The Intermediate Filament Protein Peripherin Is the Specific Interaction Partner of Mouse BPAG1-n (Dystonin) in Neurons

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Abstract. The dystonia musculorum (dt) mouse suffers from severe degeneration of primary sensory neurons. The mutated gene product is named dystonin and is identical to the neuronal isoform of bullous pemphigoid antigen 1 (BPAG1-n). BPAG1-n contains an actin-binding domain at its NH₂ terminus and a putative intermediate filament-binding domain at its COOH terminus. Because the degenerating sensory neurons of dt mice display abnormal accumulations of intermediate filaments in the axons, BPAG1-n has been postulated to organize the neuronal cytoskeleton by interacting with both the neurofilament triplet proteins (NFTPs) and microfilaments. In this paper we show by a variety of methods that the COOH-terminal tail domain of mouse BPAG1 interacts specifically with peripherin, but in contrast to a previous study (Yang, Y., J. Dowling, Q.C. Yu, P. Kouklis, D.W. Cleveland, and E. Fuchs. 1996. Cell. 86:655–665), mouse BPAG1 fails to associate with full-length NFTPs. The tail domains interfered with the association of the NFTPs with BPAG1. In dt mice, peripherin is present in axonal swellings of degenerating sensory neurons in the dorsal root ganglia and is downregulated even in other neural regions, which have no obvious signs of pathology. Since peripherin and BPAG1-n also display similar expression patterns in the nervous system, we suggest that peripherin is the specific interaction partner of BPAG1-n in vivo.

Key words: BPAG1 • dystonin • peripherin • neurofilament • intermediate filament

Intermediate filaments (IFs) are 10-nm filaments found in most eukaryotic cells and are made up of various IF proteins. Tissue-specific and developmental stage-specific expressions are intrinsic features of IF proteins. In the mammalian nervous system, five IF proteins are specifically expressed in differentiated neurons: the neurofilament (NF) triplet proteins (NF-L, NF-M, and NF-H), α-internexin, and peripherin (Fliegner and Liem, 1991; Ho and Liem, 1996; Lee and Cleveland, 1996). In the adult, the neurofilament triplet proteins (NFTPs) are widespread in the mature nervous system. They coassemble into heteropolymorphic NFs and are especially abundant in neurons with large axons. α-Internexin and peripherin are more limited to small neurons, with α-internexin primarily in the central nervous system (CNS) and peripherin predominantly in the peripheral nervous system (PNS). During the development of the CNS, the expression of α-internexin precedes that of the NFTPs (Kaplan et al., 1990; Fliegner et al., 1994). For example, at embryonic day 12 (E12) in rat, α-internexin mRNA is readily detected in the basal forebrain where neurogenesis has just begun, whereas NF-L and NF-M are still minimal or absent (Fliegner et al., 1994). NF-H appears even later in development and its expression is very limited until after birth (Shaw and Weber, 1982; Pachter and Liem, 1984). Similar to α-internexin, peripherin is expressed early in development (Leonard et al., 1988; Parysek et al., 1988; Parysek and Goldman, 1988; Escurat et al., 1990; Troy et al., 1990a). As early as E9 in mouse and E12 in rat, peripherin can be detected in the PNS, e.g., in the dorsal root ganglia (DRG), cervical ventral roots, cranial nerve ganglia, and the enteric nervous system. The expression of peripherin continues through adulthood in the PNS. In adult rat, the expression of peripherin has also been observed in the CNS. For example, a subset of brainstem reticular formation was shown to be immunoreactive to a peripherin antiserum (Brody et al., 1989).

IFs are associated with a class of cytoskeletal cross-linking proteins, known as plakins or cytolinkers (Green et al.,
detected as early as E9.5 in differentiated neurons of the development of the nervous system, BPAG1-n can be peripheral (Parysek et al., 1988; Troy et al., 1990a). During 1997). These expression patterns are similar to that of pe- present in neurons of the sympathetic nervous system, the Dowling et al., 1997). In addition, BPAG1-n is also spread in sensory neurons of adult mice, such as those in the DRG and cranial nerve ganglia (Bernier et al., 1995; Dowling et al., 1996). Three alternatively spliced isoforms of BPAG1 have been fully characterized: one epidermal iso- form (BAPG1-e) and two neuronal isoforms (BPAG1-n) (Brown et al., 1995a,b). Similar to the other members of the plakin/kytolinker family, BPAG1 exhibits a three- domain structure, consisting of a central rod domain flanked by NH₂-terminal head and COOH-terminal tail domains (Ruhrberg and Watt, 1997). The majority of the rod domain is composed of heptad repeats that mediate coiled-coil dimer formation. However, it is not known whether higher order structures can be formed. The NH₂- terminal head domain consists of six α-helical segments, whereas the COOH-terminal tail domain is made up of two homologous subdomains, known as the B and C do- mains (Green et al., 1992). The COOH-terminal tail domain has been suggested to interact with cytokeratins and NFs (Guo et al., 1995; Dowling et al., 1996; Yang et al., 1996). In addition, both of the neuronal isoforms of BPAG1 contain putative actin-binding domains at the ends of their NH₂ termini (Brown et al., 1995a).

