1 isoform 2 N-terminal unique region. Predicted sequences from rat, human, and zebrafish, identified using the TBLASTN program, show a high degree of conservation with the mouse sequence. Similar and identical amino acid residues, compared to the mouse sequence, are boxed. Human sequence that was additionally corrected with genomic information from GenBank accession #CAI12111 is underlined with a grey line. The predicted TM domain is indicated in red. **D,** Prediction of the N-terminal TM sequence using hidden Markov modeling. The probability at the core of the predicted sequence for being transmembrane is 100%. The line between 1 and 1.2 on the y axis indicates the overall prediction.

strongly predicts it to be a transmembrane domain (data not shown). The prediction score by TMHMM analysis was very high, up to 1 (which equals 100% probability) for the core of the predicted transmembrane region. For comparison, prediction of the previously demonstrated transmembrane region from Syne [18] is < 0.6 at the core of the transmembrane sequence.

Immunoblot analysis of protein extracts from Cos-1 cells transfected with Bpag1 N-terminal plasmids including the ABD region with or without the N-terminal TM region indicated appropriately sized fusion proteins were expressed, and that addition of the TM region lead to a consistent reduction in expression levels (Fig. 2A, B). Immunofluorescence analysis of these fusion proteins revealed that inclusion of the TM region directs the protein to the perinuclear area of Cos-1 cells, including staining immediately surrounding and extending several microns from the nucleus (Fig. 2C, E). This was true whether the tag was at the N-terminal (FLAG-Nterm2f) or C-terminal (Nterm2f-myc/his) end of the fusion protein (compare with Fig. 2I, K). Exclusion of the TM region from the fusion protein (FLAG-Nterm2ΔTM) resulted in loss of the perinuclear localization (Fig. 2F-H). Instead, this protein associated with actin microfilaments throughout the cytoplasm of the cell. Thus, the N-terminus of Bpag1 isoform 2 targets the protein to the perinuclear region of the cell.

To determine if the N-terminal TM region was involved in inserting into membranous structures around the nucleus, we performed two types of experiments. First, we used fractionation of cells extracted with either 0.1 M NaOH or 8 M urea. Following centrifugation at 100,000 X g, both the FLAG-Nterm2f and Nterm2f-myc/his fusion proteins fractionated with the membrane pellets (Fig. 3A). As controls,

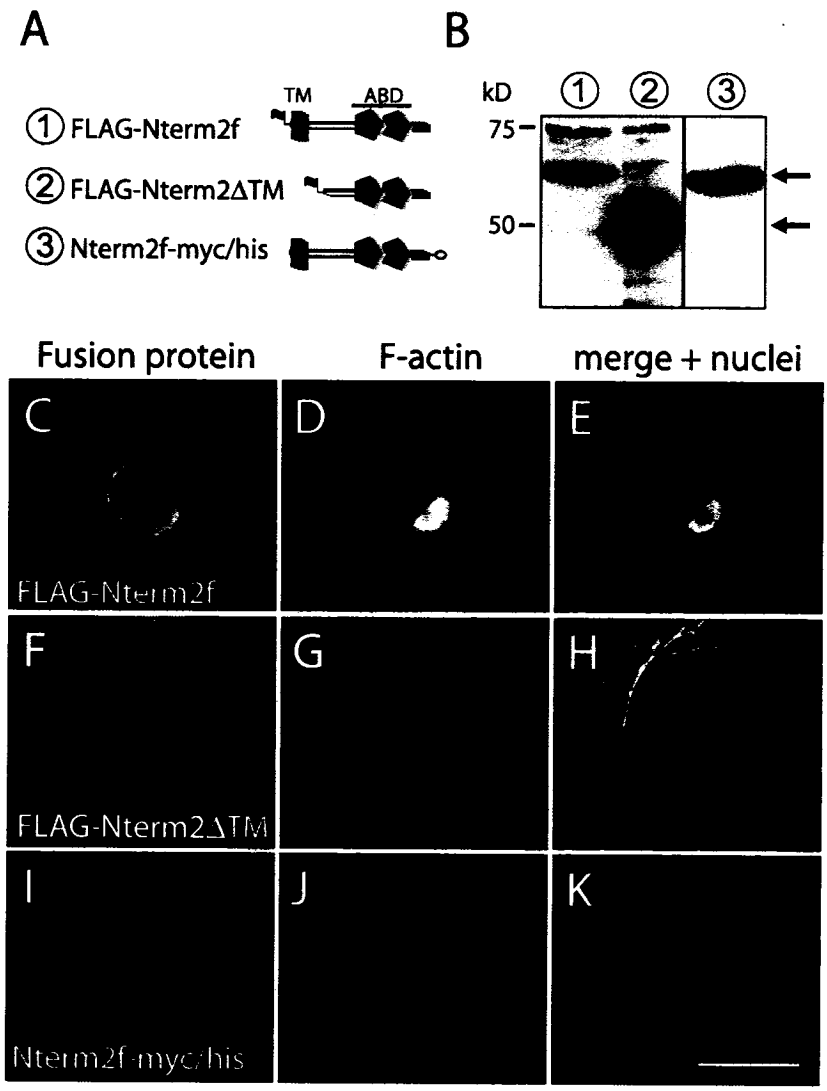

Figure 2. The Bpag1 isoform 2 N-terminus targets to the region surrounding the nucleus. **A,** Schematics of the Bpag1 fusion proteins used to observe the effect of the addition of the N-terminal region on Bpag1 localization. The FLAG-Nterm2ΔTM fusion protein is missing amino acids 1 – 125 of the sequence shown in Fig. 1B. **B,** Western blot analysis of Cos-1 lysates indicating that the appropriate sized proteins were being produced. Both the FLAG-Nterm2f and Nterm2f-myc/his fusion proteins had lower expression compared to FLAG-Nterm2ΔTM. The FLAG-Nterm2ΔTM lysate was diluted by half with loading buffer for this blot. The FLAG- and myc-tagged proteins were probed separately with the

appropriate antibodies. Weaker bands in lanes 1 and 2 were from non-specific labelling, and some minor degradation products in lane 2. **C – E,** FLAG-Nterm2f protein localized in a ring around the nucleus, typically extending out on one side. FLAG-Nterm2f also colocalized with accumulated F-actin. F-actin staining surrounding the nucleus was very bright in fusion protein expressing cells, and more peripheral F-actin staining and staining in non-expressing cells was underexposed in panels D and J. **F – H,** FLAG-Nterm2ΔTM protein never accumulated around the nucleus. Instead, it associated with actin stress fibres in lower expressing cells throughout the cytoplasm (shown), and a meshwork of bundled F-actin fibres in higher expressing cells (not shown). **I – K,** Moving the tag to the C-terminal end had little effect on localization and actin associating properties of the Nterm2f fusion protein. Bar = 20 μm in K, and is the same for all panels.

emerin, an integral inner nuclear membrane protein, and p35, a protein which can associate with membranes but which is not an integral membrane protein, were detected on the same membranes probed for the FLAG and myc fusion proteins. Emerin was observed only in the pellet fractions, and p35 was observed only in the supernatant fractions of NaOH extracted cells, with only minor contamination of p35 in the pellets of urea extracted cells (Fig. 3A). This indicates that the Bpag1 isoform 2 N-terminal fusions behave as an integral membrane protein.

As a second method to demonstrate that the Bpag1 N-terminus is inserted into membrane, and to demonstrate the orientation of membrane insertion, we used selective permeabilization of transfected cells with digitonin. Digitonin at low concentrations permeabilizes the plasma membrane, while leaving internal membranes intact [19, 32]. In cells expressing the C-terminal tagged Nterm2f-myc/his fusion protein, permeabilization with either Triton X-100 or with digitonin made no difference in the level of signal observed by immunofluorescence (Fig. 3C, E) indicating that the epitope tag is exposed to the cytoplasm and not luminal. In contrast, a much reduced level of signal was observed in FLAG-Nterm2f expressing cells permeabilized with digitonin, compared to those permeabilized with Triton X-100 (Fig. 3B, D, F, G) indicating that the N-terminal tag is on the luminal side of membranous structures. Overall, fewer stained cells were observed in the digitonin permeabilized group (230 total), versus the Triton X-100 permeabilized group (390 total) over twenty random fields from two separate transfections of FLAG-Nterm2f into Cos-1 cells. Furthermore, there was a generally much weaker signal in those cells of the digitonin permeabilized group which were stained. This was demonstrated by an inability to detect most FLAG-Nterm2f expressing

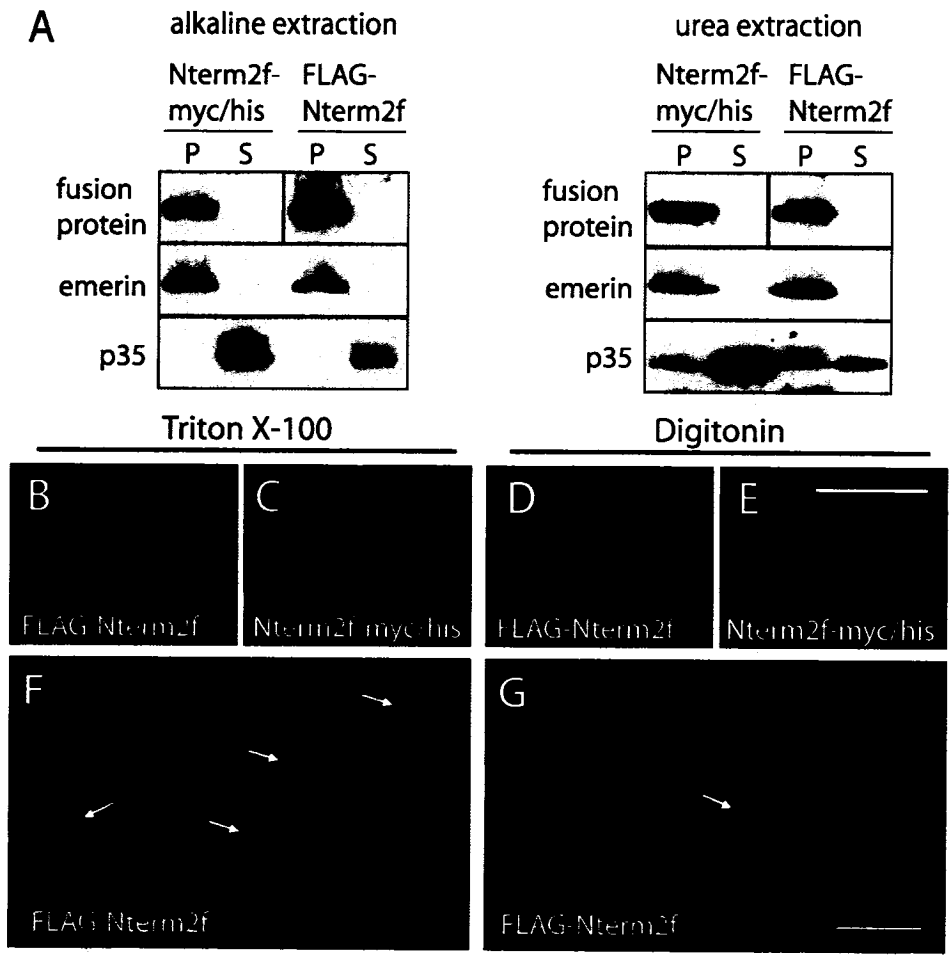

Figure 3. The Bpag1 isoform 2 N-terminus is inserted into membranes surrounding the nucleus. **A,** Cell fractionation following extraction of transfected Cos-1 cells with either 0.1 M NaOH (alkaline extraction) or 8 M urea. Both the Nterm2f-myc/his fusion protein and the FLAG-Nterm2f fusion protein were found in the pellet (P) following centrifugation at 100,000 X g. Emerin and p35 labelling served as controls for the pellet and supernatant (S) fractions, and were probed on the same blots as the fusion proteins following stripping. **B and C,** Detection of the N- and C-terminal tags of Bpag1 N-terminus fusion proteins following Triton X-100 permeabilization. **D,** Imaging of FLAG-Nterm2f fusion protein expressing cells following digitonin (40 μg/mL) permeabilization using identical exposures (20 milliseconds) to Triton X-100 permeabilized cells indicated

reduced levels of immunolabelling. Shown is a representative cell, with barely detectable expression at this exposure. **E,** No difference in signal was noted with digitonin permeabilized cells expressing the Nterm2f-myc/his fusion protein. **F and G,** A comparison of FLAG-Nterm2f expressing cells at lower magnification following either Triton X-100 (F) or digitonin (G) permeabilization. Arrows indicate the cells detected at this exposure (25 milliseconds for each). Most of the digitonin permeabilized cells were detectable only with much longer exposures. Note, no brightness or contrast adjustments were made during any step of image processing for the fusion protein signal in any of these images. Bar = 20 μm in E, and is applicable to B – D; bar = 50 μm in G, and is the same for F.

cells following digitonin permeabilization with exposure times where Triton X-100 permeabilized cells were readily visible (Fig. 3F, G). The limited staining which remained in the digitonin permeabilized cells expressing FLAG-Nterm2f may have been due to high expression of the fusion protein, and an incomplete targeting of all of it. These results indicate that the N-terminal end of the Bpag1 isoform 2 protein gets inserted into membranous structures around the nucleus, and that the C-terminal end extends into the cytoplasm.

Endogenous Bpag1 isoform 2 localization in the perinuclear region of myoblast cells

Immunostaining of endogenous Bpag1 isoform 2 protein in C2C12 myoblasts demonstrated localization consistent with the targeting of the N-terminal fusion proteins. In addition to nuclear and actin stress fibre-associated staining, anti-Bpag1 isoform 2 antibody detected protein surrounding the nucleus, with strong signal occasionally localized to one side of the nucleus [30]. Strong Bpag1 isoform 2 staining on one side of the nucleus was observed to surround the Golgi apparatus (Fig. 4A, B and E – H). Better co-localization with the GM58K Golgi marker compared to the GM130 Golgi marker was noted, though the GM58K Golgi marker stained protein in a more diffuse pattern. Perinuclear Bpag1 staining did not colocalize with strong F-actin staining detected by phalloidin, though there was phalloidin staining in this region, typically localized in a punctate pattern (Fig. 4H and see [30]). More extensive actin filament staining around the Golgi and nucleus has been described using an anti-actin antibody that preferentially detects F-actin, suggesting that phalloidin staining may under represent the F-actin in this region [33-35]. Perinuclear staining consistent with nuclear envelope or endoplasmic

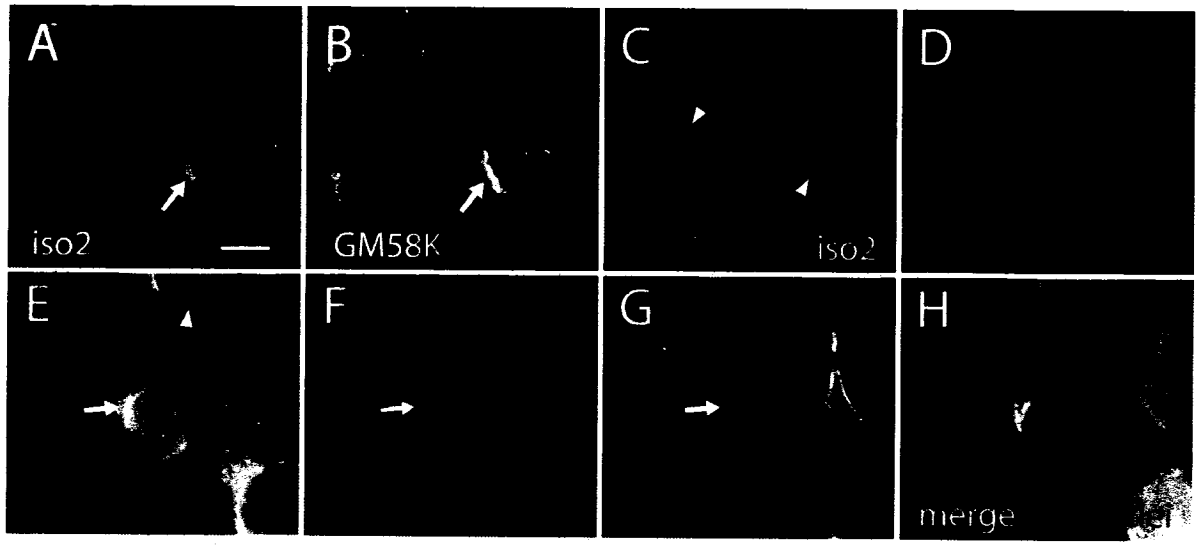

Figure 4. Perinuclear localization of endogenous Bpag1 isoform 2. **A and B,** C2C12 cells labelled with an anti-Bpag1 isoform 2 antibody (iso2) and anti-GM58K to label the Golgi. The arrow points to stronger iso2 staining co-localizing with Golgi. **C and D,** Other cells displayed a more obvious localization of iso2 staining surrounding the nuclear envelope (arrowheads). **E – H,** Co-localization of iso2 staining with anti-Golgi marker GM130 and F-actin (arrows). Only weak F-actin staining was detected overlapping with the Golgi, though actin filaments are known to regulate Golgi organization. The arrowhead in E indicates iso2 staining surrounding the nucleus. Bar in A = 10 μm, and is the same for all panels.

reticulum localization was also observed in most cells (Fig. 4C – E, H). Though a similar pattern of staining close to the nuclear envelope was noted consistently, the intensity of the staining in each specific region varied considerably from cell to cell.

Full-length Bpag1a2 reorganizes the cytoskeleton and requires the transmembrane region to target the perinuclear region of the cell

Full length Bpag1-encoding expression plasmids were used to observe the effect of the N-terminal TM region on localization in the context of the whole protein. As well, these fusion proteins allowed us to compare the distinct properties of Bpag1 protein with an isoform 1 N-terminus or an isoform 2 N-terminus. The overall structure of these fusion proteins is shown in Fig. 5A. These proteins are predicted to be 620 – 640 kD cytolinkers, interacting with actin filaments and microtubules. Though expression levels for the isoform 1 (FLAG-Bpag1a1) and isoform 2 (FLAG-Bpag1a2) fusion proteins were very low in transient transfections, and we were unable to stably transfect cells with these constructs, higher expression of the FLAG-Bpag1a2ΔTM protein allowed us to demonstrate by immunoblot the production of a high molecular weight protein (Fig. 5B). As well, each of these fusion proteins exhibited the ability to associate with actin microfilaments and microtubules (see below), indicating appropriate expression considering that the actin binding and microtubule binding domains are located near the N- and C-termini, respectively.