Unlike plectin which is expressed only in a small subset of motoneurons (Errante et al., 1994), BPAG1-n is wide- spread in sensory neurons of adult mice, such as those in the DRG and cranial nerve ganglia (Bernier et al., 1995; Dowling et al., 1997). In addition, BPAG1-n is also present in neurons of the sympathetic nervous system, the enteric nervous system, and the cerebellum (Dowling et al., 1997). These expression patterns are similar to that of pe- ripherin (Parysek et al., 1988; Troy et al., 1990a). During the development of the nervous system, BPAG1-n can be detected as early as E9.5 in differentiated neurons of mouse embryos (Dowling et al., 1997), coinciding with the expression of α-internexin and peripherin, but apparently is expressed before the NFTPs. The embryonic expression of BPAG1-n is not restricted to the developing sensory neurons; it is also detected in motoneurons. The impor- tance of BPAG1 to maintain the integrity of the nervous system has been elucidated by knockout studies of the BPAG1 gene in transgenic mice (Guo et al., 1995) and by studies of natural mouse mutants with the neurological disorder, dystonia musculorum (dt). Mice carrying muta- tions in the BPAG1 gene, as a consequence of spontane- ous mutations or gene targeting, develop dystonic move- ment, hyperextension, and hyperflexion of the limbs, and often die before weaning (Duchen et al., 1963; Duchen and Strich, 1964; Duchen, 1976; Guo et al., 1995). As a re- sult, BPAG1-n is also known as dystonin. Histopathologi- cal studies revealed that dt mice and BPAG1−/− mice suffer from severe degeneration of primary sensory neu- rons. This pathology is especially prominent in the DRG, where degenerating neurons display abnormal accumula- tions of IFs in the axons (Duchen and Strich, 1964; Janota, 1972; Sotelo and Guenet, 1988; al-Ali and al-Zuhair, 1989; Guo et al., 1995). A recent study indicated that the COOH-terminal tail domain of BPAG1 colocalized with NF-L/NF-H heteropolymeric filaments in transiently trans- fected cells and communoprecipitated with overexpressed NF-H proteins (Yang et al., 1996). Furthermore, full- length BPAG1-n protein coagulated with some of the fila- ments formed by the transfected NF-L and NF-H and the endogenous stress fibers in transfected cells. These studies led to the hypothesis that BPAG1-n is a cytoskeletal cross- linking protein, connecting NFs to microfilaments, and its functional perturbation causes NF disorganization that ul- timately leads to neuronal degeneration (Brown et al., 1995a; Yang et al., 1996). However, the distribution pat- tern of BPAG1-n is much more restricted in the nervous system as compared with the NFTPs. This difference led to the question of whether BPAG1-n might interact with other neuronal IFs (nIFs). Therefore, we investigated the interactions of this protein with all of the nIFs. Surpris- ingly, we observed that the tail domain of BPAG1 associ- ated with α-internexin and peripherin filaments, but not with filaments formed by full-length NFTPs.

Materials and Methods

Plasmid Construction
cDNAs of mouse BPAG1 COOH-terminal domain (mBPAG1-C1) were synthesized by RT-PCR based on the original partial mouse BPAG1 se- quence (Amagai et al., 1990). In brief, 1 μg of poly(A)⁺ RNA from mouse brain (Clontech) was primed with random hexamers and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s protocol (Perkin Elmer). Three pairs of primers were used to amplify three consecutive overlapping fragments of the mBPAG1-C1 cDNA. The first pair of primers included sense primer 5'- AAAACCTAGTTAAGGCAAGTT-3' and antisense primer 5'- TTAAAGTCGACTCTGATGCT-3'; the second pair of primers included sense primer 5'-AAGGTTCCTCACTCGTCTAC-3' and antisense primer 5'-GAACTAGCTACGGCAGTTGAG-3'; and the third pair of primers included sense primer 5'-CTAGGCCGCTAC-3' and antisense primer 5'-TCTGAGATAGTAACTG-3'. The last antisense primer covers the termi- nal codon of BPAG1. The three amplified fragments were subcloned into the pGEM-T vectors (Promega) and sequenced to confirm their identities. Unique restriction enzyme recognition sites, HindIII and Stul, at the overlapping regions were used to reconstruct the mBPAG1-C1 cDNA. To generate pcDNA-FLAG-mBPAG-C1, the recovered mBPAG- C1 cDNA was ligated in frame to the 3' end of the FLAG epitope tag se- quence in a pcDNA3 (Invitrogen) derived expression vector. The original FLAG epitope tag-containing vector was a generous gift from Dr. Howard Worman (Columbia University, New York). To generate pcDNA-FLAG-mBPAG-C2, the pcTOPO-mBPAG-C1 cDNA was first cloned into the pCR2.1-TOPO vector (Invitrogen). The 2.3-kb EcoRI-EcoRV fragment of pTOPO- mBPAG-C2 was then ligated to the 5.4-kb fragment of EcoRI/EcoRV digested pcDNA-FLAG-mBPAG-C1 to make pcDNA-FLAG-mBPAG-C2. All of the NFTPs, α-internexin, and peripherin expression constructs were cloned in the expression vector pRSV1 and were described previ- ously (Chin and Liem, 1989, 1990; Chin et al., 1991; Ching and Liem, 1993, 1996; Ho et al., 1995; Sun et al., 1997). To facilitate the coexpression of NF-L and NF-H in transiently transfected cells, a double expression con- struct, pcI-NFL/NFH, was created. pcI-NFL was first generated by cloning the blunt-ended 2.0-kb HindIII fragment from pRSV1-NFL into the Smal site of pcI vector (Promega). pcI-NFH was then made by cloning

The Journal of Cell Biology, Volume 144, 1999
the 3.4-kb EcoRI fragment from pGBT-NFH into the EcoRI site of pCI vector (Promega). Afterwards, the 4.7-kb BamHI-BgII fragment of pCI-NFH, which contained the expression cassette of NFH, was isolated and ligated to the BamHI digested pCI-NFL to yield pCI-NFL-NFH.

Two-hybrid and three-hybrid constructs were made with plasmids pGBT9, pGAD424, pAS2-1, and pACT2 (Clontech). pGBT-mBPAG-C1 and pAS2-mBPAG-C1 were constructed by cloning the 2.2-kb BglII-BamHI fragment from pGBT-mBPAG-C1. All of the full-length and truncated NTFP two-hybrid constructs in pGAD424 have been described previously (Leung and Liem, 1996). To engineer pGAD-α-internexin, the NcoI (blunted)-BamHI fragment comprising the full-length α-internexin cDNA was isolated from pET-α-internexin and ligated to the BamHI-digested pGAD. pGAD-Peri35-474 was engineered by inserting the 1.8-kb BamHI-SmaI fragment of pRSVí-perM1M2 (Ho et al., 1995) into the SmaI-BamHI–pGBT-Peri35-474. All of the full-length and truncated NTFP two-hybrid constructs in pGAD424 have been described previously (Leung and Liem, 1996). To engineer pGAD-α-internexin, the NcoI (blunted)-BamHI fragment comprising the full-length α-internexin cDNA was isolated from pET-α-internexin and ligated to the BamHI-digested pGAD. pGAD-Peri35-474 was engineered by inserting the 1.8-kb BamHI-SmaI fragment of pRSVí-perM1M2 (Ho et al., 1995) into the SmaI-BamHI–pGBT-Peri35-474. All of the full-length and truncated NTFP two-hybrid constructs in pGAD424 have been described previously (Leung and Liem, 1996).

To generate pACT-NFL24-542, pACT-NFM, and pACT-NFH, the HindIII fragments, containing the hybrids GAL4 activation domain (GAD)-fusion cDNA of pGAD-NFL24-542, pGAD-NFM, and pGAD-NFH, were cloned into the HindIII-digested pACT2 vector, respectively. To construct three-hybrid vector pGAD-NFL1-415/NFH1-415, pGAD-NFL1-415 was digested with SpII; and the 2.5-kb fragment containing the GAD-NFL1-415 expression cassette was then subcloned into the SpII partially digested pGAD-NFH1-415. Similar strategies were applied to create the other three-hybrid constructs, pGAD-NFL1-415/NFH1-415, pGAD-NFM1-421/NFM1-421, pGAD-NFH1-415/NFH1-415, and pGAD-NFL1-415/NFM1-421.

Prokaryotic expression constructs were made with plasmids, pET-3a, pET-3b, pET-16b, and pET-21d (Novagen). To create pET-mBPAG-C2, the 2.4-kb PCR fragment of pDNA-FLAG-mBPAG-C2 with sense primer 5'-CCACATGGACTACAAGGACGACG-3' and antisense primer 5'-ACGGGATCCTGGGAAGAATAGTAGAGG-3' was digested with BamHI and NcoI before being ligated to the BamHI-NcoI–digested pET-21d vector. To generate pET-NFL-NL cDNA in pGEM4 vector (Chin et al., 1989) was digested with StuI and the purified insert was ligated to BamHI linkers before cloning into the BamHI site of pET-3b. Similarily, full-length NF-M cDNA (Napolitano et al., 1987) was digested with EcoRI; the insert was purified, blunt-ended, and ligated to BamHI linkers. The BamHI-linked NF-M cDNA was then cloned into BamHI-digested pET-3a to make pET-NFM. For pET-NFII, the first ATG of NF-H cDNA was first mutated to an NdeI site. The NdeI-NfH NF-H fragment was then purified and cloned into NdeI-BamHI–digested pET-3a. To engineer pET-peripherin, the 1.8-kb BamHI-Smal fragment of pRSVí-perM1M2 (Ho et al., 1995) was ligated to 8-mer BamHI linkers, digested with BamHI, and cloned into the BamHI site of pET-16b vector.
Antibodies

The following primary antibodies were used for immunofluorescence staining, immunohistochemistry, and Western blots: mouse anti-FLAG M2 mAb (IBI-Kodak); rabbit polyclonal anti-α-internexin Ab(NcoA antibody and mouse anti-α-internexin mAb135 mAb (Kaplan et al., 1990); rabbit polyclonal antiperipherin Ab6264 antibody (Aletta et al., 1988) and mouse antiperipherin mAb (Chemicon); rabbit polyclonal antivimentin antibody (a gift from Dr. Eugenia Wang, Lady Davis Institute for Medical Research, Montreal, Canada); mouse anti-NFL NR4 mAb (Sigma); and rabbit polyclonal antibodies against NF-L (Kaplan et al., 1991).