Analysis of the FLAG-Bpag1a2 protein in Cos-1 cells indicated that it was primarily associated with filamentous actin (F-actin) surrounding the nucleus. FLAG-Bpag1a2 accumulated predominantly to one side of the nucleus in 30% of Cos-1 cells. In

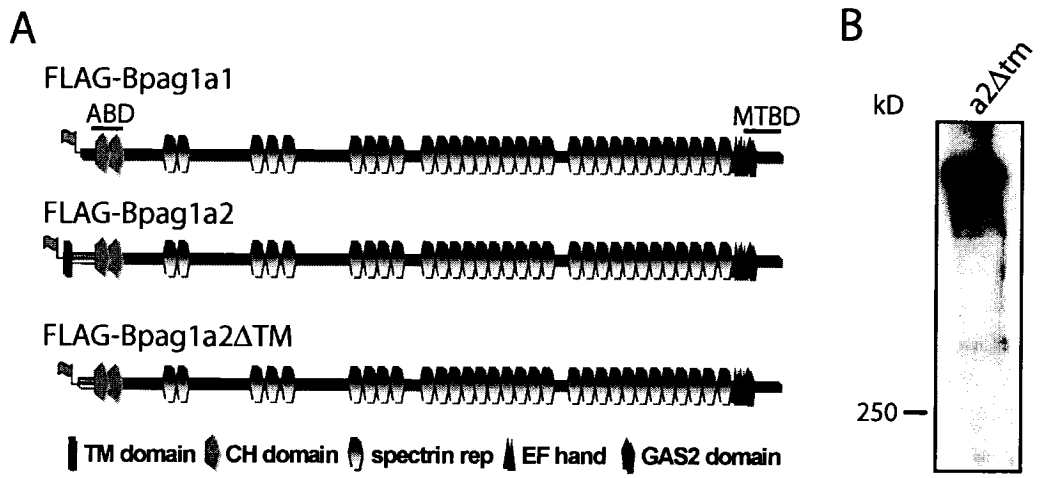

Figure 5. Full-length Bpag1 fusion proteins. **A,** Overlapping cDNAs were used to construct expression plasmids coding for full-length Bpag1a1 (FLAG-Bpag1a1), Bpag1a2 (FLAG-Bpag1a2), or Bpag1a2 lacking the TM domain (FLAG-Bpag1a2ΔTM). The positions of the actin binding domain (ABD) and microtubule binding domain (MTBD) are noted. All other domains shown are those identified by SMART analysis (smart.embl-heidelberg.de/). **B,** Immunoblot of the FLAG-Bpag1a2ΔTM protein indicated the generation of a high molecular weight fusion protein (predicted = 625 kD).

the remaining cells, the majority of the staining was either surrounding the whole nucleus in close association with it, or was distributed throughout the cell with higher expression of the fusion protein (Fig. 6; further examples of perinuclear and nuclear staining are given in Supplemental Fig. 1). The perinuclear FLAG-Bpag1a2 colocalized with accumulated actin microfilaments, indicated by staining for F-actin with phalloidin (Fig. 6 B, C). Perinuclear FLAG-Bpag1a2 exhibited a close association with the nuclear envelope, and an association with the Golgi apparatus. In cells with localization of this fusion protein closely surrounding the nucleus, most of the staining was in close proximity to the nuclear envelope (Fig. 6D – F), except where it spread out on the side of the nucleus where the Golgi apparatus was situated. In cells with a predominant localization of the fusion protein on one side of the nucleus, the fusion protein surrounded the Golgi apparatus (Fig. 6 G – I). The pattern of staining never indicated a complete co-localization with the GM130 Golgi marker, but rather that the FLAG-Bpag1a2 mainly surrounded it, with only partial overlap.

To examine if the FLAG-Bpag1a2 fusion protein could associate with both actin filaments and microtubules, we labelled cells using rhodamine-phalloidin and anti-tubulin antibody, along with detection of the fusion protein. In cells where FLAG-Bpag1a2 was localized close to the nucleus, no association with tubulin was noted (Fig. 6J – L). In fact, tubulin appeared to be excluded from this region of the cell, in comparison to the strongly accumulated F-actin. In a minority of the cells, FLAG-Bpag1a2 localized more extensively throughout the cytoplasm (Fig. 6M). In this case the fusion protein did associate with tubulin, associating with co-aligning microtubules and actin filaments (Fig.

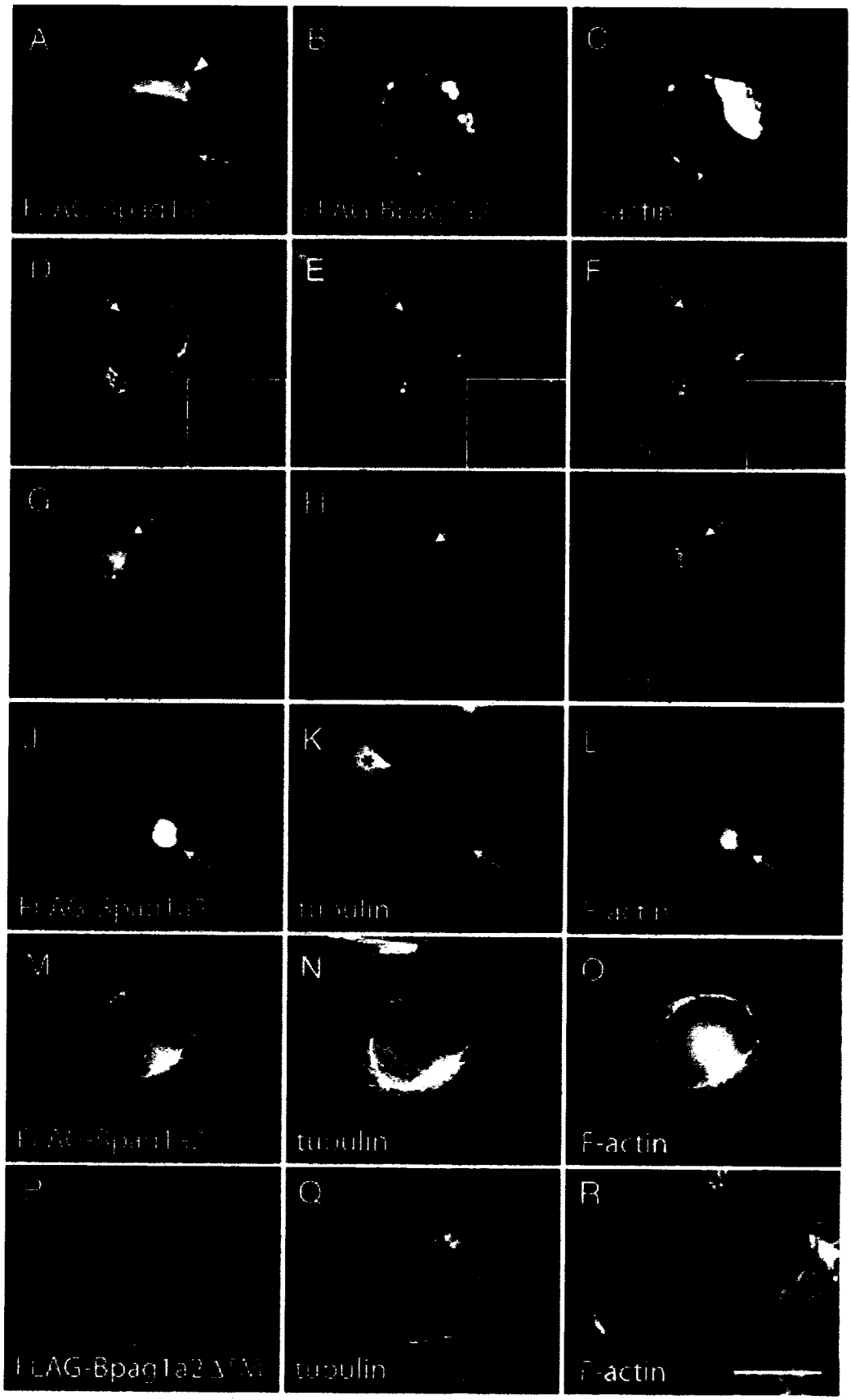
A
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Figure 6. Bpag1a2 is a cytoskeletal-associated protein that concentrates in the perinuclear region of the cell. **A,** FLAG-Bpag1a2 surrounding the nucleus of a Cos-1 cell. The arrow indicates staining in close proximity to the nuclear envelope, and the arrowhead indicates staining which extended further out from the nucleus, almost always occurring in the region where the Golgi were located. **B and C**, FLAG-Bpag1a2 surrounding the nucleus (B) was always found in association with accumulated F-actin (C). **D – F**, Double-labelling with an emerin antibody (E) demonstrated the close proximity of FLAG-Bpag1a2 (D) with the nuclear envelope. Insets are enlargements of the region of the nucleus indicated by the arrow. Panel F is a merge of D and E. **G – I**, Double-labelling with the anti-GM130 Golgi marker (arrow in H) demonstrated the proximity of the FLAG-Bpag1a2 (arrow in G) with the Golgi. Panel I is the merged image with nuclear staining included. Note, the FLAG-Bpag1a2 surrounds the GM130 staining, but only overlaps partially. **J – K**, While the FLAG-Bpag1a2 (J) colocalized with F-actin (L) surrounding and adjacent to the nuclei, tubulin was not colocalized with this fusion protein when it was in close proximity to the nucleus, and appeared to even be excluded from the region (arrow in K). The asterisk in K indicates the microtubule organizing center of an adjacent cell. **M – O**, In a few cells, FLAG-Bpag1a2 staining (M) localized throughout the cell, and was found in association with both microtubules (N) and actin filaments (O). **P – R**, Without the N-terminal TM domain present, the FLAG-Bpag1a2ΔTM fusion protein (P) was never observed to localize discretely around the nucleus. Instead, it almost always localized in a cytoskeletal-associated pattern, co-aligning with a meshwork of microtubules (Q) and actin filaments (R). Bar in R = 20 μm, and is the same for all other panels except G – I, where the bar = 10 μm.

6N, O), indicating that FLAG-Bpag1a2 was capable of an association with both types of cytoskeleton.

When the isoform 2 fusion protein was expressed without the N-terminal TM region (FLAG-Bpag1a2ΔTM), there was never a discrete perinuclear localization of the fusion protein (Fig. 6P – R). The FLAG-Bpag1a2ΔTM fusion protein localized in a cytoskeletal-associated pattern in the majority of Cos cells (99%). This truncated fusion protein was highly expressed and, as may be expected for a cytoskeletal bundling/crosslinking protein, caused the formation of an elaborate meshwork of filaments throughout the cytoplasm of the Cos-1 cells. Thus, the presence of the N-terminal TM domain was required for a restricted perinuclear localization of the Bpag1 protein.

Full-length Bpag1a1 associates with microtubules and actin filaments throughout the cytoplasm and at the plasma membrane

In comparison to FLAG-Bpag1a2, FLAG-Bpag1a1 was consistently found throughout the cytoplasm and at the cell surface, localizing in a pattern consistent predominantly with microtubule-association, and to a lesser extent with actin microfilament-association (Fig. 7A – C). In cells where FLAG-Bpag1a1 localized predominantly to microtubules, the microtubules appeared thicker compared to untransfected cells, indicating bundling by Bpag1a1 (Fig. 7A – C). This is consistent with a previous description of the Bpag1 C-terminus function [31], and was also observed in cells expressing FLAG-Bpag1a2 (see above). As well, varying degrees of actin filament co-alignment with these bundled

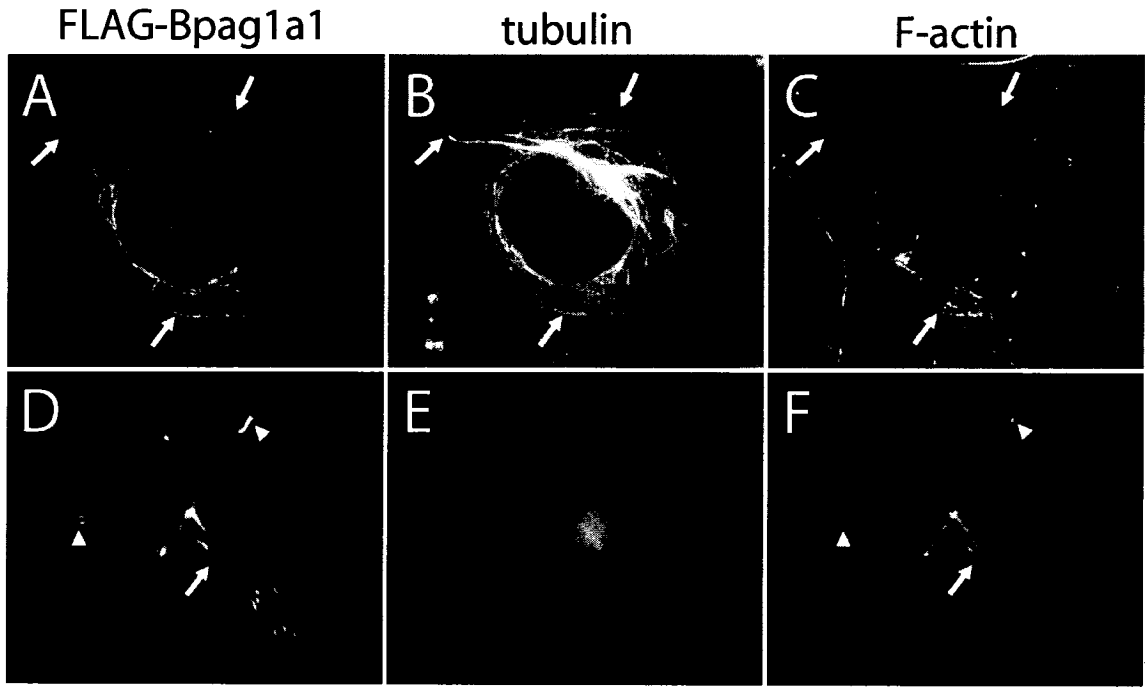

Figure 7. Bpag1 isoform 1 associates with microtubules and actin filaments throughout the cytoplasm in Cos-1 cells. **A – C,** The FLAG-Bpag1a1 fusion protein predominantly displayed a localization pattern associated with microtubules (arrows). In some cells exhibiting microtubule-associated FLAG-Bpag1a1, actin filaments were observed to co-align (arrows in C). Images were generated with extended focus imaging. **D – F,** The FLAG-Bpag1a1 fusion protein also localized to actin stress fibres (arrows) in approximately one third of the expressing cells. Strong localization at the ends of the stress fibres, the focal contact sites (arrowheads) was also noted. Microtubules are out of focus in this focal plane, close to the substratum. Bar in F = 20 μm, and is the same for all panels.

microtubules was observed, suggesting the possibility that FLAG-Bpag1a1 was cross-linking actin filaments to the microtubules (Fig. 7A – C and Supplemental Fig. 3). In approximately one third of the FLAG-Bpag1a1 expressing cells, the fusion protein predominantly localized to actin stress fibres (Fig. 7D – F). Co-aligning microtubules were not observed in this case. Interestingly, while the Bpag1 isoform 2 protein is excluded from focal contacts at the ends of the stress fibres ([30] and unpublished data), FLAG-Bpag1a1 showed clear localization to focal contact sites (arrowheads in Fig. 7D, F).

Perinuclear and nuclear FLAG-Bpag1a2 in C2C12 cells

As previously observed with endogenous Bpag1 isoform 2 protein, FLAG-Bpag1a2 was capable of co-aligning with actin stress fibres in C2C12 cells, though only a small minority of the cells displayed localization of this fusion protein along stress fibres (~1%; Fig. 8O, P). Many C2C12 cells with high expression also had a more diffuse localization of the protein throughout the cell. In the majority of cells expressing the FLAG-Bpag1a2 protein, the fusion protein was targeted to the perinuclear region, including the area surrounding the Golgi apparatus (Fig. 8A – N).

In 14% of the cells, FLAG-Bpag1a2 localized predominantly on one side of the nucleus, surrounding the Golgi apparatus where it colocalized with accumulated actin microfilaments (Fig. 8A – D). Treatment with brefeldin A, which causes a dispersal of the Golgi, did not cause a noticeable redistribution of the FLAG-Bpag1a2 (Fig. 8E – H). A similar number of FLAG-Bpag1a2 expressing cells (13%) still had the fusion protein colocalized with microfilaments on one side of the nucleus. Thus, while this fusion

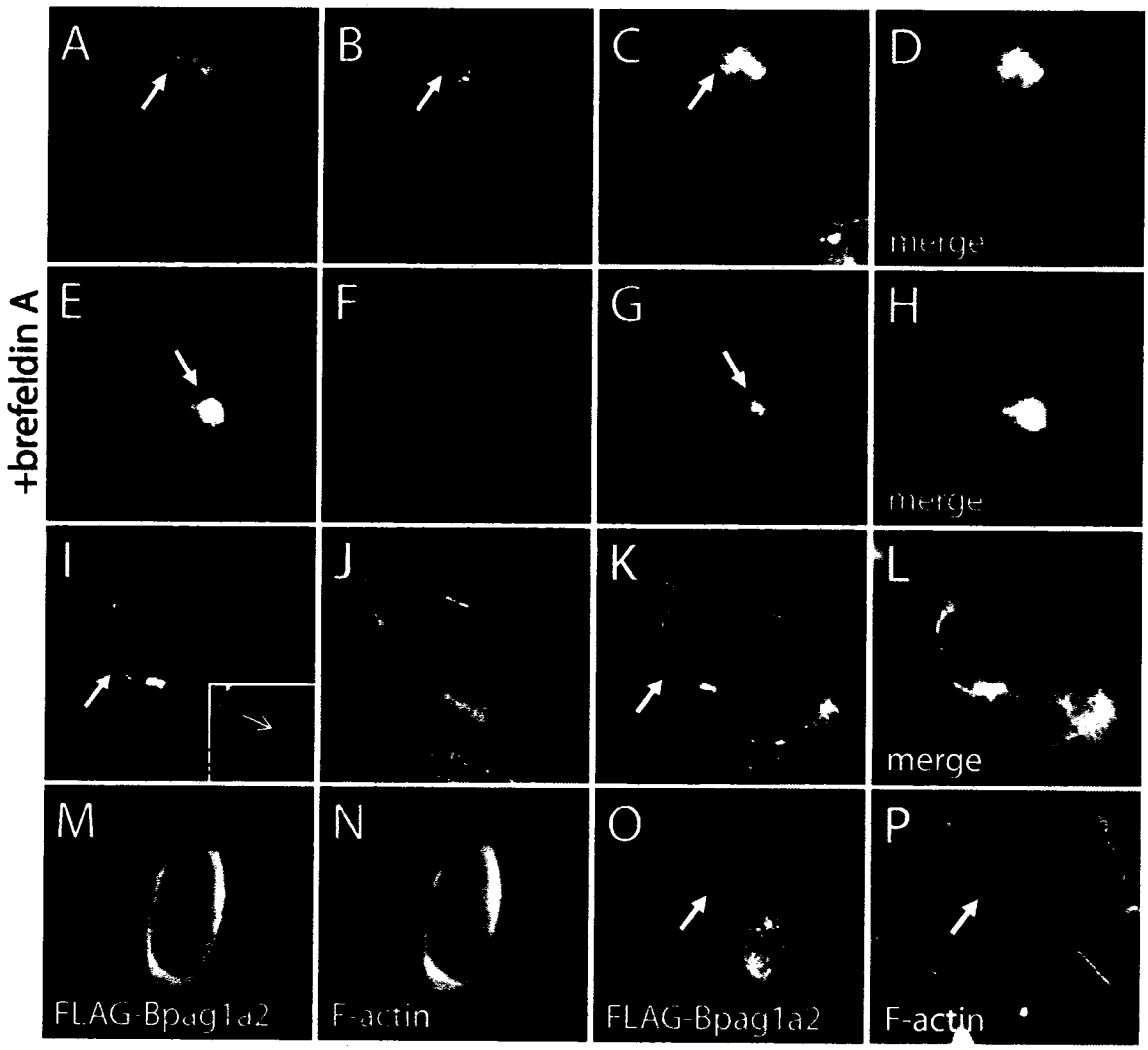

Figure 8. Perinuclear FLAG-Bpag1a2 accumulates actin microfilaments around the Golgi and nucleus in C2C12 myoblast cells. **A – D,** FLAG-Bpag1a2 colocalized with F-actin surrounding GM130 Golgi marker staining (arrows). In the merged image in D, FLAG staining is green, GM130 is purple, F-actin is red, and nuclei are blue. This is the same for H. **E – H,** When C2C12 cells were treated with brefeldin A, GM130 staining was dispersed and very weak, but FLAG-Bpag1a2 associated with F-actin could still be observed in a focal spot adjacent the nucleus (arrows in E and G) in a similar number of cells. **I – L,** FLAG-Bpag1a2 staining surrounding the nucleus (arrow in I) was also associated with an accumulation of F-actin (arrow in K), but not tubulin (J). The inset in I

shows a nuclear focal spot (open arrow) formed by FLAG-Bpag1a2. The C2C12 cells typically contained one to five of these spots. **M and N,** Flag-Bpag1a2 surrounding the nucleus associated with accumulated F-actin .**O and P,** Flag-Bpag1a2 co-aligning with actin stress fibres in some cells. Bar in P = 10 μm, and is the same for all panels.

protein associated around the Golgi apparatus, its localization was not dependant upon the Golgi.