Results

Isolation of a cDNA Clone Encoding the Mouse BPAG1 COOH-terminal Domain

To study the interactions of BPAG1 with nIFs, we isolated three consecutive overlapping pieces of the BPAG1 cDNA by RT-PCR on mouse brain mRNA. The three amplified DNA fragments were ligated together to yield a ~3.0-kb partial mouse BPAG1 cDNA. Sequencing of this cDNA showed that it encoded 988 amino acids, including the entire tail domain (768 amino acids) and the COOH terminus of the rod domain (220 amino acids). Because the complete mouse BPAG1 cDNA sequence had not been fully characterized (Amagai et al., 1990), the nomenclature of the mouse BPAG1 COOH-terminal proteins was designated according to the sequence of the human BPAG1 epidermal isoform (Fig. 1). We compared our sequence with several EST clones using BLAST. The sequences of a series of I.M.A.G.E. clones (clones 1262090, 931676, 1227492, 1242939, 975299, 317229, and 975617) are identical to the sequence of mBPAG-C2 and cover 75% of the sequence. Furthermore, the mBPAG-C1 sequence is identical to the deduced protein sequence of BPM1, a partial mouse BPAG1 cDNA (accession number 321215; Amagai et al., 1990). The accession number for the mouse BPAG-C1 sequence is AF115383.

As deduced from the respective cDNA sequences, the amino acid composition of the mouse BPAG1 tail domain is ~80% identical to that of the human BPAG1 tail domain. Similar to the human orthologue, the tail domain of mouse BPAG1 contains two homologous subdomains, B and C.

Mouse BPAG1 COOH-terminal Protein Associates with α-Internexin and Peripherin, but Not with NFTPs in the Yeast Two-Hybrid System

A recent study on the interactions of desmoplakin with cytokeratins indicated that the yeast two-hybrid system is a useful tool to characterize associations between plakins/cytolinkers and IF proteins (Meng et al., 1997). Therefore, we initially studied the associations of mBPAG-C1 with nIF proteins by a similar approach. mBPAG-C1 was fused with the GBD in vector pGBT9 (Fig. 1), and was tested in pairwise combinations with various GAD-fused nIF proteins that were expressed by the pGAD424 vector. Interactions between mBPAG-C1 and nIF proteins were determined by qualitative β-galactosidase filter lift assays of the yeast cotransformants. Surprisingly, mBPAG-C1 interacted with α-internexin and peripherin, but not with vimentin, NF-L, NF-M, or NF-H in the two-hybrid studies (Table I). To determine whether the last 220 amino acids of the mouse BPAG1 rod domain in mBPAG-C1 interact nonspecifically with the IF proteins in the two-hybrid system, we also prepared GBD-mBPAG-r220 and tested for its binding with each of the GAD-nIFs. No interactions between GBD-mBPAG-r220 and any one of the GAD-nIFs were observed (Table I). Because the expression levels of fusion proteins might play a role in detecting weak interactions, we also cloned cDNAs of mBPAG-C1 and each of the NFTPs into higher expression two-hybrid shuttle vectors, pAS2-1 and pACT2, and reexamined their interactions. However, we were still not able to detect any interactions between mBPAG-C1 and any of the NFTPs (Table I).
Mouse BPAG1 COOH-terminal Proteins Colocalize with α-Internexin and Peripherin Filaments, but Not with NFTP Filaments in Transiently Transfected Cells

The results of the two-hybrid experiments prompted us to repeat some of the transient transfection assays performed by Yang et al. (1996) on mBPAG-C1 and NFTPs. In addition, we wanted to confirm the interactions of mBPAG-C1 with α-internexin and peripherin. To facilitate the detection of mBPAG-C1 in the transient transfection assays, the NH$_2$ terminus of mBPAG-C1 was fused to a FLAG epitope tag, which also provided the translational start codon (Fig. 1). All transfections were performed in a human adrenal carcinoma cell line, SW13.cl.2Vim and vimentin filament networks in transient transfection studies. Since none of the NFTP subunits can self-assemble into normal filamentous networks in transiently transfected cells (Fig. 2, A and B). In contrast, mBPAG-C1 appeared to disrupt the normal filament formation of α-internexin. α-Internexin has been shown previously to self-assemble into normal filamentous networks in transiently transfected cells from transient transfections of pRSVi-α-internexin (E and F), or vimentin (H and I). mBPAG-C1 associated with the filament network of peripherin, but disrupted the normal filament network of α-internexin. The perturbed α-internexin filament bundles were decorated with mBPAG-C1. The expressed mBPAG-C1 also appeared to affect the morphology of the vimentin filament network. Bar, 10 μm.

Table I. Two-Hybrid Analysis of the Interactions between GBD-mBPAG1 Proteins and NFT Proteins

|          | mBPAG-C1 | mBPAG-r220 |
|----------|----------|------------|
| NF-L     | -        | -          |
| NF-M     | -        | -          |
| NF-H     | -        | -          |
| α-Internexin | +       | -          |
| Peripherin | +       | -          |
| Vimentin | -        | -          |

SFY526 cells cotransformed with pGHT-mBPAG-C1 or pGHT-mBPAG-r220 and various pGAD-NFs were selected in minus Trp-Leu media. The plus and minus signs represent the results of the β-galactosidase filter lift assays on cotransformants. The NF-L and peripherin constructs code for amino acids 24–542 of NF-L and amino acids 35–474 of peripherin, respectively. In the case of NFTPs studies, identical results were obtained from GAD-fused NFTPs expressed by vector pACT2 and GBD-fused mBPAG-C1 expressed by vector pAS2-1.