FLAG-Bpag1a2 which localized close to the nuclear envelope always colocalized with accumulated microfilaments, but not with microtubules (Fig. 8I – L), similar to what was observed in Cos-1 cells. The ability of FLAG-Bpag1a2 to influence nuclear envelope architecture was indicated by the anecdotal observation that this protein could be associated with nuclear invaginations (Supplemental Fig. 3). Inside the nucleus, small FLAG positive foci associated with nucleoli were frequently observed (open arrow in Fig. 8I inset; Supplemental Fig. 2). Thus, localization of FLAG-Bpag1a2 in C2C12 cells was consistent with this protein playing a role in nuclear envelope and intranuclear organization.

Discussion

Attachment of organelles to the cytoskeleton is critical for maintaining proper organization in the cell and for proper organelle function (reviewed in [36-38]). Along with neurofilament aggregation in axons, a hallmark of several neurodegenerative diseases, improper placement of the nucleus in affected neurons was noted early on as a characteristic pathological feature in Bpag1 deficient mice [27-29]. Here, we show that transcript for an isoform of Bpag1 codes for a highly conserved membrane protein that is involved in cytoskeletal organization surrounding the nucleus. We demonstrate that a Bpag1 isoform 2 N-terminal sequence containing a previously undescribed transmembrane region is responsible for targeting the protein to the perinuclear region of the cell. Endogenous Bpag1 isoform 2 protein in myoblasts demonstrated strong

perinuclear localization in many cells, along with intranuclear and actin cytoskeleton localizations (this study and [30]). Overexpression of a full-length Bpag1 isoform 2 protein, which was predominantly perinuclear, resulted in accumulation of microfilaments surrounding the nucleus and Golgi apparatus. This indicates that Bpag1 isoform 2 proteins may be involved in cytoskeletal organization and membrane attachment in the region surrounding the nucleus of cells.

The isoform 2 transmembrane region is necessary for targeting of the Bpag1 protein to membranes surrounding the nucleus

While we had previously presented the observations that Bpag1 could actively transport into the nucleus in a non-epithelial cell type, and that it surrounded the nucleus and was associated with stress fibres adjacent to the nucleus, we had no explanation for the extensive perinuclear localization [30]. Fusion proteins used in this previous study also lacked the N-terminal TM domain. In this study, we have demonstrated that a Bpag1 isoform 2 N-terminal sequence is responsible for perinuclear localization of the Bpag1 N-terminus, and was necessary for the accumulation of full-length fusion protein corresponding to the Bpag1a isoform 2 surrounding the nucleus and Golgi. That the strongly predicted transmembrane domain inserts into membrane was demonstrated by the close association of Bpag1a2 fusion proteins with the nuclear envelope and membranous Golgi stacks, by the demonstration that the N-terminus was pelleted with membrane following alkaline or urea extraction of Cos-1 cells, and by the demonstration that the N-teriminal end was poorly detected by immunofluorescence labelling following selective permeabilization of the plasma membrane with digitonin. This indicates an

orientation of Bpag1a2 with a short N-terminal sequence on the luminal side of organelles, and the remainder of the protein extending out into the cytoplasm. Membrane attachment may include attachment to nuclear envelope, Golgi, and endoplasmic reticulum, though further investigation is necessary to determine where specifically this attachment is.

Though Bpag1 contains a nuclear localization signal and can be found in the nucleus ([30]; Fig. 4 and Supplemental Figs. 1 and 2), a limited intranuclear localization of the FLAG-Bpag1a2 fusion protein was noted in this study. This may have been due to the use of transient transfections for the fusion protein analysis, whereas the protein may have shown a slightly different localization with stable expression. As well, the Bpag1 gene and corresponding transcripts are very large, with multiple alternative splicing patterns having been described in the literature [6, 8, 39] and indicated by multiple alternately spliced EST sequences. The use of one variant representing a Bpag1a protein with the isoform 2 N-terminus is likely not entirely representative of all of the endogenous Bpag1 isoform 2 protein. The C2C12 cells used for immunolabelling of endogenous isoform 2 protein, for instance, contain two predominant Bpag1 transcripts [10], likely corresponding to Bpag1a and Bpag1b proteins, and potentially containing multiple small splicing variations within each. Thus, associations with membrane surrounding the nucleus may include both outer and inner nuclear membrane, though further analysis is required to determine this.

The Golgi apparatus consists largely of stacked membranous enclosures adjacent to the nucleus. The extent of the association of the Bpag1a2 TM domain with Golgi was unclear, however. Neither the FLAG-Bpag1a2 fusion protein nor the endogenous Bpag1

isoform 2 appeared to colocalize completely with the Golgi, as labelled by the GM130 marker, but rather were consistently observed to surround the Golgi. Further, dispersal of the Golgi with brefeldin A did not cause a dispersion of FLAG-Bpag1a2 in cells where it was predominantly localized to one side of the nucleus. Since the strong discrete localization of the Bpag1 isoforms 2 fusion proteins around the Golgi in both Cos-1 and C2C12 cells was not observed with staining of the endogenous protein, the extent of this localization may have been partly an artifact of overexpression. That some C2C12 cells did have discretely localized isoform 2 staining around the Golgi suggests that at least some of this protein is normally targeted there, however.

While the N-terminal TM domain of Bpag1 may not be involved in direct interactions with much of the Golgi apparatus, limited interactions via this domain and other regions of Bpag1 may occur with the Golgi . Bpag1 may also associate with membrane not labelled by the anti-GM130 antibody. In Syne-1, a region in the central coiled-coil part of the protein was demonstrated to target to Golgi [21], whereas its C-terminal transmembrane domain has only been implicated in targeting to the nuclear envelope, and not to the Golgi. A C-terminal region prior to the Gas2/GAR22 homology domain in MACF1, the closest relative to Bpag1, interacts with the Golgi p230 protein [22]. This region is 95% similar (89% identical) to the C-terminal end of Bpag1a and Bpag1b. This sequence was present in our full-length fusion proteins, though evidently cannot target Bpag1 to the Golgi since fusion protein containing this sequence, but lacking the N-terminal TM domain, did not accumulate in the perinuclear region. As well, a region specific to the MACF1b isoform has recently been demonstrated to target to Golgi, and MACF1b has been implicated in Golgi organization [40]. Thus, although

multiple regions in Bpag1 may be involved in Golgi interactions, the N-terminal isoform 2 TM domain was necessary to target the full-length fusion protein used in this study to the region surrounding the Golgi.

Bpag1a2 is likely involved in microfilament organization and microfilament attachment to membranes surrounding the nucleus

Expression of FLAG-Bpag1a2 in Cos-1 and C2C12 cells caused the accumulation of actin microfilaments surrounding the nucleus and the Golgi. F-actin staining colocalizing with the fusion protein was typically very strong relative to F-actin staining in the rest of the cell and in untransfected cells. A growing number of studies indicate that F-actin is important in nuclear positioning and nuclear envelope structuring [19, 41, 42] as well as with organization of the Golgi apparatus [33-35]. As such, Bpag1 may be important in the bundling and attachment of actin filaments that are involved in nuclear and Golgi positioning and organization. FLAG-Bpag1a2 in the perinuclear region did not colocalize with strong microtubule staining, despite the fact that Golgi are located close to the microtubule-organizing centre. This appeared to be due, in part, to an actual exclusion of microtubules from the region where FLAG-Bpag1a2 accumulated around the nucleus. The lack of FLAG-Bpag1a2 accumulation of, or obvious association with, microtubules in this region indicates that either this protein does not use its C-terminal microtubule binding domain (MTBD) when present in the perinuclear region, or that it lacked the C-terminal end, possibly due to proteolytic cleavage. Flag-Bpag1a2 that localized throughout the cytoplasm did associate with microtubules, indicating that full-length protein containing the C-terminal MTBD was being produced. As with Syne proteins

located on the outside of the nucleus, association with actin filaments appeared to be the major cytoskeletal interaction of Bpag1 isoform 2 proteins.

Bpag1 associates with microfilaments and microtubules in an isoform-specific manner

While Bpag1a2 may interact primarily with the actin cytoskeleton in the perinuclear region, FLAG-Bpag1a1 protein localized extensively with microtubules and actin microfilaments throughout the cytoplasm, and at focal contact attachments at the plasma membrane. Given that the Bpag1 ABD directly binds actin [43], and the MTBD associates with and likely binds directly with microtubules [31], it is possible that Bpag1 can serve as a linker protein connecting microfilaments and microtubules. Such a role has previously been demonstrated for MACF1 [44]. However, co-alignment of microtubules with FLAG-Bpag1a1 localized along actin stress fibres was not observed, suggesting that Bpag1a1 protein that associated with actin structures may not use its MTBD, or may be truncated. Truncated N-terminal isoform 1 fusion proteins up to 183 kD (1578 amino acids long) do not co-align with stress fibres, but rather strongly aggregate actin into large foci ([30] and unpublished data). Thus, it is possible that the full-length Bpag1a1, or at least most of the length of it, is required for a normal interaction with actin structures. Focal contact localization of the FLAG-Bpag1a1 protein, but not isoform 2 fusion proteins or endogenous isoform 2 protein [30] indicated that the isoform 2 protein is restricted from actin-association in these plasma membrane structures, possibly to aid in its localization to membranous structures in the perinuclear region.

The multiple localization patterns of individual isoforms of the Bpag1 proteins suggest that these proteins may actively re-locate during different times in the cell cycle,

or to facilitate cellular events requiring architectural reorganization such as cell movement. Alternate isoforms that are expressed in the same or different tissues likely play different roles in cytoskeletal (and possibly nucleoskeletal) organization dependent upon the cell type and context-specific conditions in which the cell is found (e.g. in a migrating cell during development versus a postmitotic adult cell). Currently, we are performing transgenic rescue experiments in mice deficient for non-epithelial Bpag1 isoforms to examine if the isoforms described in this paper can compensate for loss of endogenous Bpag1 function. This should help to elucidate the functional *in vivo* significance of each of these proteins, and explain in more detail how Bpag1 is necessary for the maintenance of neuronal and muscle cytoarchitecture.

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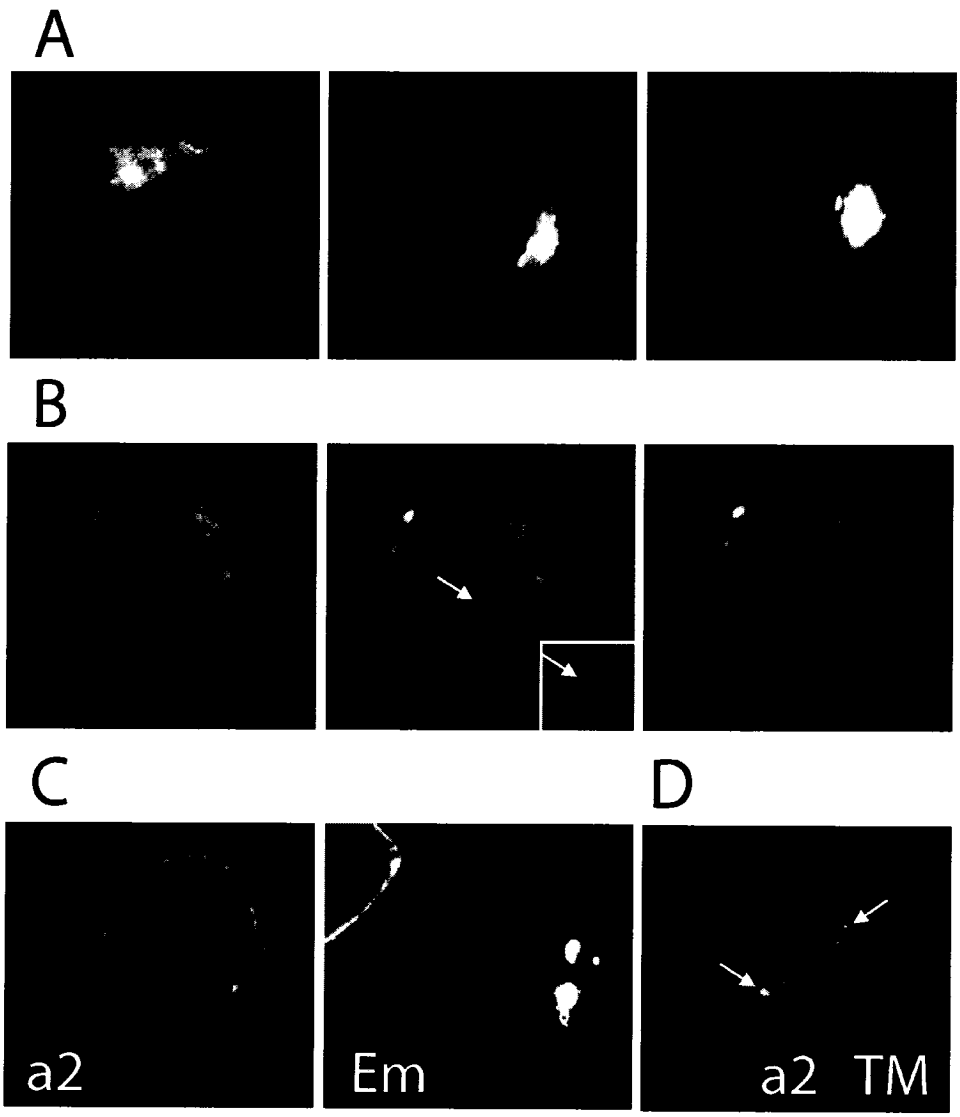
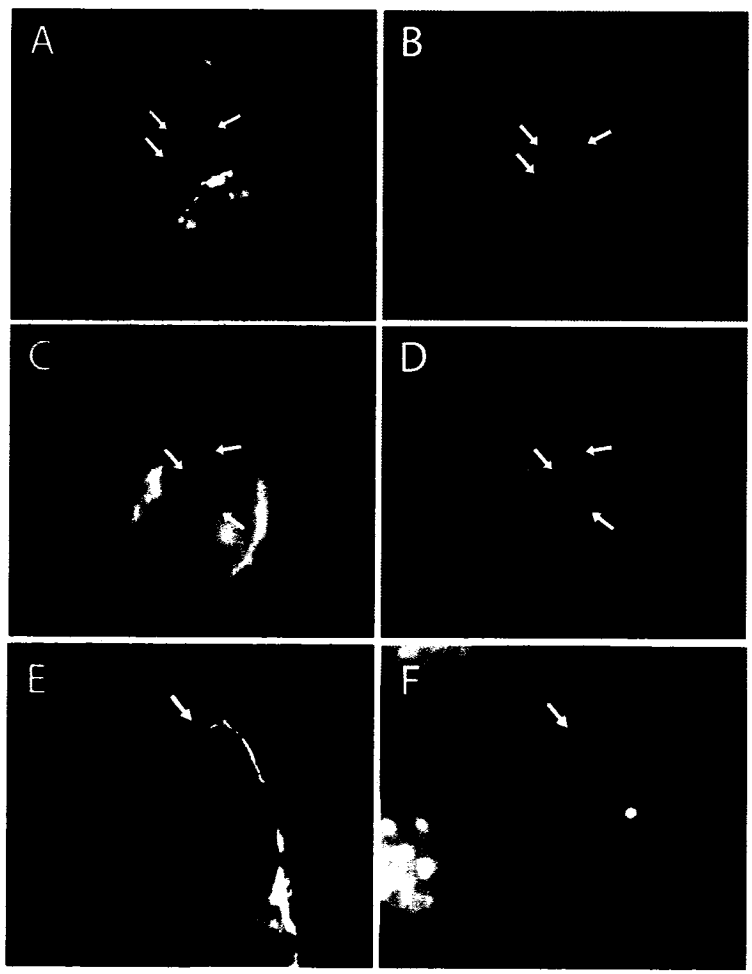
Supplemental material



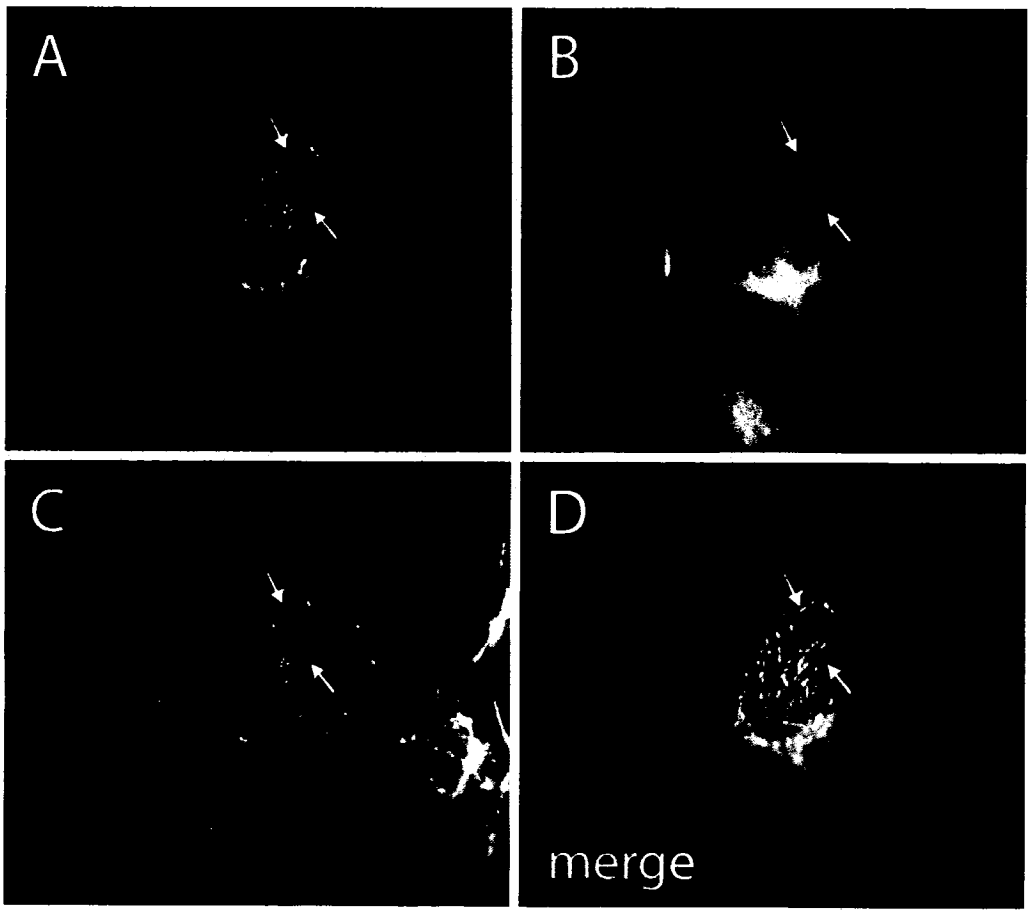
Supplemental Figure 1. FLAG-Bpag1a2 nuclear and perinuclear localization in Cos-1 cells. **A,** Three examples of FLAG-Bpag1a2 surrounding and inside of the nucleus. **B,** Focusing through the nucleus with 1 μm Z steps. Note the spot within the nucleus that is present in the middle focal plane (arrow; enlarged in the inset). **C,** Emerin (Em) and FLAG-Bpag1a2 (a2) appeared in very close proximity to each other in many cells. **D,**

Compared to the FLAG-Bpag1a2 fusion protein, FLAG-Bpag1a2ΔTM (ΔTM) was better localized within the nucleus of approximately 1% of the cells expressing this fusion protein. Note the aggregate spots, which colocalized with nucleoli. The nuclear-localized fusion protein typically surrounded the nucleoli diffusely, with a variable number of spots appearing to be colocalized with nucleoli (see also Supplemental Figure 2).



Supplemental Figure 2. FLAG-Bpag1a2 nuclear foci and perinuclear FLAG-Bpag1a2 in C2C12 myoblasts. **A and C,** FLAG staining in C2C12 cells expressing FLAG-Bpag1a2

showing the presence of nuclear foci (arrows). **B and D,** Images from A and C were merged with the respective phase contrast images of the cells. Focal staining in the nuclei consistently co-localized with nucleoli (arrows). **E and F,** Association of FLAG-Bpag1a2 with an abberant nuclear invagination in a C2C12 cell (arrows).



Supplemental Figure 3. Microtubule-associated FLAG-Bpag1a1 cross-linking with F-actin. **A,** FLAG-Bpag1a1 in a Cos-1 cell displaying a punctate localization co-aligning with microtubules, which are shown in **B. C,** F-actin co-aligned with microtubules only in association with the FLAG-Bpag1a1 fusion protein. **D,** Merged images from A – C.

Chapter 5

Dystonin/Bpag1 is a necessary endoplasmic reticulum/nuclear envelope protein in sensory neurons

Contributions of additional authors

Gilbert Bernier produced the RNase protection assay data shown in Figure 1. I had written the original manuscript independently, and Rashmi Kothary and Gilbert provided comments during the editing.

Dystonin/Bpag1 is a necessary endoplasmic reticulum/nuclear envelope protein in sensory neurons

Running title: dystonin is an ER/NE-associated organizing protein

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Abstract

Dystonin/Bpag1 proteins are cytoskeletal linkers whose loss of function in mice results in the neuromuscular disorder, *dystonia musculorum* (*dt*). While previous evidence indicated that cytoskeletal defects in the axon are a primary cause of *dt* neurodegeneration, more recent data suggests that this may not be the case. In the present study, we demonstrate perikaryal defects in dorsal root ganglia (DRG) neurons prior to the onset and at the beginning of the phenotype stage in *dt* mice. Dystonin in DRG localized within the perikarya of a subset of neurons, primarily around the nucleus and extending out in a reticular pattern associated with the endoplasmic reticulum (ER). Dystonin-a2 fusion protein associated with and reorganized the ER in cell culture, indicating a role in the normal organization of this organelle. This isoform also localized around the nucleus, and retention at the nuclear envelope may be facilitated by an interaction with nesprin-3α. ER defects in *dt* mice, as demonstrated here, may ultimately result in pathogenesis involving an ER stress response and contribute significantly to the *dt* phenotype.

Introduction

Common themes in the study of neurodegeneration are the involvement of neurofilament aggregation (reviewed in Al-Chalabi and Miller, 2003; Cairns et al., 2004), and the vulnerability of neuronal axons (Garcia-Fresco et al., 2006; Pun et al., 2006). In mice, the neurodegenerative disorder *dystonia musculorum* (*dt*) has been demonstrated to involve axonal neurofilament aggregation and microtubule disorganization in sensory neurons (Janota, 1972; Dalpé et al., 1998; de Repentigny et al., 2003). The *dt* disorder is caused by mutations in the *dystonin* (*Dst*; *Bpag1*) [1] gene, which codes for cytoskeletal cross-linking proteins (Brown et al., 1995a; Leung et al., 2001; Young et al., 2006). Axonal aggregates of neurofilaments in *dt* mice, however, have been demonstrated to be irrelevant to the progression of this disorder (Eyer et al., 1998), and accumulation of neurofilaments in the perikaryon does not in itself cause overt pathology (Eyer and Peterson, 1994). Disorganization of axonal microtubules has also been reported to be absent in cultured *dt* dorsal root ganglion (DRG) neurons (Pool et al., 2006). Given that perikaryal abnormalities including nuclear eccentricity and chromatolysis are present in *dt* mice (Duchen and Strich, 1964; Hanker and Peach, 1976; Messer and Strominger, 1980; Sotelo and Guenet, 1988), it is possible that abnormalities aside from cytoskeletal disorganization in the axon initiate the *dt* pathology.

The overall picture of the *dystonin* gene has taken over a decade to resolve. Originally, only cDNA sequence for the epithelial *dystonin* gene product, Bpag1, had been described (Stanley et al., 1988; Sawamura et al., 1991). Upstream actin binding

[1] While we have used the *Bpag1* nomenclature in the past, *dystonin* has been asserted as the official gene name by the Mouse Genomic Nomenclature Committee and the HUGO Gene Nomenclature Committee, and we therefore refer to the gene as *dystonin*, and gene products as dystonin.

domain (ABD) coding sequence and alternatively spliced 5' exons specifying neuronal isoforms 1 and 2 were subsequently demonstrated (Brown et al., 1995a). Much later, roughly half of the exons at the 3' end of the gene, which code for the C-terminal halves of the neuronal and muscle isoforms, were described (Leung et al., 2001). Importantly, the neuronal and muscle isoforms either do not contain, or at least rarely contain, the C-terminal intermediate filament-binding domain of the epithelial isoform, though several studies had already ascribed a high importance to this domain's function in neurons and muscle (Yang et al., 1996; Dalpé et al., 1999; Leung et al., 1999; Yang et al., 1999). The updated dystonin gene structure has lead to a re-evaluation of results from studies which presumed that the neuronal and muscle transcripts largely resembled the epithelial transcript, and that a loss of neurofilament or peripherin intermediate filament interactions with neuronal dystonin proteins was important to the etiology of the *dt* disorder.

Recently, we have described an isoform of dystonin (dystonin-a2) that is affected in *dt* mice as being an integral membrane protein targeted to membranes surrounding the nucleus (Young et al., 2006). This isoform is primarily neuronal, with its strongest expression in sensory ganglia (Bernier et al., 1995). In the current study, we elaborate on the role of this isoform at the endoplasmic reticulum (ER) and nuclear envelope (NE), and suggest that loss of this unique isoform in particular is an important contributing factor to the *dt* phenotype. As with multiple human neurodegenerative disorders (Rao and Bredesen, 2004), ER stress may be a key feature of the *dt* disease.

Materials and Methods

RT-PCR, ribonuclease protection assays, and sequence alignments

For RT-PCR experiments, total RNA was collected from P3 dt^{tg4} or wild-type littermate mouse brain using a standard Trizol (Invitrogen, Carlsbad, CA) extraction protocol, and equal amounts were reverse transcribed with MuMLV reverse transcriptase (Invitrogen). The resulting cDNA was PCR amplified using Taq DNA polymerase (Invitrogen) with the following primers: 5'-CTGCTCTAGACAGAGTGAACAAGAGTC-3' for the isoform 2 forward primer; 5'-GTCTCCAAGGATGCACCTAGGGAT-3' for the isoform 3 forward primer; and 5'-CATCGTTTGCACCAATGCC-3' for the common reverse primer (shown in Fig. 1B). Ribonuclease protection assays were performed as before (Bernier et al., 1995). A plasmid containing a fragment corresponding to the *Dst* isoform 2 exon deleted in dt^{tg4} mice was used for generating the riboprobe. For alignment of the 5' plakin sequences, published mouse and human plectin, Macf1, and dystonin sequences (Brown et al., 1995a; Brown et al., 1995b; Bernier et al., 1996; Fuchs et al., 1999; Zhang et al., 2004; Young et al., 2006) were aligned along with genomic translations identified using the TBLASTN program (www.ncbi.nlm.nih.gov/BLAST/), including those from zebrafish and pufferfish. Sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw/).

Recombinant protein constructs

Detailed methods are available in Supplementary Materials.

Animals and cell culture

dt^{tg4} mutant mice and control littermates were used at early phenotype stage (P11 – P14), just after the onset of neuromuscular dysfunction in the affected mice, or well prior to the

onset of phenotype (P3 – P5). The generation of this line and characterization of the mutation have been described previously (Kothary et al., 1988; Brown et al., 1995a). The onset of phenotype was generally assessed by the appearance of limb clasping after picking the mice up by the tails. Brain samples were collected from dt^{Alb} and dt^{24J} mice following the onset of phenotype. For DRG sections, lumbar DRG were quickly dissected, washed in PBS, dipped in a solution of 0.4% trypan blue in Hank's buffered salt solution to lightly stain the sheath, and then were rapidly frozen in OCT (Sakura, Torrance, CA) using liquid nitrogen. The embedded ganglia were kept at -80°C until they were sectioned frozen at 8 μm thickness with a cryostat, and placed on gelatin-subbed slides.

Cos-1 and C2C12 cells were maintained in DMEM (Wisent, St. Bruno, QC) + 10% FBS (Invitrogen) and 1% pen/step/antimycotic (Invitrogen). Cells were passaged at 80 – 90% confluency in 10 cm plastic Petri dishes, and plated onto glass coverslips for use in immunofluorscence assays. Cell transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's directions.

Co-immunoprecipitations and immunoblotting

Confluent Cos-1 cells in 10 cm Petri dishes were transfected at 24 - 36 hours prior to collecting lysates for co-immunoprecipitations. Lysates were collected on ice by scraping the cells into an igepal lysis buffer (1% igepal, 150 mM NaCl, 1% glycerol, 1mM EDTA, 50 mM Tris, pH 7.4, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL pepstatin A, 30 μg/mL aprotonin, 20 mM leupeptin), and subjected to immunoprecipitation using mouse monoclonal anti-myc (Invitrogen) or rabbit polyclonal anti-GFP (Invitrogen) antibodies,

as previously described (Young et al., 2003). Protein samples were resolved on standard 10% SDS-PAGE gels, and transferred onto PVDF membranes for immunoblotting with the anti-myc or anti-GFP antibodies, as before (Young et al., 2003). DRG protein samples were collected from 10 DRGs/animal and similar amounts of protein were resolved on 10% gels. Antibodies used were anti-protein disulfide isomerase (Stressgen, Ann Arbor, MI) at a 1:2000 dilution, anti-KDEL (Stressgen) at 1:250 to detect GRP78, anti-calreticulin (Research Diagnostics, Concord, MA) at 1:1000, anti-ERp29 (Stressgen) at 1:1000, and anti-β-tubulin supernatant (Developmental Studies Hybridoma Bank, University of Iowa) at 1:50.

Nissl staining

For all tissue section staining, air-dried DRG sections were first washed quickly with PBS, fixed for 10 minutes in 4% paraformaldehyde (PFA) buffered with PBS, and washed again with PBS for 3 x 10 minutes. For Nissl staining, 0.5% w/v cresyl violet acetate (dissolved in 0.3% acetic acid) was used in a basic staining protocol. Nissl stained sections were observed on a Zeiss Axioplan 2 using DIC optics.

Immunofluorescence analysis

For immunofluorescence staining, following PFA fixation and washing in PBS, the tissue sections and cultured cells were processed essentially as described before (Young et al., 2003). Dystonin was detected in tissue sections using a monoclonal antibody (clone 1B10; Abnova, Taipei City, Taiwan)(1:400 dilution) directed against a 100 amino acid human sequence common to the N-terminal end of each of the known major dystonin

isoforms. The sequence targeted is 98% identical to mouse dystonin. Other antibodies used were anti-lamin B polyclonal (M-20; Santa Cruz, Santa Cruz, CA) at 1:200, anti-phosphorylated neurofilament heavy monoclonal supernatant (RT-97; Developmental Studies Hybridoma Bank) at 1:3, anti-GM130 monoclonal (clone 35; BD Transduction, Franklin Lakes, NJ) at 1:500, anti-PDI polyclonal (Stressgen) at 1:100, anti-myc monoclonal (Invitrogen) at 1:500, anti-myc polyclonal (Bethyl, Montgomery, TX) at 1:100, anti-FLAG monoclonal (M5; Sigma, St. Louis) at 1:500, and anti-nesprin-3 polyclonal (a kind gift from Dr. Arnoud Sonnenberg, Netherlands Cancer Institute) at 1:200. Appropriate secondary antibodies labelled with Alexa Fluor 488 (Invitrogen), FITC, Cy3, or Cy5 (Jackson Immunoresearch, West Grove, PA) were used for fluorescence detection. Alexa Fluor 546-phalloidin (Invitrogen) was used to visualize F-actin and Hoechst 33342 (Sigma) was used to visualize nuclear chromatin. The endogenous fluorescence was visualized for GFP.

Tissue sections and cells were observed using either a Zeiss Axioplan 2 equipped with epifluorescence illumination, or with a Zeiss LSM 410 invert laser-scanning microscope. Digital images were captured using Axiovision or LSM software (Zeiss, Thornwood, NY), and processed with Adobe Photoshop CS and Adobe Illustrator 10. For quantifications of F-actin punctae and nuclear eccentricity from DRG sections, neuronal cell bodies at least 15 μm wide from P3 – P5 mice, and at least 20 μm wide from P11 – P14 mice were used. For dystonin immunostaining, DRG sections from two P3 and one P4 wild-type animal with one P4 *Dst -/-* littermate, and three P14 wild-type animals, one P14 heterozygote, and two P14 *Dst-/-* littermates were observed.

Results

Dystonin isoform 2 is a unique member of the plakin family affected in dystonia musculorum *mice*

With the recent descriptions of the deletion mutation in the dt^{Alb} line of *dystonia musculorum* mice (Pool et al., 2005; Goryunov et al., in press), there are now three characterized mutations in the dystonin gene that cause the *dt* disorder (Fig. 1A). Two of these, the dt^{Alb} deletion (Messer and Strominger, 1980) and the *Bpag1* targeted knock-out deletion (Guo et al., 1995), remove exons coding for sequence common to all major dystonin isoforms (dystonin isoforms are summarized in Supplemental Fig. 1). The dt^{Alb} deletion likely results in a complete null, having been demonstrated to be null for dystonin in the brain and for the epithelial Bpag1 protein (Guo et al., 1995; Pool et al., 2005; Goryunov et al., in press). The third known deletion, the dt^{tg4} mutation (Brown et al., 1995a), causes a deletion of 5' exons specific to isoforms 1 and 2, which are generated by alternative splicing (Fig. 1A and B). Accordingly, transcript for isoforms 1 and 2 cannot be amplified by RT-PCR from dt^{tg4} mice (Fig. 1C; Dalpé et al., 1999). The isoform 2 transcript is, in addition, a primarily neuronal transcript expressed most prominently in sensory ganglia (Bernier et al., 1995) whose levels of expression are affected in different *dt* mouse lines (Fig. 1C). This implicates loss of neuronal isoform 2 (dystonin-a2) as being important in the generation of the *dt* disorder.

Despite the fact that isoform 3 (originally called Bpag1n3) was described as being essential for the stabilization of microtubules in peripheral neurons, with its loss contributing significantly to the *dt* phenotype (Yang et al., 1999), isoform 3-specific transcript can be amplified normally from dt^{tg4} mice (Fig. 1C). Further, the original

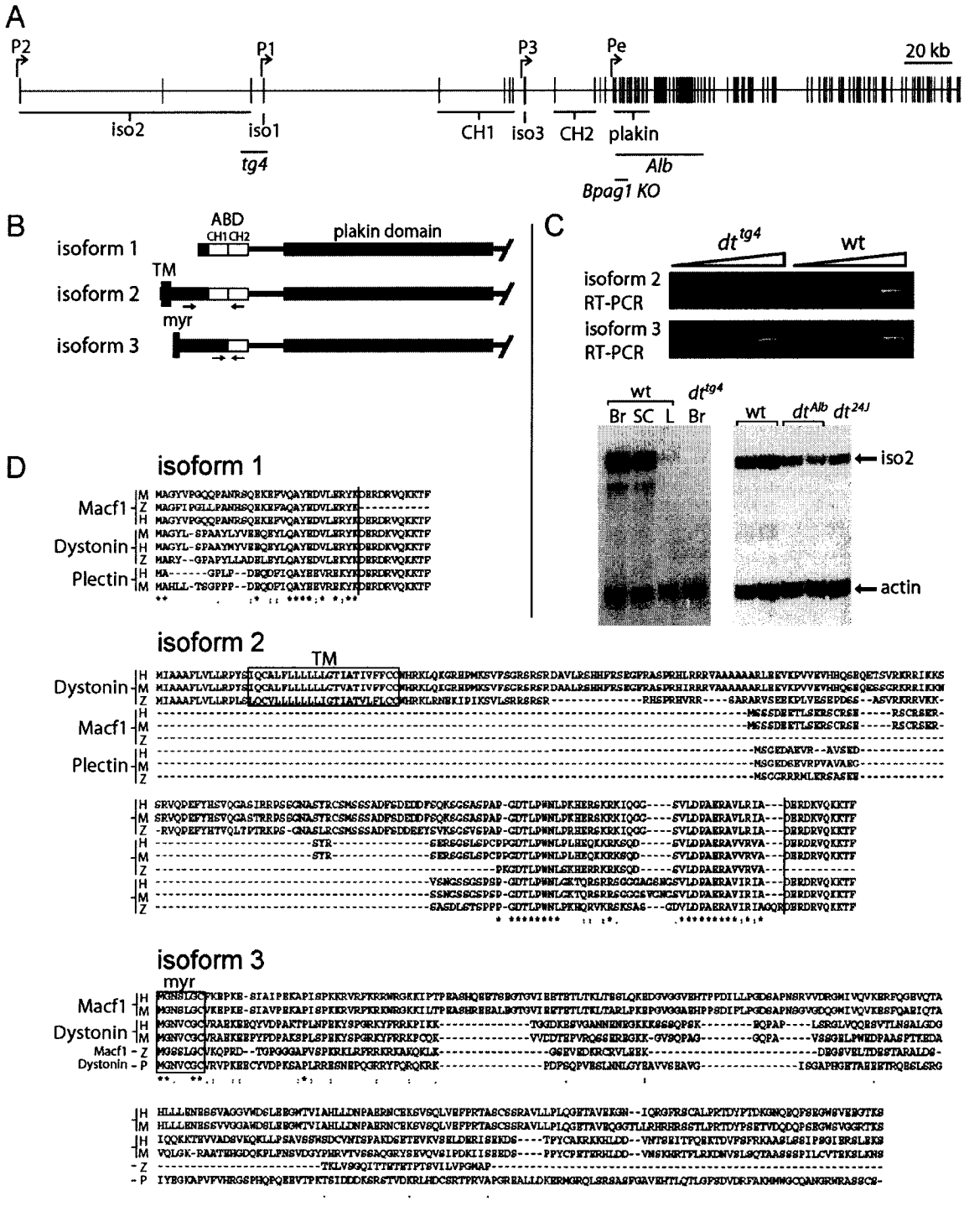

Figure 1. Dystonin isoform 2 is a unique plakin protein, affected in *dystonia musculorum* (*dt*) mice. **A,** Overview of the mouse *dystonin* gene, and the location of known deletions in *dt* mutant mice. The *tg4* mutantion deletes exons unique to dystonin isoforms 1 and 2

(iso1 and iso2). The Albany (*Alb*) and Bpag1 knock-out (*Bpag1 KO*) mutants contain deletions in exons common to all known major isoforms of dystonin. CH1 and CH2 = calponin homology domains 1 and 2, respectively; iso3 = dystonin isoform 3 (Bpag1n3); P1, P2, P3, Pe = promoter regions for the isoforms 1, 2, 3 and the epithelial isoform, respectively. **B,** Schematics of the 5' ends of the non-epithelial mRNA transcripts, including coding sequence for the actin binding domain (ABD), plakin domain, transmembrane (TM) domain, and a putative myristoylation motif (myr). Approximate locations of primers used for RT-PCR are indicated by the arrows. The isoform 2 primers flank the sequence deleted within the *tg4* mutation. **C,** RT-PCR results using total brain RNA are representative of two dt^{tg4} and two wild-type littermates; increasing PCR cycles for semi-quantitatve analysis were used (25, 30, or 35 cycles). One band from the dt^{tg4} isoform 3 RT-PCR was excised, cloned, and sequenced to confirm its identity. RNase protection assays indicated that the isoform 2 transcript, absent in dt^{tg4} brain samples, is also greatly decreased in the dt^{Alb} and dt^{24J} mutant alleles. Br = brain; SC = spinal cord; L = liver. The right-hand blot was performed using brain samples. **D,** Alignment of the dystonin isoform 1, 2, and 3 N-terminal peptide sequences with corresponding MACF1 and plectin sequences. Sequences used were from human (H), mouse (M), zebrafish (Z), or pufferfish (P). Identical amino acids in all sequences are indicated with an asterisk, with conserved amino acids indicated by one or two dots (semi-conserved or conserved, respectively). The isoform 2 TM region and isoform 3 myristoylation motif (boxed) are both conserved between species. The isoform 3 myristoylation motif is also found in Macf1, but the isoform 2 N-terminus is unique among the plakin proteins in containing the conserved TM region.

description of the isoform 3 5' end (Yang et al., 1999) contained a sequencing error as indicated by alignment to the human and mouse genomic sequences (Jefferson et al., 2006) and 5' RACE analysis (Supplemental Fig. 2). This error resulted in over 200 N-terminal amino acids being disregarded. The corrected amino acid sequence, which aligns at the N-terminal end of the equivalent Macf1 isoform 3 (Bernier et al., 1996), is shown in Fig. 1D. The conserved N-terminal end consists of a putative myristoylation site and adjacent positively charged region, indicating the possibility of plasma membrane localization via a myristoyl lipid modification (Bentham et al., 2006). Observations of mouse isoform 3 N-terminal fusion proteins expressed in cell culture support this (Jefferson et al., 2006; Young and Kothary, unpublished observations). Our preliminary 3' RACE analysis also indicates that this isoform has a 3' end corresponding to that described by Leung and colleagues (2001), not that described by Yang and colleagues (1999) (Supplemental Fig. 2).