Figure 2. Analysis of mBPAG-C1 with peripherin, α-internexin, and vimentin filament networks in transient transfection studies. SW13.cl.2Vim$^-$ cells from transient transfections of pRSVi-peripherin (C), pRSVi-α-internexin (F), or pRSVi-vimentin (I), and cotransfections of pcDNA-FLAG-mBPAG-C1 with (A and B) pRSVi-peripherin, (D and E) pRSVi-α-internexin, or (G and H) pRSVi-vimentin were double-labeled with mouse anti-FLAG M2 mAb (A, D, and G) and rabbit polyclonal antibodies against peripherin (B and C), α-internexin (E and F), or vimentin (H and I). mBPAG-C1 associated with the filament network of peripherin, but disrupted the normal filament network of α-internexin. The perturbed α-internexin filament bundles were decorated with mBPAG-C1. The expressed mBPAG-C1 also appeared to affect the morphology of the vimentin filament network. Bar, 10 μm.

were indeed distinct, we performed confocal microscopy on the cells cotransfected with mBPAG-C1, NF-L, and NF-H. As shown in Fig. 3, C and D, the filamentous staining pattern observed for NF-L/NF-H filaments did not colocalize with the diffuse mBPAG-C1 staining pattern.

The discrepancy between our results and Yang et al. (1996) could be due to the presence of the partial rod domain in our mBPAG-C1 protein. To investigate this possibility, transient transfections with pcDNA-FLAG-mBPAG-C2 construct were carried out. mBPAG-C2 contains only the mouse BPAG1 tail domain (Fig. 1) and is therefore the mouse equivalent of the human BPAG1 tail domain used by Yang et al. (1996). As shown in Fig. 4 by confocal microscopy, mBPAG-C2 also colocalized with the peripherin filament network, but not with the NF-L/NF-H filament network. Moreover, mBPAG-C2 disrupted the α-internexin filament network and showed no correlation with the NF-L/NF-M filament network (data not shown). Because identical results were obtained by using either mBPAG-C1 or mBPAG-C2, we concluded that the presence of the partial rod domain in mBPAG-C1 did not.

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affect its interaction with IF networks in transient transfection assays.

**Mouse BPAG-C2 Interacts with Peripherin, but Not with NFTPs in Overlay Binding Assays**

To confirm the specificity of the interaction between mBPAG-C2 and peripherin, we also used in vitro overlay binding assays. Peripherin was chosen, instead of α-internexin, because its in vivo expression pattern resembles that of BPAG1-n; therefore, their interaction is of more physiological significance (Parysek and Goldman, 1988; Troy et al., 1990a; Dowling et al., 1997). Recent studies on the interactions of plectin and desmoplakin with IF proteins demonstrated that the association between these proteins can be monitored by overlay binding assays (Nikolic et al., 1996; Meng et al., 1997). Hence, we examined the interactions between mBPAG-C2 and IF proteins by similar strategies. Bacterially expressed IF proteins and mBPAG-C2 were successfully purified by column chromatography (Fig. 5 A). Purified IF proteins were resolved by SDS-PAGE and transferred onto a nylon membrane. After the removal of SDS by washing in PBS (0.05% Tween 20 and 5% BSA), the membrane was incubated with mBPAG-C2 protein. The bound mBPAG-C2 was detected by an anti-FLAG mAb. As illustrated in Fig. 5 B, mBPAG-C2 associated with peripherin protein, but not with the NFTPs.

Since it is possible that the interaction of mBPAG-C2 and NFs occurs only with polymerized IFs, we reassembled peripherin, as well as combinations of NF-L/NF-M and NF-L/NF-H, in vitro by dialysis against PBS. The reassembled filaments were then examined for possible interactions with mBPAG-C2 using a slot-blot overlay assay. The relative amounts of mBPAG-C2 bound to the various polymerized IFs were measured by densitometric analysis. As shown in Fig. 5 C, the BPAG1 tail domain interacted with polymerized peripherin, but not with polymerized NF-L/NF-H filaments. Bar, 10 μm.

**Mouse BPAG-C1 Interacts with Tailless NFTPs in the Two-Hybrid Assays**

Since we have shown that NF-H and NF-M prefer to form heterodimers with NF-L (Leung and Liem, 1996), we performed three-hybrid studies to examine whether mBPAG-C1 could associate with heterodimers of NF-L/NF-M (L/M) or NF-L/NF-H (L/H). We took advantage of the yeast strain HF7c, which contained a His3 reporter gene, as well as a weak lacZ reporter gene. We reasoned that if mBPAG-C1 could interact with heterodimers of L/M or L/H, triple transformants of pGBT-mBPAG-C1/pGAD-NF-L/pGAD-NF-M or pGBT-mBPAG-C1/pGAD-NF-L/pGAD-NF-H in HF7c would grow on minus histidine-tryptophan synthetic dropout plates. The selective pressure of histidine-free medium would keep all three plasmids in the same cells, provided that the expressed proteins interacted together as a unit. Under these conditions, there were still no interactions between
were also detected in cotransformants of pGBT-mBPAG-C1, because very slow growing small colonies of mBPAG-C1 interacted strongly with the L/M (L/M), and NF-L/NF-H (L/H) were polymerized in vitro, doted onto a nitrocellulose membrane, and incubated with FLAG-mBPAG-C2-His. Bound FLAG-mBPAG-C2-His was visualized with the anti-FLAG M2 antibody and quantified as described. Data shown are averages of triplicate determinations.