Since the loss of dystonin isoform 1 and/or 2 proteins appears to be important to the etiology of the *dt* mice, we examined if any mRNA transcript data, or translations from genomic DNA, exist that indicates homologous proteins which could compensate for the loss of each dystonin isoform. Macf1 and plectin are related plakin family members which have N-terminal regions that most closely resemble dystonin, with Macf1 appearing to be largely redundant in structure over the length of the protein (Brown et al., 1995a; Bernier et al., 1996; Fuchs et al., 1999; Leung et al., 2001; Lin et al., 2005). While there are both Macf1 and plectin N-terminal regions homologous to dystonin isoform 1, and a homologous Macf1 isoform 3 protein, the plectin and Macf1 N-termini which most closely resemble the dystonin isoform 2 N-terminus differ at the start,

and are dissimilar from dystonin isoform 2 in that they lack any possible transmembrane domain (Fig. 1D). The transmembrane domain and most of the isoform 2 specific N-terminal sequence is highly conserved in the vertebrates, indicating an important conserved function for this sequence. This provides genetic evidence that loss of dystonin isoform 2 is unlikely to be compensated for by any closely related protein, and is likely a significant factor in the etiology of the disease in *dt* mice.

Perikaryal defects in dt *DRG neurons*

Previous defects described within the neuronal perikarya of *dt* mice consist of nuclear eccentricity and chromatolysis (loss of chromophilic material from around the nucleus, caused by rough ER disorganization, and a swelling of the perikaryon) (Duchen and Strich, 1964; Hanker and Peach, 1976; Messer and Strominger, 1980; Sotelo and Guenet, 1988). In agreement, we found that Nissl substance stained by cresyl violet was greatly reduced in most neurons throughout the DRG following the onset of phenotype stage (P11 – P14) (Fig. 2F). Strong staining in the nucleoli and the surrounding satellite cells was not affected. Prior to the phenotype stage (P3 – P5), while there may be some reduction of Nissl staining (Fig. 2C), the difference was not as apparent compared to the phenotype stage DRGs. Nuclear eccentricity was also apparent following the onset of the phenotype, with 49% of *dt* DRG neurons compared to 21% of control neurons displaying peripherally located nuclei. No difference in nuclear positioning was noted at the pre-phenotype stage. Overall, the *dt* ganglia at phenotype stage appeared less well organized, with more spaces between the neurons.

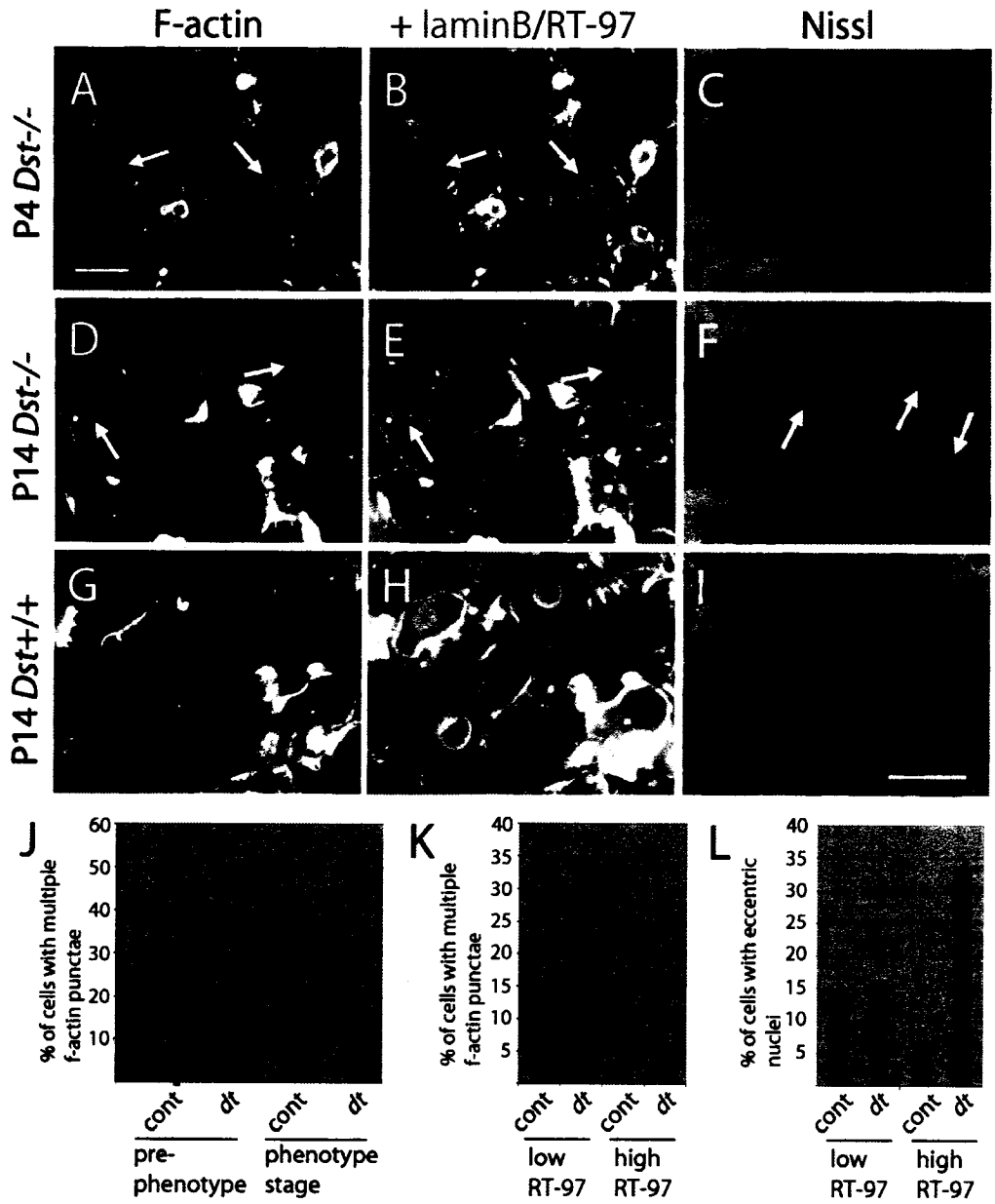

Figure 2. Neuronal defects in *dt* mice include early abnormal F-actin punctae and a widespread loss of Nissl staining. **A, B, D, E, G, and H,** F-actin (A, D, G), and the merged image of F-actin (white), lamin B and phorphorylated neurofilament heavy (RT-97) staining (B, E, H) in DRG sections. F-actin punctae (arrows) were predominantly observed in perikarya with weak, or no, RT-97 staining in the pre-phenotype (A, B) and phenotype stage (D, E) *Dst-/-* DRG neurons. F-actin punctae in wild type (*Dst+/+*)

neurons were fewer and less prominent. **C, F, I,** Nissl staining in P4 *Dst-/-* (C), P14 *Dst-/-* (F) and P14 wt (I) DRG neurons. There was a marked reduction in Nissl staining in the cytoplasm of the *Dst-/-* P14 neurons (arrows). Note that the nucleolar staining, and staining in the surrounding satellite cells is still strong. **J,** Quantification of the F-actin punctae observed in DRG neurons from P3 – P5 (pre-phenotype) and P11 – P14 (phenotype stage) dt^{tg4} mutants (red circles) and control littermates (wild-type = blue squares; heterozygotes = green triangles) (a minimum of 150 neurons from 2 – 3 ganglia/animal were observed for each age group and genotype). Cells with ≥2 discrete F-actin punctae in the cytoplasm were counted. **K,** Quantification showing that the majority of perikarya with aberrant F-actin punctae occurred in a subset of neurons with little or no RT-97 labelling. **L,** Increased nuclear eccentricity was prominent in neurons strongly positive for RT-97. Averages (with standard error of the mean) from three phenotype stage dt^{tg4} mutants and three control littermates are shown for K and L. Scale bars = 20 μm; bar in A also applies to B, D, E, G, H, and the bar in I also applies to C, F.

Although neuronal dystonin isoforms were originally demonstrated to contain an N-terminal ABD (Brown et al., 1995a), no aberrant F-actin organization in *dt* neurons has been previously described. Here, we show that DRG neurons from *dt* mice contain abnormal accumulations of F-actin punctae in a subset of neurons (Fig. 2). These typically appeared as multiple punctae within the central region of the perikaryon. While aggregation of phosphorylated neurofilament has been associated with degenerative neurons (reviewed in Al-Chalabi and Miller, 2003), and perikaryal accumulation has been used as an indicator of affected neurons in the *dt* mouse spinal cord (Campbell and Peterson, 1992), the monoclonal antibody RT-97 directed against phosphorylated neurofilament heavy (NF-H) also detects a normal subpopulation of generally larger myelinated primary afferent neurons within the DRG (Pomonis et al., 2001; Xu et al., 2005). The NF-H positive subpopulation is among the affected neurons in *dt* mice, though they appear much less affected compared to the smaller, unmyelinated neurons (Carlsten et al., 2001). Interestingly, multiple F-actin punctae were predominantly observed in perikarya with weak RT-97 staining (Fig. 2K), while eccentric nuclei were predominantly observed to be present in perikarya with strong RT-97 staining (Fig. 2L). Thus, the different subpopulations of DRG neurons appear to be differently affected by the *dt* mutation.

Dystonin is an ER-associated protein in DRG neurons

Using a monoclonal antibody directed against an N-terminal region common to multiple dystonin isoforms, we analyzed the localization of dystonin in DRG sections. In DRG from P14 mice, this antibody produced primarily perikaryal staining in a subset of

smaller neurons, as well as staining in some of the cells found within the central neuropil region (Fig. 3). Staining using RT-97 on adjacent sections indicated that the dystonin stained neurons were weakly RT-97 positive (Fig. 3B, C). Neurite staining with the dystonin antibody was notably scarce, though was present in some fibres. The perikaryal dystonin was often concentrated around the nucleus, and extended out into the cytoplasm in a reticular pattern (Fig. 3E – H; Fig. 7A, D). This staining pattern overlapped with ERp29 staining, used as a marker for the ER (Fig. 3H, I). Light staining in the nucleus was also present in many of the neurons, with this being more pronounced in a few. In sections from P3 and P4 mice, the dystonin staining was very limited, with only a few neuronal perikarya being lightly stained (Fig. 3D). Staining in sections from P14 dt^{tg4} mutants served as a control for the specificity of the antibody. There was a weak diffuse background staining generally being present throughout these sections (Fig. 3A). Occasional brighter staining in the mutant sections appeared to be primarily interstitial, and some small cell bodies within the neuropil region were lightly stained. Staining in neuronal perikarya similar to that of the normal littermates was completely absent.

If the ER is affected in *dt* mice, alterations to ER organization may be reflected in an ER stress response. To examine if ER stress response proteins were up-regulated in dt^{tg4} DRGs, we analyzed DRG lysates for the expression of several ER stress response-associated proteins in early phenotype stage (P14) mice. Both protein disulfide isomerase (PDI) and GRP78 showed clear upregulation in dt^{tg4} DRGs compared to control littermates (Fig. 3J, K). Levels of calreticulin were only marginally different, and levels of ERp29 were similar. Immunoblot analysis of ERp29, however, showed an additional higher band (~5 kD heavier) present in the *dt* DRG samples, which was only faintly

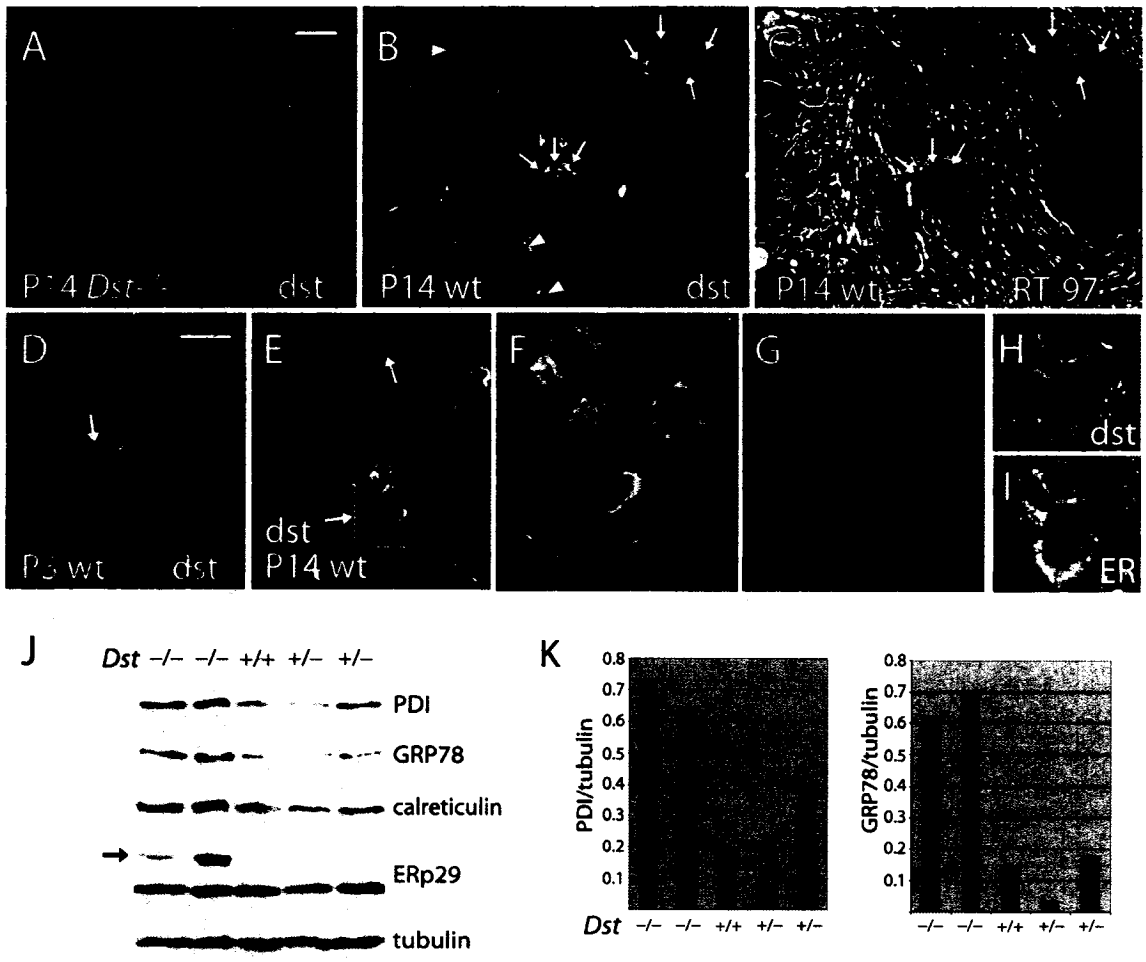

Figure 3. Dystonin expression in the dorsal root ganglia. **A,** In *Dst-/-* (*dt*^{tg4}) DRG ganglia, labelling with dystonin antibody primarily produced a weak diffuse staining. **B,** In a DRG section from a wild-type P14 littermate, the dystonin antibody is shown labelling the perikarya of a small subset of neurons (arrows) as well as smaller cell bodies (arrowheads) within the neuropil. **C,** RT-97 staining in a section adjacent to the one shown in B. The dystonin positive neurons were generally labelled only weakly, or not at all, by the RT-97 antibody (arrows). **D,** Expression of dystonin in P3 DRG neurons was weakly detected in only a few perikarya, indicating low expression in younger mice (arrow). **E,** Dystonin labelling in neurons (arrows) had a generally reticular appearance in the cytoplasm. **F,** An enlarged 1 μm thick confocal section of the cluster of neurons

boxed in E. Reticular cytoplasmic staining and light nuclear staining was observed. The merged image with Hoechst chromatin staining is shown in **G. H and I,** Confocal images of two neurons from a *Dst* heterozygous P14 mouse DRG section showing dystonin (H) and ERp29 (I) staining. **J,** DRG lysate was immunoblotted for various ER stress response-associated proteins. The same blot was probed and reprobed with each antibody, and stripped when appropriate. An additional band on the ERp29 blot associated with the *Dst-/-* lysates is indicated by an arrow. Tubulin is shown as a loading control. **K,** Quantification of the band intensity ratios of PDI/tubulin and GRP78/tubulin for each lane of the blot shown in J. Scale bars = 40 μm; bar in A also applies to B, C; bar in D also applies to E.

detected with long exposures in the normal control samples (Fig. 3J). This may represent an abnormally processed form of the protein. Thus, there is an altered expression of proteins indicative of an ER stress response in mutant DRGs.

Dystonin immunostaining co-localizes with ER and outer nuclear membrane markers in C2C12 cells

We have previously demonstrated that various dystonin isoforms are well-expressed in the C2C12 cell line (Dalpé et al., 1999; Young et al., 2003). While we had found that both endogenous dystonin and fusion proteins localize surrounding the Golgi apparatus, they did not completely co-localize with this organelle, and we found no conclusive evidence that they associated directly with it (Young et al., 2006). The dystonin monoclonal antibody and our previously used anti-isoform 2 polyclonal showed similar patterns of staining in C2C12 cells, with some additional staining with the monoclonal and minor variations in staining intensities in different regions between the two (Fig. 4A – D). Good co-localization of endogenous dystonin was observed with an ER marker, PDI (Fig. 4D – F). In cells in which the PDI staining was more condensed around the nucleus, so too was the dystonin staining. Dystonin protein also localizes more prominently immediately surrounding the nucleus in some cells (Young et al., 2006), and showed co-localization with the outer nuclear membrane marker, nesprin-3 (Fig. 4G – I).