mBPAG-C1 and L/M or L/H (Table II). Therefore, we postulated that the failure of NFTPs to interact with mBPAG-C1 might be inherent in the structure of the molecules. Since the greatest differences among nIF proteins are at their tail domains, we prepared tailless NFTPs (L, M, H, L) and tested for their interactions with mBPAG-C1 in yeast strain HF7c. We observed fast growing colonies in triple transformants of pGBT-mBPAG-C1/pGAD-L/D/pGAD-H/Δ. Cotransformants of the three-hybrid vectors and pGBT-mBPAG-C1 really preferred to bind to the heterodimer of tailless NF-L and tailless NF-H, we constructed pGAD424-based three-hybrid vectors that contained two expression cassettes. By these means, two GAD fusion proteins could be expressed in the same transformant with single selection. Cotransformants of the three-hybrid vectors and pGBT-mBPAG-C1 would express the three fusion proteins simultaneously. Quantitative measurements of β-galactosidase activities were used to determine the relative strength of each interaction. The yeast transformants with pGBT-mBPAG-C1/pGAD-L/Δ/pGAD-H/Δ consistently displayed a high β-galactosidase activity, further confirming that mBPAG-C1 interact strongly with the L/Δ/H/Δ heterodimer (Fig. 6).

Table II. Two-Hybrid Interactions of mBPAG-C1 and NFTPs in the Yeast Strain HF7c

|   | GAD-L | M | H | L + M | L + H | LΔ | MΔ | HΔ | LΔ + MΔ | LΔ + HΔ |
|---|-------|---|---|------|------|----|----|----|---------|---------|
| GAD-H |      |   |   |      |      |    |    |    |         |         |
| HIS+ | -     | - | - | -    | -    | -  | -  | -  | -       | -       |
| LacZ+ | ND    | ND | ND | ND   | ND   | ND | ND | ND | +       | +       |

HF7c cells cotransformed with pGBT-mBPAG-C1 and GAD-fused NFTPs constructs were selected in minus Trp-Leu-His media for 7 d. SC and FC correspond to the appearance of slow growing and fast growing colonies, respectively; minus sign, no colonies. β-Galactosidase filter lift assays were also performed for the growing colonies; plus and minus signs represented the results of the filter assays; ND, not determined. L, M, H, LΔ, MΔ, and HΔ refer to NFL1-415, NFM1-421, and NFH1-415, respectively. GAD-NFL24-542, GAD-NFM, and GAD-NFH were expressed from the higher expression vector pACT2, whereas GAD-fused tailless NFTPs were expressed from pGAD424 vector.

Mouse BPAG-C1 Associates with Nonfilamentous Structures Formed by Tailless NFTPs in Transiently Transfected Cells

To determine if the tail domains of NFTPs also interfered with the association of mBPAG-C1 with filaments formed by NF-L/NF-M or NF-L/NF-H in the transient transfection assays, we performed cotransfections of mBPAG-C1 with various combinations of tailless and full-length NFTPs. As described previously, normal filament networks are observed from coassembly of tailless NF-L and wild-type NF-M or coassembly of wild-type NF-L and tailless NF-M in transiently transfected SW13.cl.2Vim- cells (Ching and Liem, 1993). However, mBPAG-C1 did not associate with any of the filamentous networks formed from these coas-
Assemblies and maintained a diffuse staining pattern (Fig. 7, A–D). mBPAG-C1 only colocalized with aggregates formed by coassembly of tailless NF-L and tailless NF-M (Fig. 7, E and F). These results implied that the tail domains of NF-L and NF-M interfered with the binding of mBPAG-C1 to the filament networks formed by these proteins. We transfected wild-type NF-H with tailless NF-L in SW13.cl.2Vim\textsuperscript{2} cells, we observed filamentous staining as reported previously (Ching and Liem, 1993). However, mBPAG-C1 did not colocalize with these filament networks. mBPAG-C1 only associated with the aggregates formed by tailless NF-L and tailless NF-M. Bar, 10 μm.

Pathological Changes of Peripherin in dt Mice

Since the COOH-terminal domain of mouse BPAG1 is able to associate with peripherin and the two proteins share a similar in vivo expression pattern, we wanted to determine whether there was any peripherin involvement in the development of dt pathology. We first looked at the peripherin immunostaining in the DRG of dt mice and their normal littermates. As expected, peripherin was present in the axonal swellings of dt mice (Fig. 9). We then compared the amounts of various nIF proteins, actin, and tubulin in nervous tissues of 3-wk-old homozygous dt mice and their normal littermates. Although the amounts of NF-L, α-internein, actin, and tubulin in nervous tissues of 3-wk-old homozygous dt mice and their normal littermates, peripherin was generally downregulated in the dt mice (Fig. 10). The other two NFTPs, NF-M and NF-H, were present in comparable amounts between the homozygous dt mice and their normal littermates (data not shown). It should also be noted that the sciatic nerves of dt mice were smaller in diameter and yielded less protein than their normal littermates. These changes in the sciatic nerves are probably the result of the neuronal degeneration observed in dt mice.
Discussion

Interactions of BPAG1 COOH-terminal with the NFTPs: Interference by the NFTP Tail Domains

The biochemical and cellular mechanisms underlying the neurological degeneration of dt mice have received a lot of attention recently in the IF field (Bousquet and Coulombe, 1996; Fuchs and Cleveland, 1998; Houseweart and Cleveland, 1998). The presence of an IF-binding domain in BPAG1 (dystonin), the mutated gene product, and the abnormal accumulations of NFs in the degenerating sensory neurons of the dt mice resulted in the hypothesis that BPAG1-n is an important organizing element of NFs in sensory neurons (Brown et al., 1995a; Yang et al., 1996). Our studies have demonstrated that the IF-binding tail domain of mouse BPAG1 does not interact with full-length NFTPs. This finding poses a disparity with a recent study which showed that the tail domain of human BPAG1-n associated with the heteropolymeric filaments formed by murine NF-L and NF-H in transiently transfected SW13.2Vim- cells (Yang et al., 1996). We did not observe any colocalization of mouse BPAG1 COOH-terminal proteins with the filaments formed by rat NF-L and NF-H or by NF-L and NF-M. It should be noted that a similar FLAG-tag was used for the detection of the BPAG1 proteins in both studies. The three-hybrid assays and cotransfection experiments in our studies indicated that mBPAG-C1 interacted strongly with tailless NF-L and tailless NF-H, whereas the presence of the tail domains of any of the NFTPs appeared to prevent the assembled filaments from interacting with mBPAG-C1 (Table III). Yang et al. (1996) also showed that mouse NF-H protein overexpressed in COS cells was coimmunoprecipitated with the tail domain of human BPAG1-n, suggesting a direct interaction between NF-H protein and BPAG1-n. However, in our two-hybrid studies and overlay analysis, no interactions were detected between the BPAG1 COOH-terminal proteins and full-length NF-H. This discrepancy could be due to the presence of other IF proteins in the COS cells, which might serve as mediators between BPAG1 and NF-H. To circumvent this possibility, we repeated the immunoprecipitation experiments by using the SW13.2Vim- cell line that is devoid of cytoplasmic IFs, but were not able to observe the coimmunoprecipitation of

Figure 9. Peripherin can be detected in the axonal swellings of dt mice. Cryosections of the DRG from (A) dt mouse and (B) normal littermate were immunostained with peripherin mAbs. Arrows point to the peripherin staining in axonal swellings, whereas arrowheads point to peripherin staining in normal looking axons. Bar, 10 μm.

Figure 10. Peripherin is downregulated in nervous tissues from dt mice. Western blots of nervous tissues from dt mice (DT) and normal littermates (WT) were immunostained with various antibodies as indicated. Loadings of same amounts of total protein in each set of tissue samples from dt mice and normal littermates were confirmed by Ponceau red staining (data not shown). In the case of peripherin and NF-L immunostainings, the blots were first probed with antiperipherin antibody, and then were stripped and reprobed with anti-NF antibody. The amounts of the protein samples on the blots immunostained with anti-α-internexin antibody were the same as those used for the NF-L and peripherin immunostainings, while one-half and one-third of the amounts were used for the blots immunostained with antiactin and antitubulin antibodies, respectively. 43–58% reduction of peripherin was detected in various nervous tissues of the dt mice as determined by densitometric analysis.
| Transfection     | IF phenotype       | BPAG colocalization |
|------------------|--------------------|---------------------|
| BPAG/vimentin    | Filaments          | No                  |
| BPAG/α-internexin| Filament bundles, punctate | Yes          |
| BPAG/peripherin  | Filaments          | Yes                 |
| BPAG/L/M         | Filaments          | No                  |
| BPAG/L/H         | Filaments          | No                  |
| BPAG/L1-421/M    | Filaments          | No                  |
| BPAG/M1-415/L    | Filaments          | No                  |
| BPAG/L1-421/M1-415| Aggregates      | Yes                 |
| BPAG/L1-421/H    | Filaments          | No                  |
| BPAG/H1-415/L    | Filaments          | No                  |
| BPAG/L1-421/H1-415| Aggregates     | Yes                 |

Table III. Summary of Transient Transfection Studies on BPAG1 COOH-terminal Proteins and nIFs

mBPAG-C1 with NF-H, either in the presence or absence of NF-L. We are at a loss to explain the discrepancies of our experiments with those described by Yang et al. (1996). The only difference between our experiments is the source of BPAG1. Our COOH-terminal domain constructs were cloned from mouse brain mRNA, whereas the constructs made by Yang et al. (1996) were obtained from a human cDNA library. However, BPAG1 is highly conserved between human and mouse; the primary sequence is 80% identical.

Interactions of BPAG1 with Peripherin: Relationship to the Pathogenesis of the dt Mouse