Full-length dystonin-a2 can reorganize the ER

Using a C-terminal tagged myc/his isoform 2 fusion protein (dyst a2-myc/his) expressed in Cos-1 cells, dystonin isoform 2 localized surrounding the nuclei in a majority (>50%)

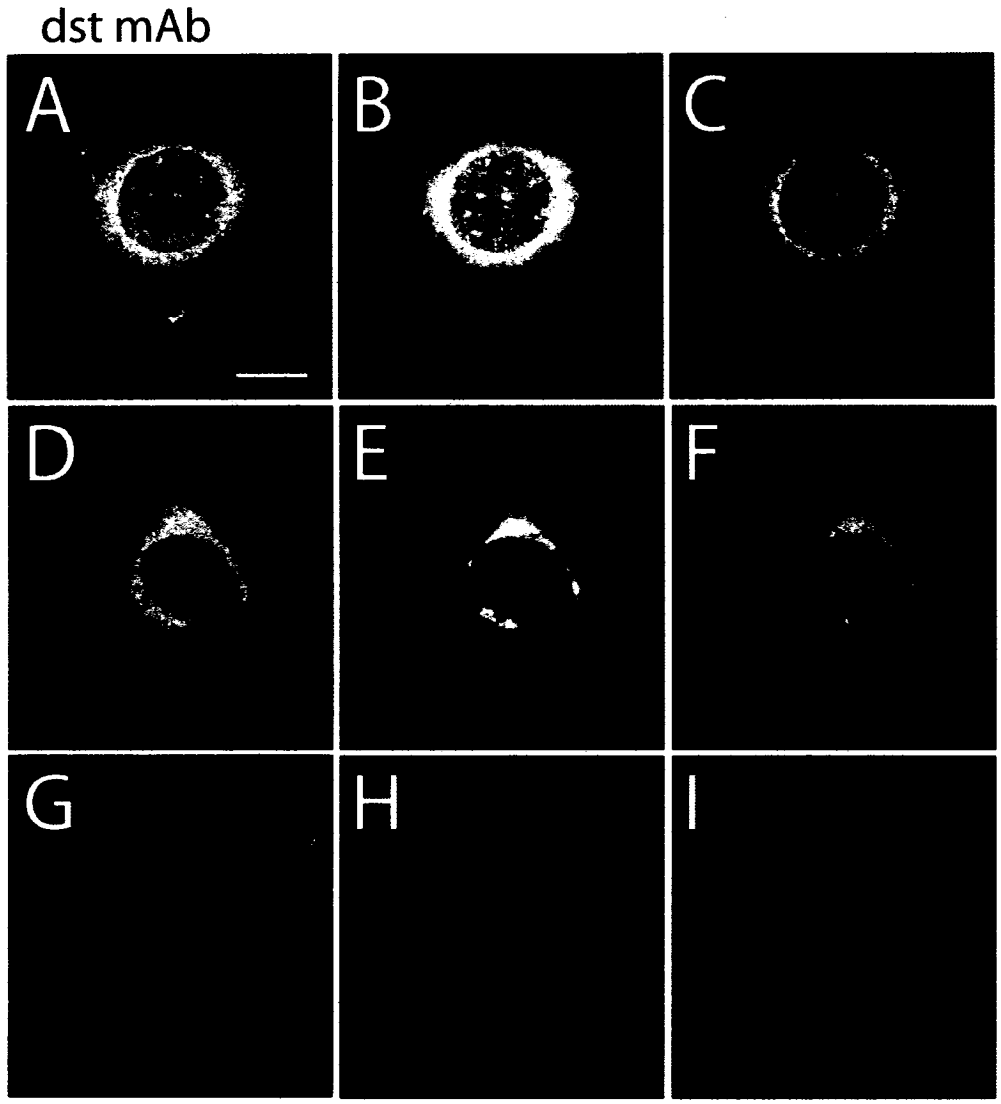

Figure 4. Dystonin in C2C12 cells co-localizes with ER and NE markers. **A – C,** Confocal images indicating that the dystonin monoclonal antibody used for DRG labelling co-localizes extensively with a previously characterized dystonin isoform 2 polyclonal antibody (Young et al., 2003). Some additional staining was observed with the monoclonal antibody, and the intensities of the staining showed differences in some areas. It is likely that the monoclonal antibody would recognize other dystonin isoforms in these cells. The majority of the staining overlapped, though. **D – F,** Staining with the monoclonal antibody extensively co-localized in the cytoplasm with PDI staining, used to

mark the ER. **H – J,** In cells where the relative intensity of the dystonin monoclonal antibody staining was higher immediately surrounding the nucleus, this staining co-localized with nesprin-3 staining, used to mark the outer nuclear membrane. Scale bar = 20 μm, and applies to all images.

of the cells (Fig. 5). Fusion protein that localized away from the nuclear envelope in these cells was generally found in association with a reorganized, reticular ER structure, observed by anti-PDI staining (Fig. 5C – F). PDI staining in untransfected cells was always more amorphous, with staining commonly concentrated on one side of the nucleus. ER-associated dystonin also showed an association with accumulated F-actin (Supplemental Fig. 3). In comparison to the ER, Golgi organization appeared largely unaffected in cells transfected by the dyst a2-myc/his fusion (Fig. 5E). An N-terminal FLAG-tagged isoform 2 fusion protein (FLAG-dyst a2) did not show a similar reorganization of the ER structure, though it did co-localize with PDI staining accumulated around the nucleus (Fig. 5G – I). Expression of a C-terminal tagged fusion lacking the C-terminal half of the protein (a2Nterm-myc/his) also localized only in close association with the nucleus (Fig. 5B). Thus, the N-terminal epitope tag may interfere with normal interactions of this protein, and the C-terminal end of the protein is likely required for normal function.

The dystonin isoform 2 N-terminus can associate with nesprin-3α

The recently-described nesprin-3α protein, an outer NE protein, interacts with both the plectin ABD region and the similar dystonin ABD by yeast two-hybrid analysis (Wilhelmsen et al., 2005). We followed-up on these results by investigating whether nesprin-3α and the dystonin isoform 2 N-terminus associated in cell culture. In a reciprocal co-immunoprecipitation assay, a dystonin isoform 2 N-terminal myc/his fusion protein, but not an isoform 3 N-terminal fusion protein, associated with a GFP-nesprin3α fusion protein co-expressed in Cos-1 cells (Fig. 6B).

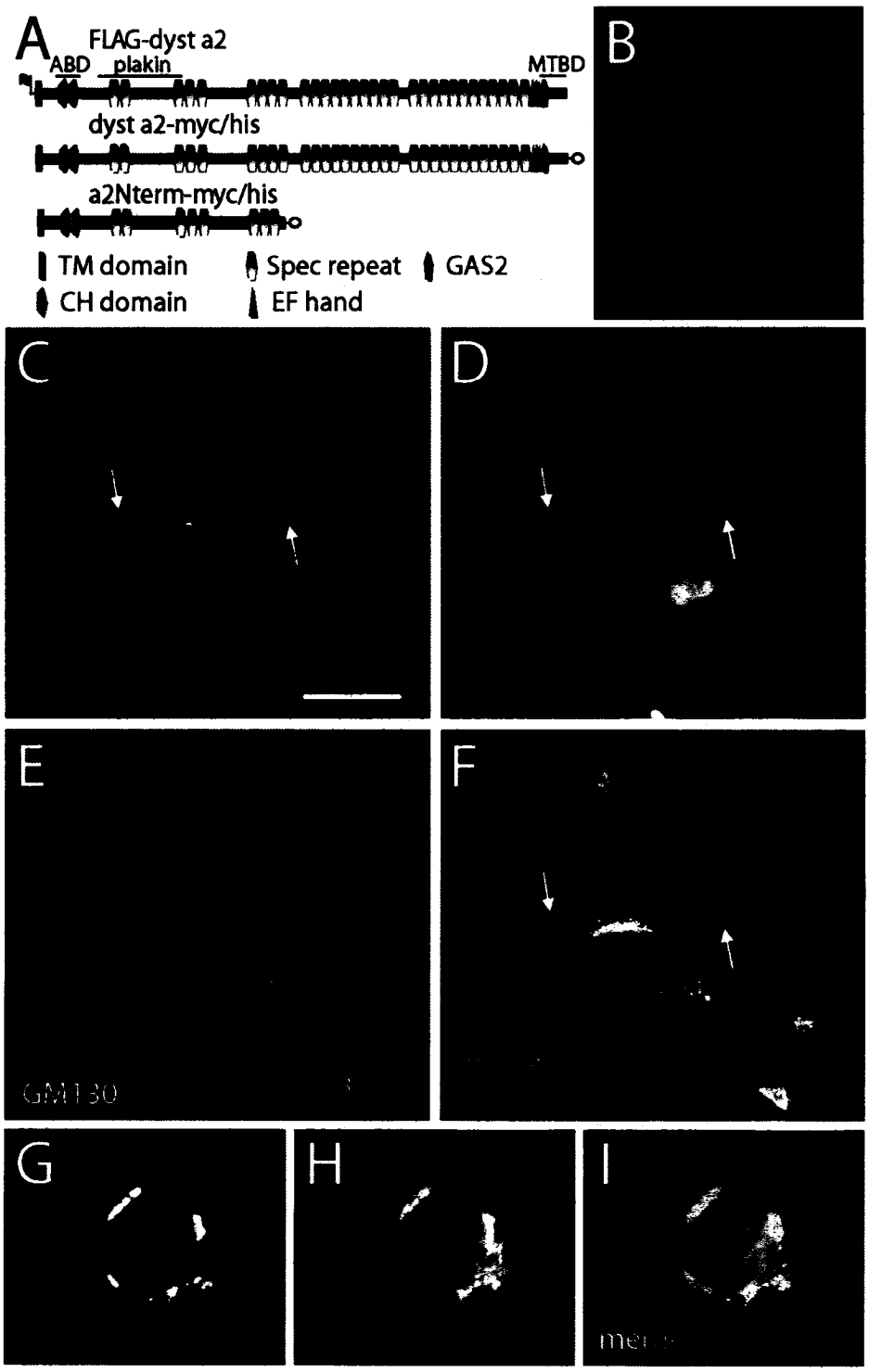

Figure 5. Full-length dystonin isoform 2 fusion protein expressed in Cos-1 cells reorganizes the endoplasmic reticulum (ER). **A,** Schematics of the full-length and N-terminal dystonin isoform 2 fusion proteins used. Domains shown are those determined

by SMART analysis (http://smart.embl-heidelberg.de/), and the actin binding domain (ABD), plakin, and microtubule binding domain (MTBD) are indicated. **B,** Extended focus image showing that the a2Nterm-myc/his fusion containing the N-terminal half of the dystonin-a2 protein localized primarily in a discrete pattern surrounding the nucleus. **C – F,** Extended focus image showing that the full-length C-terminally tagged dyst a2-myc/his fusion protein (C) localized primarily around the nucleus and in association with an altered ER structure, observed by anti-PDI staining (D). While the fusion protein also typically surrounded the Golgi apparatus (GM130 staining in E), there was no consistently obvious association with the Golgi. The merged image is shown in F along with Hoechst staining for the nuclei. **G – I,** Full-length fusion protein with an N-terminal FLAG tag (FLAG-dyst a2 in G) did not display the same consistent reorganization of the ER (H) as the dyst a2-myc/his fusion, but was associated with condensed PDI staining. The merged image is shown in I. Scale bar = 20 μm, and applies to all images.

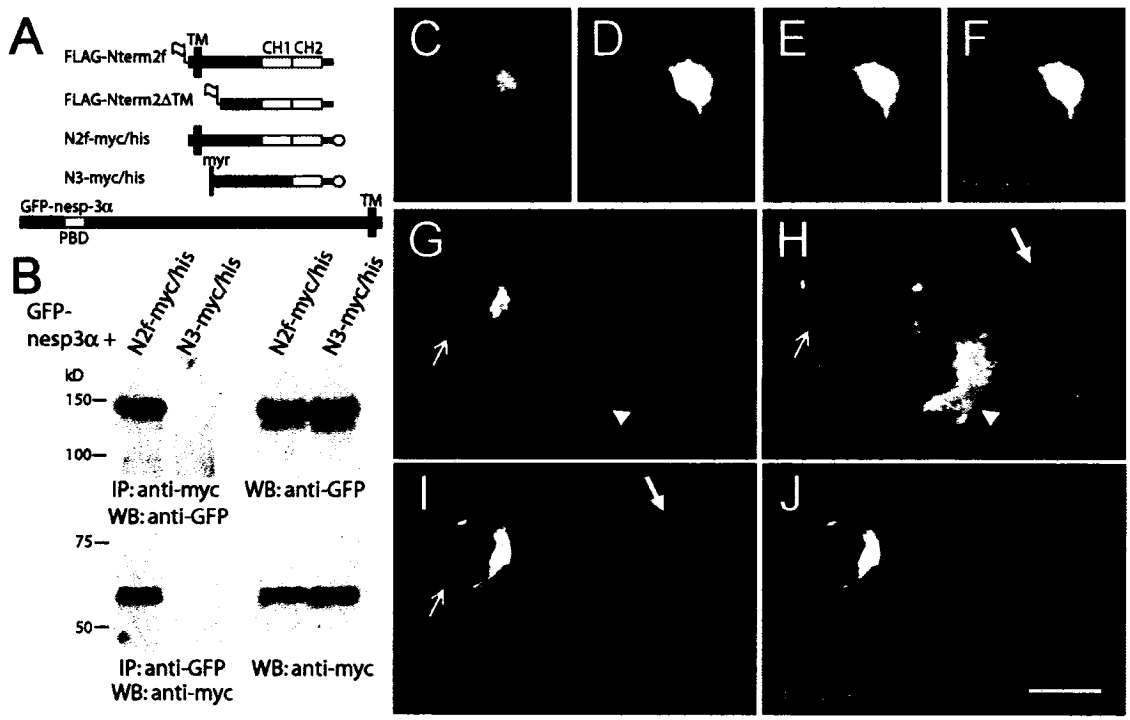

Figure 6. The dystonin isoform 2 N-terminus can associate with nesprin-3α. **A,** Schematics of dystonin isoform 2 and isoform 3 N-terminal constructs, and the nesprin-3α construct used in this figure. PBD = plectin binding domain; other abbreviations and symbols are as in previous figures. **B,** Co-immunoprecipitation assays demonstrating that the dystonin isoform 2 (N2f-myc/his), but not the isoform 3 (N3myc/his), N-terminal fusion interacts with GFP-nesprin-3α when co-expressed in Cos-1 cells. The left-hand side blots show reciprocal co-immunoprecipitation results, and the right-hand side blots show the respective whole cell lysate blots. **C – F,** When co-expressed, FLAG-Nterm2f always overlapped in its distribution around the nucleus with the GFP-nesprin-3α and F-actin. **G – J,** The FLAG-Nterm2ΔTM fusion protein did not localize discretely around the nucleus on its own (arrowheads), but was recruited around the nucleus when co-expressed in cells with GFP-nesprin-3α (open arrows). GFP-nesprin-3α expressed on its own did not recruit F-actin around the nucleus (closed arrows) while F-actin was only

weakly recruited around the nucleus in cells co-expressing the FLAG-Nterm2ΔTM fusion protein (open arrows). Scale bar = 20 μm and applies to all images.

By immunofluorescence analysis, dystonin isoform 2 N-terminal FLAG (shown) or C-terminal myc/his (not shown) fusion protein always co-localized with GFP-nesprin-3α (Fig. 6C – F). Moreover, when the GFP-nesprin-3α fusion protein was co-expressed with dystonin isoform 2 FLAG fusion protein lacking the N-terminal transmembrane domain (FLAG-Nterm2ΔTM), which localized throughout the cytoplasm along with bundled F-actin filaments and never localized discretely around the nucleus on its own (Young et al., 2006), the dystonin fusion protein was now always recruited around the nucleus (Fig. 6G – J). The FLAG-Nterm2ΔTM protein which localized around the nucleus in association with GFP-nesprin-3α was not associated with the strong F-actin staining that the longer N-terminal fusion proteins were associated with (Fig. 6C – J). This indicates a competition between nesprin-3α and F-actin for binding to the dystonin ABD. As well, GFP-nesprin-3β, which lacks the ABD interaction domain, did not show obvious association with dystonin isoform 2 fusion proteins (not shown). Thus, similar to the case with plectin (Wilhelmsen et al., 2005), nesprin-3α has the ability to recruit dystonin protein around the nucleus.

In DRG sections from P14 wild-type mice, endogenous nesprin-3 and dystonin co-localized at the NE in a small subset of neurons (Fig. 7). Nesprin-3 localized at the NE in many of the neuronal and non-neuronal cell bodies throughout the ganglia, and its level of expression varied widely. The expression of dystonin did not display a pattern resembling that of nesprin-3, though the two did co-localize in a subset of neurons at the NE (Fig. 7D – F). Dystonin was also observed in cells with no obvious nesprin-3 localized at the NE, and the reciprocal was also true, with strong nesprin-3 staining occurring in cells with no obvious dystonin staining. Thus, nesprin-3 and dystonin are

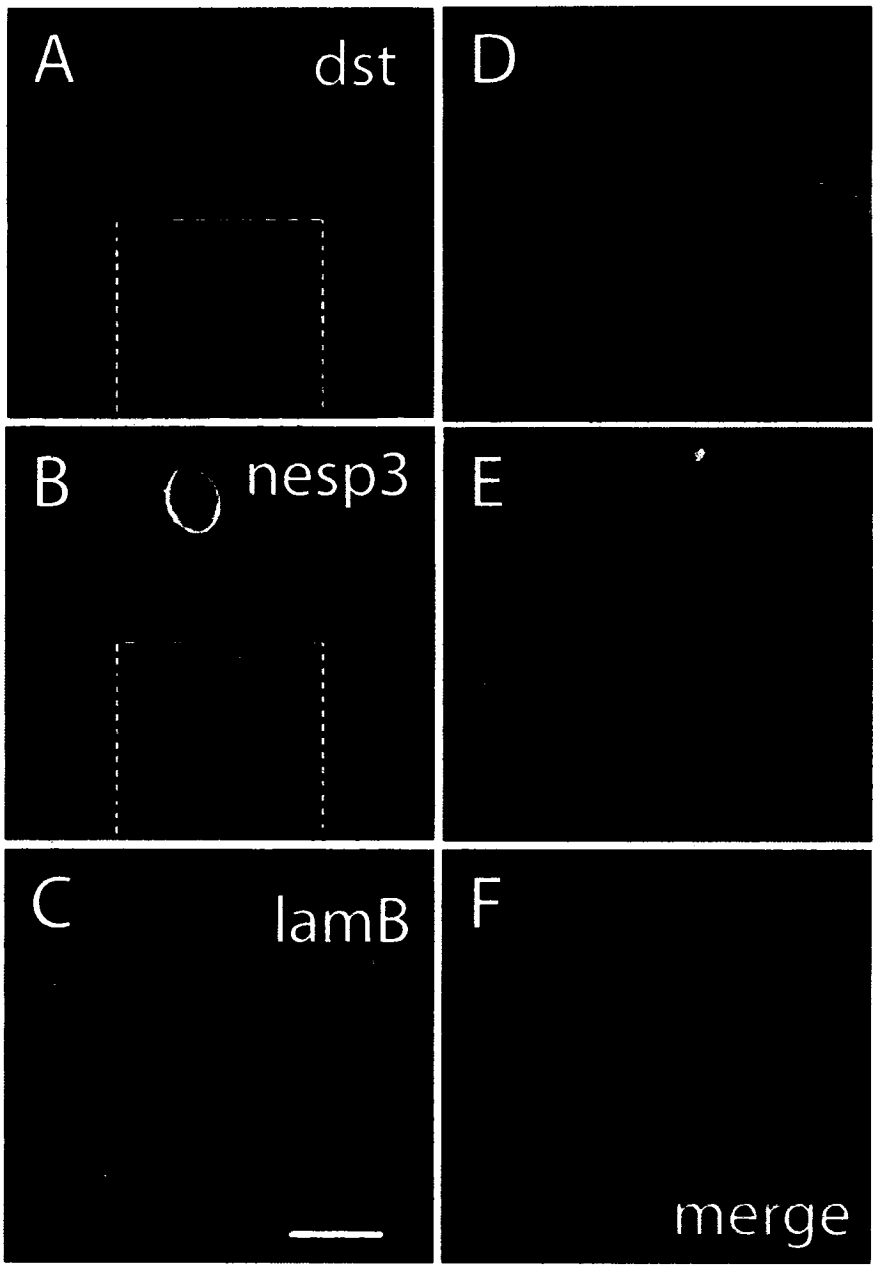

Figure 7. Nesprin-3 and dystonin co-localize at the nuclear envelope (NE) in a small subset of DRG neurons. **A,** Dystonin (dyst) labelling in two neuronal perikarya within a P14 DRG section. **B,** The same section labelled for nesprin-3 (nesp3), showing staining within the same two neurons, plus additional cells. **C,** Lamin B (lamB) labelling in the same section indicating the NE localization of nesprin-3. Not all cells displayed the NE-localized nesprin-3, however, and the levels of nesprin-3 staining varied widely from cell

to cell. **D – F,** Confocal sections of dystonin (D) and nesprin-3 (E) imaged from the areas boxed in A and B. The merged image is shown in F. Scale bar = 20 μm and applies to A – C.

both expressed in the DRG and co-localize in a subset of neurons, though they are not ubiquitously associated with each other.

Discussion

As with many neurological disorders, murine *dystonia musculorum* is associated with multiple cytological abnormalities, including the presence of axonal swellings, neurofilament aggregates, and chromatolysis with nuclear eccentricity. Therefore, the initial cause of the neurodegeneration and sensory ataxia is unclear. While axonal cytoskeleton disorganization and axonal transport defects had been strongly implicated as being a primary cause of the disease (Dalpé et al., 1998; Yang et al., 1999; de Repentigny et al., 2003; Liu et al., 2003), more recent analysis of axonal transport in cultured *dt* neurons (Pool et al., 2006) and of dystonin isoform 3 (Jefferson et al., 2006; and this study), whose loss was implicated in causing microtubule destabilization, does not support this. In this study, we find that an isoform that is affected by each of the known *dt* mutations, and which is the least likely to be compensated for as indicated by the lack of a similar homolog, is capable of reorganizing the ER, reorganizes the actin cytoskeleton associates with the ER and the nucleus, and can associate with the recently-described outer NE protein, nesprin-3α. Along with the perikaryal localization of dystonin in DRG sensory neurons, and the observations in *dt* neurons of aberrant perikaryal F-actin punctae and the loss of Nissl staining, this indicates that loss of organizing functions of the neuronal dystonin-a2 isoform within the perikaryon are involved in causing the *dt* phenotype.

Dystonin-a2 is a conserved, unique membrane protein associated with the endoplasmic reticulum and outer nuclear membrane

Cytological abnormalities in *dt* DRG neurons indicate that the organization of F-actin and the ER within the cell body may be directly affected due to loss of the dystonin-a2 isoform. The upregulation of ER stress proteins in *dt* DRG (Fig. 3) implicates ER dysfunction in contributing to the phenotype of the mice. When expressed in Cos cells, used due to the abnormally large size of our expression constructs and the easily transfectable nature of this cell type, dystonin-a2 fusion protein associated with and reorganized the ER (Fig. 5). As we have previously demonstrated that a conserved N-terminal transmembrane domain in dystonin-a2 is responsible for insertion into membranes surrounding the nucleus (Young et al., 2006), this indicates that dystonin may directly insert into ER membrane, and affect ER organization via interactions with the cytoskeleton (Fig. 8). A normal retention of dystonin within outer nuclear membrane, which is contiguous with the ER, may be facilitated by an interaction with nesprin-3α, as indicated by the association of dystonin-a2 and nesprin-3α fusion proteins in Cos cells, and the co-localization of these proteins in some DRG neurons. The limited co-expression of these two proteins in the DRG, however, indicates that this may not be a common interaction in neurons. None-the-less, dystonin-a2 clearly has the ability to localize to the ER and NE, and likely functions predominantly in these subcellular locations.

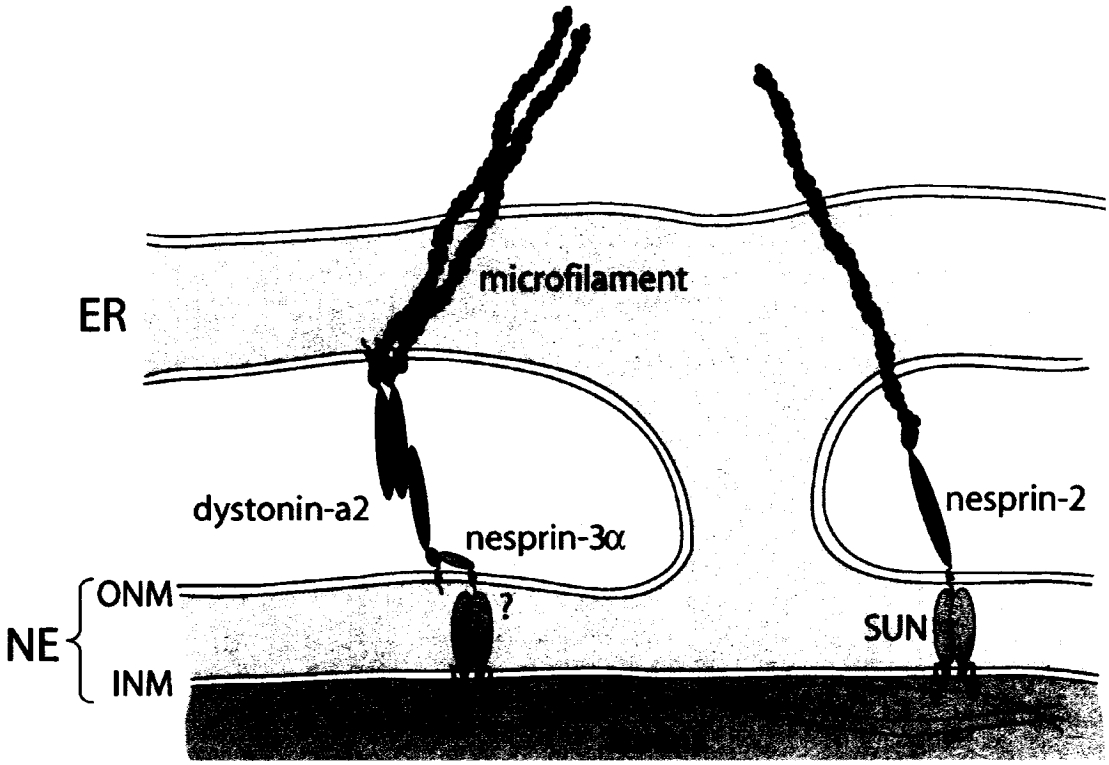

Figure 8. Putative model indicating associations of dystonin-a2 at the ER and the contiguous outer NE. As with nesprin-2 (Zhen et al., 2001; Padmakumar et al., 2005), dystonin-a2 has been implicated in tethering F-actin around the nucleus, and can bundle actin microfilaments via self-association (Young et al., 2003; Young et al., 2006). While nesprin-2, and potentially nesprin-3 (?), proteins can be retained at the NE via interactions with SUN proteins (Padmakumar et al., 2005; Crisp et al., 2006; Haque et al., 2006), dystonin-a2 may be retained at the NE via an interaction with nesprin-3α, and also localize extensively to the ER, as indicated by the staining of endogenous protein extending out several microns from the nucleus (this study and Young et al., 2003; Young et al., 2006). Dystonin-a2 has the potential to self-associate via the N-terminus, and may also self-associate via downstream spectrin repeat sequences (based on homology to other spectrin-related proteins). ONM = outer nuclear membrane; INM = inner nuclear membrane.

Different subsets of DRG neurons are differently affected by the dt *mutation*

Given the results of previous *in situ* hybridization studies using sequence from the dystonin-a 5' or 3' transcript ends as probes (Bernier et al., 1995; Yang et al., 1996; Leonova and Lomax, 2002), and immunohistochemical analysis (Bernier et al., 1995; Yang et al., 1996; Dalpé et al., 1998) it was somewhat surprising to observe such a limited amount of dystonin immunoreactivity in the DRG. Prior expression analysis has indicated that dystonin is more ubiquitous in the DRG neurons. The dystonin isoform 2 transcript, specifically, has been well-demonstrated to be primarily expressed in neuronal tissue, with expression in the DRG starting as early as E10.5 (Bernier et al., 1995). While many of the neurons may express the dystonin protein at levels too low for immunodetection with the antibody used in the present study, clearly there is a subpopulation of DRG neurons expressing an ER-localized protein which is absent in dt^{tg4} mice. It is also possible that, due to epitope inaccessibility, the monoclonal dystonin antibody used in the present study is preferentially recognizing only some of the neuronal dystonin.

The limited detection of dystonin in the present study may account for the heterogeneity of neuronal defects observed. We suggest that F-actin aggregation, present in weakly RT-97 positive neurons, may represent a cell-intrinsic defect, whereas nuclear eccentricity, which occurred primarily in the larger myelinated subset of neurons, may be reflective of the gross myelination defect known to develop in the *dt* mice (Bernier et al., 1998). The widespread loss of Nissl staining at the onset of the phenotype stage indicated a common defect with the different subsets of neurons. This loss, reflective of ER disorganization, is commonly found in chromatolytic neurons with eccentric nuclei,

generally resulting from axonal damage (McIlwain and Hoke, 2005). We suggest, therefore, that ER disorganization in the different *dt* sensory neuron subtypes may occur via both cell-intrinsic defects and axonal damage due to abnormal myelination.

Myelination defects may also account for some axonal cytoskeleton disorganization previously observed in *dt* neurons from phenotype stage mice (Janota, 1972; Sotelo and Guenet, 1988; de Repentigny et al., 2003). It has recently been reported that abnormal myelination results in the disorganization of the axonal cytoskeleton (Garcia-Fresco et al., 2006). While dystonin proteins clearly contain cytoskeletal interacting domains (Brown et al., 1995; Leung et al., 2001), and full-length isoform-specific dystonin fusion proteins are capable of reorganizing both actin microfilaments and microtubules in cell culture (Young et al., 2006), the nature of the various dystonin cytoskeletal interactions in neurons is unclear. It is possible that axonal cytoskeleton defects are caused directly by the loss of a particular dystonin isoform (e.g. dystonin-a1), and cytoskeletal defects observed in embryonic neurons (Bernier and Kothary, 1998) cannot be accounted for by dysmyelination, as full myelination occurs postnatally. Thus, further analysis is needed to clarify any direct role of dystonin in axonal organization.

While discrepancies concerning the expression of dystonin, the importance of dystonin and its relevance to neurodegeneration, and the very nature of what dystonin is exist in the literature (e.g. Yang et al., 1996, Dalpé et al., 1998; Dalpé et al., 1999; Leung et al., 1999; Yang et al., 1999; Liu et al., 2003), we have found consistent evidence that the dystonin-a2 isoform is an integral membrane protein associated with the endoplasmic reticulum and nuclear envelope. Defects observed in the dt^{tg4} DRG are consistent with

both cell-intrinsic defects affecting structure in the perikaryon, including the ER, as well as abnormalities arising due to aberrant myelination. ER dysfunction along with axonal protein aggregates in *dt* mice indicates similarities in the etiology of the *dt* neuropathy and human neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (Rao and Bredesen, 2004; Paschen and Mengesdorf, 2005). Classical ultrastructural analysis of the originally-described *dt* line had many years ago concluded that there is "clear evidence of the involvement of the perikaryon of certain sensory ganglion cells early in the disease" (Hanker and Peach, 1976). This disorganization may result in pathogenic responses associated with ER dysfunction including aberrant protein folding and production and/or altered calcium homeostasis, and contribute to the subsequent neurodegeneration.

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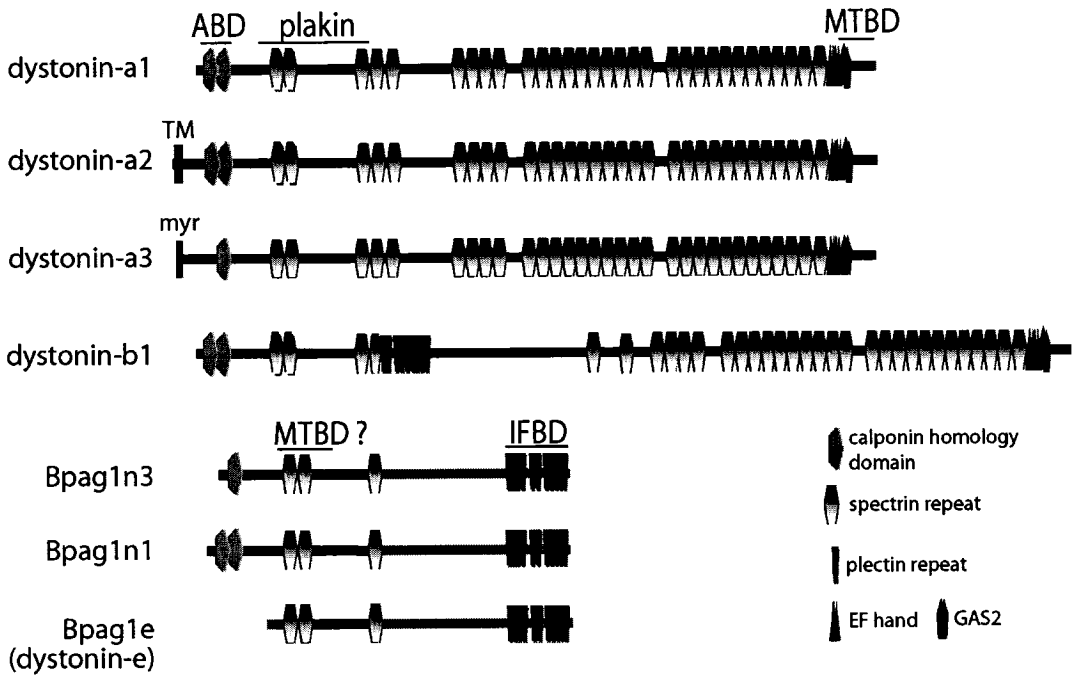
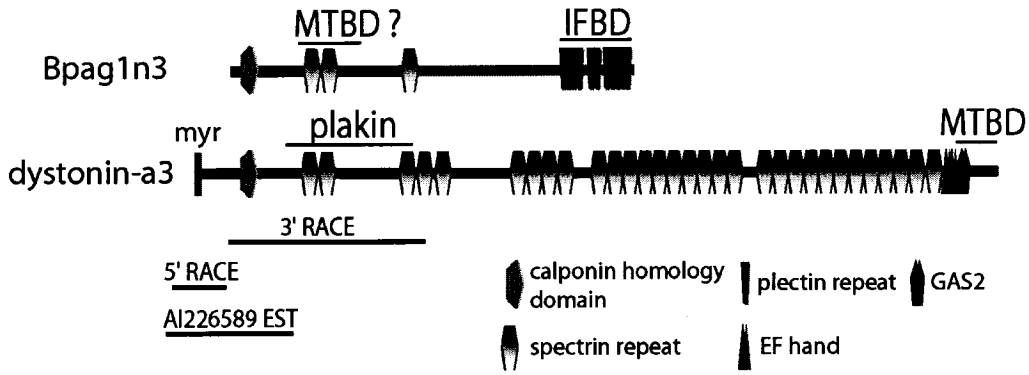
Supplementary Materials

Recombinant protein constructs

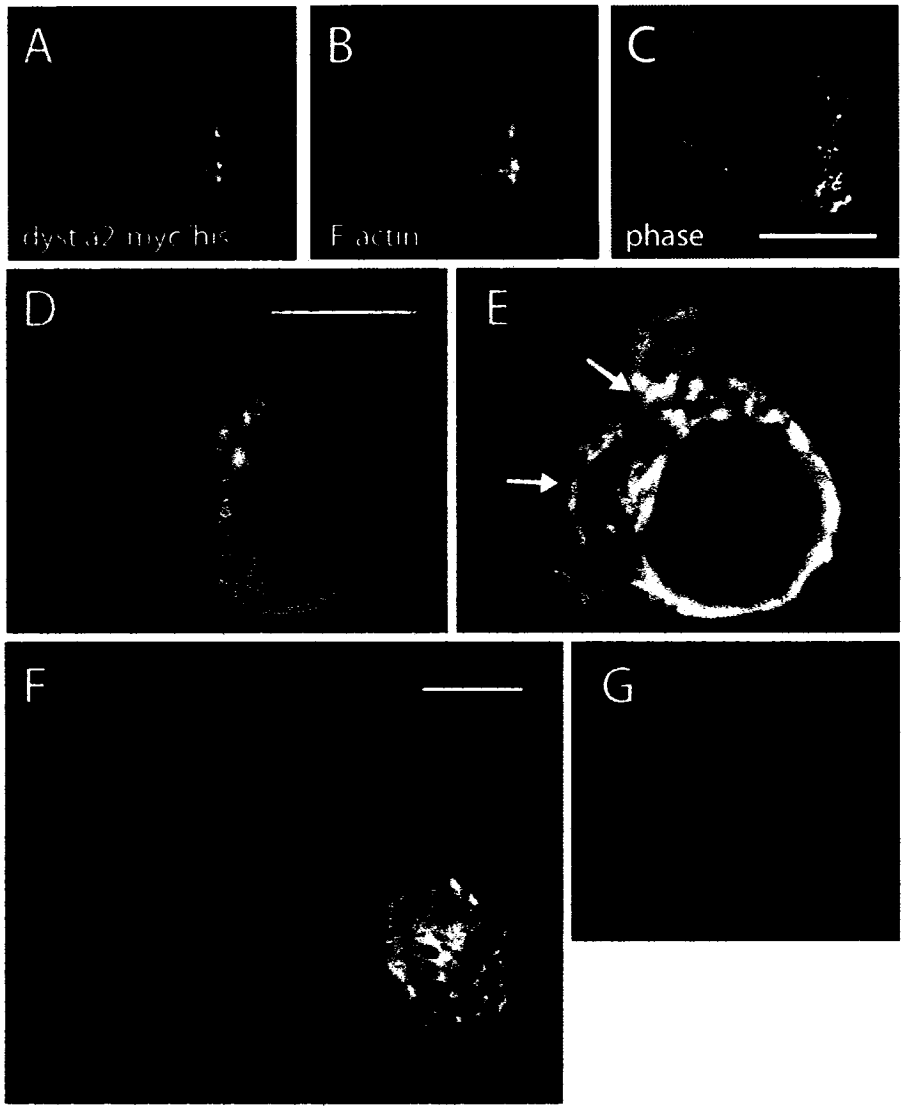
The full-length dystonin isoform 2-myc/his construct (dyst a2-myc/his) was generated by inserting cDNA from between the unique *SalI* and *BstBI* restriction sites of the previously described full-length FLAG-Bpag1a2 (FLAG-dyst a2) into the Nterm2f-myc/his (N2f-myc/his) plasmid (Young et al., 2006). An adapter oligonucleotide, 5'-CGTCGATCGA-3', was inserted at the *BstBI* site at the 3' end of the dystonin open reading frame (ORF) to remove the endogenous stop codon and to put the myc/his tag in frame. This results in sequence coding for the terminal two amino acids being replaced by the coding sequence for the C-terminal myc/his tag. Cloning of the full-length constructs was done using StablII bacteria to prevent rearrangements of these large (>22 kb) expression plasmids. The C-terminal myc/his fusion plasmid encoding only the N-terminal half of dystonin isoform 2 (a2Nterm-myc/his) was generated by inserting ORF sequence up to the second *XbaI* site from the FLAG-Bpag1a2 ORF into the Nterm2-myc/his plasmid. The N3-myc/his plasmid was made by inserting 5' sequence coding for the dystonin isoform 3 variant, including the Kozak sequence, into the Nterm2f-myc/his plasmid using an *EcoR1* – *EcoRV* restriction fragment. The primers used to amplify the isoform 3 5' end were: 5'-GAATCAAATGGGGAATGTCTGTG-3' for the forward primer and the common reverse primer described above. The other dystonin N-terminal fusion plasmids used have been described previously (Young et al., 2003; Young et al., 2006). GFP-nesprin-3α and GFP-nesprin-3β plasmids were generous gifts from Dr. Arnoud Sonnenberg (Netherlands Cancer Institute).



Supplementary figure 1. Dystonin isoforms. Domain structure for dystonin-a (predominant neuronal isoform) and dystonin-b (predominant muscle isoform) as described in Leung et al. (2001). The dystonin-a isoforms are depicted with alternate N-terminal ends (as in Brown et al., 1995, Young et al., 2006, and Jefferson et al., in press), specifying dystonin-a1, dystonin-a2, and dystonin-a3. The Bpag1n1 isoform is as described in Brown et al. (1995), though the 3' end of the transcript was not confirmed in this study. Bpag1n3 is a similar isoform which lacks the full N-terminal ABD, as described in Yang et al., 1999. Bpag1e (dystonin-e) is the epithelial isoform, whose transcript was initially characterized (Stanley et al., 1988; Sawamura et al., 1991). TM = transmembrane domain, myr = myristoylation motif, ABD = actin binding domain, MTBD = microtubule binding domain, IFBD = intermediate filament binding domain.