Although we were unable to show interactions between BPAG1 COOH-terminal proteins and the full-length NFTPs, we clearly demonstrated that the COOH-terminal domain of BPAG1-n can associate with the other two nIFs, peripherin and α-internexin, in the yeast two-hybrid and cotransfection experiments (Tables I and III). The interaction of BPAG1 with peripherin is of particular interest, because the two proteins exhibit similar expression patterns in the mouse PNS (Parysek et al., 1988; Troy et al., 1990a; Bernier et al., 1995; Dowling et al., 1997). We confirmed these interactions by overlay binding assays. We tested interactions of the mouse BPAG1 tail domain with NFTPs and peripherin that had been separated by SDS-PAGE and transferred onto a nylon membrane, as well as with repolymerized filaments that had been immobilized on a nitrocellulose membrane. In both cases, specific interactions were observed only between peripherin and BPAG1. The strong and specific interactions between peripherin and BPAG1 in the two-hybrid and overlay binding assays, the colocalization of BPAG1 COOH-terminal proteins with peripherin filaments in transient transfection studies, and the similarity in their in vivo expression patterns strongly suggest that peripherin is an important interaction partner of BPAG1-n. We also performed preliminary studies on the pathological changes associated with peripherin in dt mice. Axonal swellings of sensory neurons in the DRG are the most significant histopathological hallmarks of dt mice (Duchen et al., 1963; Duchen and Strich, 1964; Sotelo and Guenet, 1988; al-Ali and al-Zuhair, 1989; Guo et al., 1995). Immunostaining with antiperipherin antibody in the DRG revealed its presence in these axonal swellings (Fig. 9), although not all swellings were immunoreactive to the peripherin antibodies (data not shown). At present, it is not clear whether peripherin is absent in some of these swellings or just too scarce to be detected immunohistochemically. We also observed a general downregulation of peripherin in dt mice. The reduction in the peripherin levels is not restricted to the CNS. It is also detected in the CNS (Fig. 10), although no overt structural abnormalities have been observed in the CNS (Duchen et al., 1963). Presumably, peripherin is important in maintaining the integrity of neurons, therefore its disorganization and downregulation would ultimately cause neuronal degeneration that may account for the pathology of dt mice.

Interactions of BPAG1 with NFTs: Potential Role in the dt Pathogenesis

We have demonstrated in the two-hybrid assays and overlay studies that the COOH-terminal domain of BPAG1 does not interact with wild-type NFTPs. Furthermore, mBPAG-C1 and mBPAG-C2 do not colocalize with the filaments formed by transfected NF-L/NF-M or NF-L/NF-H. However, we cannot exclude the possibility that full-length BPAG1-n can associate with native NFs in axons under normal physiological conditions. Although the tail domains of NFTPs appeared to prevent the proteins from binding to mBPAG-C1 in vitro, it is possible that post-translational modifications of NFTPs and the presence of other linker proteins may expose the BPAG1-binding domain of NFTPs on the surface of NFs in axons, thereby allowing interactions between BPAG1-n and NFTPs. Furthermore, peripherin has been shown to copolymerize with NFTPs (Parysek et al., 1991) and therefore interactions between BPAG1-n with NFTPs could occur indirectly through peripherin.

During the initial phase of dt pathogenesis, most of the motoneurons in the BPAG1-deficient mouse look normal, indicating that BPAG1 is not necessary for the initial organization of NFs in motoneurons (Duchen and Strich, 1964; Janota, 1972; Duchen, 1976; al-Ali and al-Zuhair, 1989). To study the role of axonal NFs on the pathogenesis of dt axonopathy, Eyer et al. (1998) produced homozygous dt mice carrying a NFH-lacZ transgene. Because of the impairment in the axonal transport of NFs, these transgenic mice contain sensory neurons with axons devoid of NFs. Neuronal degeneration is still observed in these mice even though very few, if any, axonal swellings (devoid of NFs) are present, suggesting that abnormal accumulations of NFs in axonal swellings are not the only factor in dt pathogenesis. Nevertheless, the double transgenic mice displayed a longer life span, indicating that axonal swellings with neurofilamentous accumulations play a secondary role in promoting the neuronal degeneration in dt mice. However, it is not yet known whether the abnormal accumulations of NFs in the axons are directly caused by the absence of BPAG1-n, disorganization and downregulation of peripherin, or by the impairment of axonal transport.

The Functions of BPAG1-n and Peripherin: A Lesson from Plectin

Why would peripherin need to be “organized” by BPAG1-n? What causes the downregulation of peripherin in dt mice? Why does the dt pathology occur late in develop-
ment, even though peripherin and BPAG1-n are expressed early in differentiated neurons? These are some of the questions that remain unanswered. Before we are able to answer these questions, we must first understand the functions of BPAG1-n and peripherin. BPAG1-n is structurally very similar to plectin, although it has a shorter tail domain (Ruhrberg and Watt, 1997). Plectin is known for its ability to associate with many cytoplasmic structures, including IFs, actin filaments, microtubules, myosin filaments, and membrane proteins (Ptetela and Wiche, 1980; Svitkina et al., 1996; Wiche, 1998). It is possible that BPAG1-n also interacts with some of these components in neurons. Therefore, perturbations of BPAG1-n may not only affect microfilaments and peripherin, but also other neuronal structures. Until we have identified all the interaction partners of BPAG1-n, it will be difficult to determine the precise cause of the neuronal degeneration in dt mice. The function of peripherin is even less understood. Peripherin has been suggested to have a different function than NFTPs, since it is upregulated after nerve injury, while the NFTPs are downregulated (Troy et al., 1990b; Belecy-Adams et al., 1993). However, downregulation of peripherin by antisense oligonucleotides in cultured PC12 cells did not result in any observable effects on process outgrowth (Troy et al., 1992). Nevertheless, our present study shows that BPAG1-n is able to bind peripherin and that the association of BPAG1-n with peripherin appears to have physiological significance and may contribute to the development of dt pathology.

We thank Ms. Beth Rosen for technical assistance, Ms. Theresa Swayne for the help with the confocal microscopy, Dr. Gee Ching for editorial assistance, and Dr. Chung-Liang Ho for the preparation of the original peripherin two-hybrid constructs.

This work was supported by grant NS15182 from the National Institutes of Health. C.L. Leung and D. Sun were supported as trainees on training grant AG00189.

Received for publication 8 May 1998 and in revised form 23 December 1998.

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