Supplementary figure 2. Cloning of the dystonin isoform 3 5' end. Schematic of the N-terminal ends of dystonin-a3, Bpag1n3 (Yang et al., 1999), and sequence derived from 5' and 3' RACE analysis. Isoform-specific 5' sequence for dystonin isoform 3 5' end is shown if Figure 1. The dystonin isoform 3 5' coding sequence was based on 5' RACE analysis using mouse brain RNA, as well as the sequencing of a kidney-derived mouse EST (GenBank accession #AI226589), and is identical to cDNA sequence already identified in GenBank accession #DQ463750 (Jefferson et al., 2006). 5' RACE was carried out using the SMART RACE Advantage 2 kit (BD Biosciences) as previously described (Young et al., 2006), with the following gene specific primer sequence: 5'-ACTTGAGCTGGCTGCCGTTTGGGAA-3'. The same reagents, along with the isoform 3 forward primer described in the Material and Methods, were also used for 3' RACE analysis. Labelling is as in Supplemental Figure 1.



Supplementary figure 3. Full-length dystonin isoform 2 fusion protein expressed in Cos-1 cells primarily localizes around the nucleus and ER in association with F-actin. **A,** Conventional epifluorescence image showing the dyst a2myc/his fusion in a meshwork pattern, focussed at the bottom of the nucleus. **B,** F-actin staining in the same cell. **C,** Using phase contrast, the nucleus of this cell displayed multiple vesicles (black dots) associated with it. **D and E,** 1um thick confocal sections focussed at the bottom of a nucleus (D) and at the middle of the nucleus showing overlapping dyst a2-myc/his fusion

protein localization with F-actin surrounding the ER (arrows) and nucleus. **F,** A confocal micrograph of two cells showing distinct localizations of the dyst a2-myc/his fusion protein. While the cell on the right side had fusion protein localized around the nucleus and ER, as with the majority of cells, the cell on the left side exhibited fusion protein localized in a pattern indicating microtubule association, and showed no obvious F-actin association. **G,** A confocal micrograph showing the discrete intranuclear localization of the dyst a2-myc/his fusion protein observed in a minority of cells. Scale bars = 20 μm; bar in C also applies to A, B; bar in D also applies to E; bar in F also applies to G.

Chapter 6

General Discussion

6.1 Dystonin and human disease

The diversity of genetic mutations which give rise to neuromuscular disorders indicates there are many cellular mechanisms, affecting differing subsets of neurons, glia, and muscle, which can ultimately lead to muscle weakness, incoordination, ataxic movement, spasticity, and a multitude of other neuromuscular deficits. Although the characteristics of the *dystonia musculorum* mice resemble to some degree human dystonia, the affected genes in each code for highly dissimilar proteins. The affected protein in early onset torsion dystonia, an endoplasmic reticulum/nuclear envelope resident protein, has been recently implicated in affecting neuronal NE structuring (Goodchild and Dauer, 2004; Naismith et al., 2004; Goodchild and Dauer, 2005; Goodchild et al., 2005), however, and the general findings of my thesis work have lead me to conclude that an important isoform affected in murine *dystonia musculorum* is an ER/NE structural protein. Whether this is coincidental or not would require further investigation.

The specific neurons affected in early onset torsion dystonia, thought to be primarily in the basal ganglia of the brain (Rostasy et al., 2003), are different than the sensory neurons affected in *dystonia musculorum* mice (though human histopathological analysis is notably somewhat more difficult, and this may not be definitive). While a sensory ataxia may evidently resemble the motor incoordination caused by basal ganglia dysfunction, the meaningfulness of similarities in the disorders at the cellular level is unclear. Gaining an understanding of dystonin function and its relation to neuropathology may be more likely to shed light on the importance of cytoskeletal and organelle disorganization in neurodegeneration in general, rather than on the exact mechanisms of any specific human neurological disorder.

The *dystonin* gene itself has been found to be mutated in one case of human disease (Giorda et al., 2004). A chromosomal translocation in a young girl was recently identified that removed the 3' end of the *dystonin* gene, affecting specifically the neuronal/muscle isoforms of dystonin and leaving the epithelial isoform intact. The girl suffers from severe motor deficits and mental retardation, and was born with tracheo-oesophageal atresia which was corrected at birth. The nature of the genetic mutation suggests that it is unlikely to occur commonly, though, and this case may provide an isolated example of the deleterious consequences of dystonin dysfunction in a human.

6.2 What form of dystonin is the most relevant to understanding neurodegeneration?

Following the detailed characterization of dystonin transcripts in brain, heart, and skin, three basic structures, for dystonin-a (Bpag1a), dystonin-b (Bpag1b), and dystonin-e (Bpag1; Bpag1e) were elucidated (Intro Fig. 2) (Leung et al., 2001). Additionally, the dystonin-a/b isoforms contain at least three alternate 5' ends specifying dystonin-a1, dystonin-a2, etc.... A summary of the subcellular localizations of dystonin-a isoforms is shown in Figure 1. Evidence presented over a decade ago excluded the epithelial dystonin-e protein as being important for understanding the neuromuscular phenotype observed in dystonin mutant mice, since it is expressed normally in the dt^{j} line of *dystonia musculorum* mice (Guo et al., 1995). The fact that dystonin-a/b isoform 3-specific transcript can be amplified normally from dt^{tg4} mice, and the distant location of the dt^{tg4} deletion from the isoform 3 promoter and coding region, indicates that its loss is also unlikely to be involved in generating the *dt* phenotype. This is despite prior claims that it

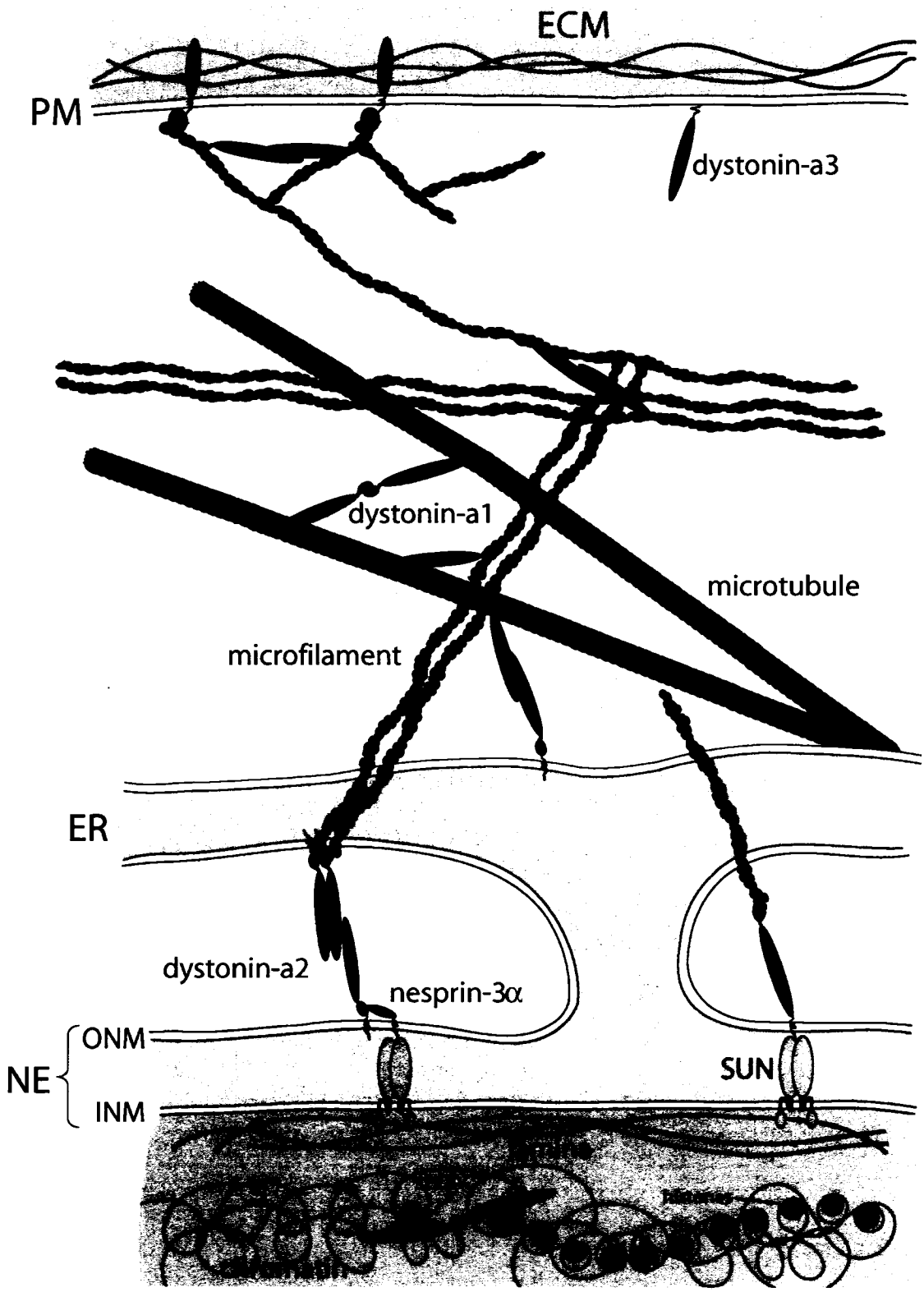


Figure 1. Summary of the demonstrated and putative localizations and interactions of dystonin-a proteins. Dystonin-a1 fusion protein expressed in Cos-1 cells indicated that it

can associate with, bundle, and likely cross-link microtubules and actin microfilaments throughout the cytoplasm and at adhesion structures at the plasma membrane (PM). The dystonin-a2 isoform contains an N-terminal transmembrane domain which inserts into membranes surrounding the nucleus. This likely includes endoplasmic reticulum (ER) and outer nuclear membrane (ONM). This isoform may be retained in the ONM by an association with nesprin-3α. The predominant cytoskeletal interaction of this isoform is with actin filaments. As well, dystonin-a2 can localize inside the nucleus via a nuclear localization signal. Inside the nucleus, it may interact with histone deacetylase (HDAC) proteins, indicating a possible involvement in regulating chromatin structure and gene transcription. Dystonin-a3 contains a putative myristoylation motif at its N-terminus, and expression of the dystonin-a3 N-terminus in Cos-1 cells indicates that this may facilitate its localization to the plasma membrane. Other proteins drawn indicate focal contact proteins (green and light and dark purple), transcription regulation complexes (light and dark pink), and large isoform nesprins (dark green/orange). ECM = extracellular matrix; INM = inner nuclear membrane; NE = nuclear envelope.

is very important for axonal microtubule stabilization and its loss leads to the *dt* neurodegeneration (Yang et al., 1999). It is more likely that the dystonin-a/b isoforms 1 and/or 2 are most relevant to understanding the *dt* phenotype, since these are the isoforms affected by each of the characterized *dt* mutations.

Since the *dt* phenotype has been characterized primarily as a neuropathy, with defects in sensory neurons being repeatedly demonstrated in different lines of *dt* mice, I have focussed on the neuronal dystonin-a isoforms. In particular, the dystonin-a2 isoform appears to be the most unique and least likely to be compensated for by any related protein (Chapter 5). The isoform 2 variant was also early-on characterized as being predominantly neuronal, with its expression being most prominent in sensory ganglia (Bernier et al., 1995). At the subcellular level, dystonin localization in DRG neurons strikingly resembles the localization of dystonin-a2 fusion protein expressed in Cos cells. As well, defects in the neuronal perikarya of dt^{tg4} mice appear to be reflective of the loss of function of dystonin-a2 associated with the ER. Though a rigorous analysis using transgenic rescue is needed to prove the point definitively, it seems likely that dystonin-a2 is a necessary neuronal protein absent in *dt* mice which is at least partly responsible for the generation of the *dt* phenotype.

Prior to the elucidation of the dystonin-a transcript structure and the isoform 2 transmembrane domain coding region, studies had indicated that loss of dystonin likely resulted directly in disorganization of the axonal cytoskeleton due to the loss of a physical link between different types of cytoskeletal filaments (Yang et al., 1996; Leung et al., 1999a; Yang et al., 1999). Originally, loss of neuronal dystonin (Bpag1n) was proposed to result in the loss of an essential link between neurofilaments and actin

microfilaments (Yang et al., 1996). It was subsequently demonstrated, however, that the dystonin intermediate filament binding domain, even if present in neuronal dystonin, does not interact with neurofilaments (Leung et al., 1999a). Rather, this binding domain was demonstrated to interact with peripherin, an intermediate filament protein expressed in peripheral neurons (Leung et al., 1999a). This interaction may be important to neuronal dystonin function in maintaining axonal cytoskeleton organization. However, the same author who demonstrated this subsequently demonstrated that the predominant neuronal dystonin isoform (dystonin-a; Bpag1a) does not contain the C-terminal intermediate filament binding domain, and thus is not likely to interact with any neuronal intermediate filaments at all (Leung et al., 2001a). All of the early studies demonstrating important dystonin functions likely to be absent in *dt* mice failed to provide any proof that restoring the putative missing functions could restore *dt* neurons to normal, however (e.g. through transgenic rescue, or rescue experiments in cell culture experiments). It is likely that a lack of knowledge of the complete dystonin transcript structures lead to severe misinterpretations of the data, and only with transgenic rescue experiments based on our current knowledge of the dystonin transcripts will we be able to definitely assess what necessary dystonin functions are lost in *dt* neurons.

6.3 Is dystonin in the nucleus important?

Earlier results of mine indicated that dystonin contains a nuclear localization signal, and can actively translocate into the nucleus (Chapter 3). With the use of full-length fusion proteins expressed in Cos cells, I have repeatedly observed nuclear localization of the dystonin-a2 isoform, though only in a relatively small number of cells (Chapters 4 and 5).

Dystonin-a1 fusion protein does not localize to the nucleus (Chapter 4), and the dystonin isoform 3 N-terminus appears to direct the protein to the plasma membrane (Jefferson et al., in press; Young and Kothary, unpublished observations). In DRG neurons, while most of the dystonin appeared to be localized outside the nucleus, relatively stronger intranuclear staining was observed in a few neurons (Chapter 5). It is therefore possible that dystonin-a2 does play a role in the nucleus *in vivo*, though what its importance may be is unclear.

Interestingly, we have recently observed by bioinformatics analysis the presence of a putative histone deacetylase (HDAC) interacting domain within the conserved plakin domain of dystonin (Intro Fig. 2). HDAC proteins are key regulators of gene expression via their interactions with histones (Hisahara et al., 2005). Structural similarities to SMC ATPase proteins, involved in DNA repair, are also present in the different dystonin isoforms (as indicated, for instance, in GenBank entries for dystonin proteins). As discussed in Chapter 2, other spectrin superfamily members are known be functional within the nucleus, and an involvement in nuclear structure and function may represent a recurrent feature within this group of large structural proteins. Most recently, α-spectrin ($\alpha SpII\Sigma^{*}$) has been demonstrated to interact with multiple types of nuclear proteins (Sridharan et al., in press), further indicating that spectrin superfamily members can serve as scaffolding proteins to help organize the highly compartmentalized nucleus. An analysis to assess the functionality of the putative HDAC interacting domain, as well as an analysis of when and where dystonin is most prominently found in the nucleus should help to shed light on the importance of nuclear dystonin.

6.4 What is the relevance of neurofilament aggregates and axonal swellings in *dt* mice?

The development of axonal swellings in *dt* neurons has been demonstrated through the use of chimaeric mutants to be a cell-intrinsic phenomenon (Campbell and Peterson, 1992). Though they have been associated with aggregates of hyperphosphorylated neurofilaments, the swellings occur independently of neurofilament aggregates (Eyer et al., 1998). And, though it has been suggested that cytoskeletal disorganization may occur throughout the *dt* axon (Yang et al., 1999; De Repentigny et al., 2003; Liu et al., 2003), earlier descriptions of axonal cytoskeleton disorganization were limited to neurofilament accumulation within the axonal swellings (Janota, 1972; Hanker and Peach, 1976; Sotelo and Guenet, 1988). It seems plausible that neurofilaments appear disorganized within these restricted sites along the axon only as a result of a generally altered morphology and accumulation of other cellular debris, such as clustered mitochondria, within the swellings.

What, then, causes the axonal swellings? Axonal swellings are not unique to *dt* mice, and also develop in neurodegenerative diseases including amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, Alzheimer's disease, and cerebrospinal ataxia (Garcia-Fresco et al., 2006). Transgenic mice containing defects in normal axonal-glial interactions also display focal swellings containing disorganized neurofilaments and accumulations of mitochondria (Griffiths et al., 1998; Garcia-Fresco et al., 2006). Since *dt* mice clearly contain myelination defects (Duchen and Strich, 1964; Kothary et al., 1988; Bernier et al., 1998), altered interactions of the *dt* sensory neurons with myelin may result in the formation of the swellings. However, as noted, the *dt* swellings can

occur in a cell-intrinsic manner. Therefore, while aberrant myelination may be a contributing factor to the axonal swellings, it is unlikely the main cause.

Other causes that have been suggested for axonal swellings include defects in mitochondrial function and axonal transport (Ferreirinha et al., 2004), and NMDA receptor signalling following a neurotoxic stimulus (Takeuchi et al., 2005). Axonal transport has been demonstrated to be affected in *dt* mice (De Repentigny et al., 2003), though short range transport in cultured neurons is unaffected in cultured *dt* sensory neurons (Pool et al., 2006). Swellings have never been reported in cultured *dt* neurons, and it is possible that this is a phenomenon limited to mature neurons in the animal. Intrinsic axonal transport defects in *dt* mice may cause the accumulation of cytoskeletal filaments and organelles along the axon, and result in the focal swellings. In combination with altered myelin interactions, this could account for the consistent observation of axonal swellings as being one of the more prominent features of the *dt* pathology.

6.5 What is the relevance of perikaryal abnormalities in *dt* mice?

The first indications that the neuronal cell body is directly affected by the *dt* mutations were the observations in early phenotype stage mice of Nissl substance displacement, nuclear eccentricity, and cell body swelling – all features of chromatolytic neurons (Hanker and Peach, 1976). Since chromatolysis is a feature observed following axonal damage, it is possible that these features may occur, at least in part, as a result of the *dt* myelination defects. This is consistent with my observation that neurons in early phenotype stage mice which display increased nuclear eccentricity belong to the larger, myelinated subpopulation (as indicated by RT-97 staining) (Chapter 5).

These cells did not, however, contain the increased numbers of F-actin punctae that were observed in the smaller, weakly RT-97 positive neurons. Since the neuronal perikarya which stained with dystonin antibody were also those that were weakly RT-97 positive, it is possible that this represents an intrinsic defect due to loss of ER-associated dystonin. As the loss or displacement of Nissl substance in the cytoplasm represents a disorganization of the rough ER, a common feature in the *dt* sensory neurons may be death and dysfunction due to activation of the ER stress response and/or altered calcium homeostasis.

Our work demonstrating the localization and possible functions of a dystonin isoform that is well-expressed in sensory ganglia, and which is affected in each of the characterized *dt* lines, indicates that disorganization associated with internal membranous structures is a possible cause of the cell-intrinsic defects in sensory neurons. Aberrant organization of the ER could explain the progressive neurodegeneration observed in these mice. A further analysis using transgenic rescue of neurons with the dystonin-a2 protein should prove the necessity of this particular protein to sensory neuron function. Rescue experiments aimed at expression of rescue transgenes in the myelinating Schwann cells versus the sensory neurons should help delineate the extent to which dysfunction results from intrinsic neuronal defects or aberrant myelination. The use of transgene expression in neurons should also facilitate observations of protein localization and function of the specific dystonin isoforms *in vivo*.

Since the *dt* mice share cellular defects common to several neurodegenerative disorders, this work should help define exactly what mechanisms are responsible for

generating the commonly observed characteristics of neurodegeneration. This may help clarify the cellular processes that should be targeted by potential treatments for neurodegenerative disorders, and should stand as one of the long-term goals of this research.

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