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A Role for Epsin N-terminal Homology/AP180 N-terminal Homology (ENTH/ANTH) Domains in Tubulin Binding*

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Natasha K. Hussain‡§, Montarop Yamabhai¶, Asha L. Bhakar‡‖, Martina Metzler, Stephen S. G. Ferguson‡‡, Michael R. Hayden**, Peter S. McPherson‡§§, and Brian K. Kay¶¶**

*From the ‡Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada, ¶School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand, **The Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia V5Z 4H4, Canada, ‡‡Cell Biology Research Group, Robarts Research Institute, London, Ontario N6A 5K8, Canada, and ¶¶Biosciences Division, Argonne National Laboratory, Argonne, Illinois 60439*

The epsin N-terminal homology (ENTH) domain is a protein module of ~150 amino acids found at the N terminus of a variety of proteins identified in yeast, plants, nematode, frog, and mammals. ENTH domains comprise multiple α-helices folded upon each other to form a compact globular structure that has been implicated in interactions with lipids and proteins. In characterizing this evolutionarily conserved domain, we isolated and identified tubulin as an ENTH domain-binding partner. The interaction, which is direct and has a dissociation constant of ~1 μM, was observed with ENTH domains of proteins present in various species. Tubulin is co-immunoprecipitated from rat brain extracts with the ENTH domain-containing proteins, epsins 1 and 2, and punctate epsin staining is observed along the microtubule cytoskeleton of dissociated cortical neurons. Consistent with a role in microtubule processes, the over-expression of epsin ENTH domain in PC12 cells stimulates neurite outgrowth. These data demonstrate an evolutionarily conserved property of ENTH domains to interact with tubulin and microtubules.

The epsin N-terminal homology (ENTH)[1] domain is an evolutionarily conserved globular module of ~150 amino acids that occurs at the amino terminus of a variety of proteins (1, 2). Originally noted in the plant protein, Af10 (3), the ENTH domain has subsequently been characterized in epsins (2) and enthoprotin (4) (also termed epsinR (5, 6) or Clint (7)), and in yeast proteins including Ent1p/Ent2p (8, 9) and Ent3p (10). Adaptor protein 180 (AP180), clathrin assembly lymphoid myeloid leukemia protein (CALM), Huntingtin-interacting protein-1 (HIP1) and HIP12, and the yeast proteins yAP180 and Sla2p contain a module that is so similar in structure to the epsin ENTH domain that they were initially denoted ENTH-bearing proteins (11–13). However, recent structural studies have refined our understanding such that ENTH-like domains from these proteins have been re-designated ANTH domain-containing proteins in accordance with their higher structural similarity to AP180 rather than epsin (14). In an effort to simplify the nomenclature applied in this study, we refer to these homologous structures as E/ANTH domains when collectively discussing proteins bearing either domain, but maintain the ENTH or ANTH nomenclature when discussing individual proteins.

A common feature among many E/ANTH domain-bearing proteins is that their C termini contain peptide motifs, indicative of a functional role in clathrin-mediated membrane budding including clathrin and clathrin adaptor protein-binding elements (1, 15). In addition to their interactions with multiple endocytic components, many of the currently characterized E/ANTH proteins, including epsin, AP180, and HIP1/12, are localized to clathrin-coated pits where they function in clathrin-mediated endocytosis (2, 16–25). Interestingly, enthoprotin is unique in this group because it is predominantly localized to the *trans*-Golgi network rather than the plasma membrane, and it appears to regulate clathrin-mediated budding events occurring specifically along the *trans*-Golgi network and endosomal pathway (4–7, 10).

Recent studies have demonstrated that the E/ANTH domains of epsin and AP180 can mediate lipid binding, particularly to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), and that this interaction is required for efficient clathrin-mediated endocytosis in COS-7 cells (12, 13). Furthermore, upon PtdIns(4,5)P_2 binding, the epsin ENTH domain was shown to drive the curvature of clathrin-coated pits on the plasma membrane, whereas the AP180 ANTH domain appears to be more involved in regulating the diameter of emergent vesicles (14). This functional difference in ENTH and ANTH domains is likely imparted by the structural variances between them. In the AP180 ANTH domain, the specific residues involved in lipid binding reside within α-helices α1 and α2 and in the loop between them (12, 13). However, the epsin ENTH domain

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§§ A CIHR Investigator, a Killam Scholar of the Montreal Neurological Institute, and a McGill University William Dawson Scholar. To whom correspondence should be addressed: Dept. of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, 3801 University Ave., Montreal, Quebec H3A 2B4, Canada. Tel.: 514-398-7355; Fax: 514-398-8106; E-mail: peter.mcpherson@mcgill.ca.

[1] The abbreviations used are: ENTH, epsin N-terminal homology; ANTH, AP180 N-terminal homology; E/ANTH, epsin/AP180 N-terminal homology; DH, Dbl homology; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HIP, Huntingtin-interacting protein; MAP, microtubule-associated protein; MES, 4-morpholineethanesulfonic acid; MP90, mitotic phosphoprotein of 90 kDa; PH, pleckstrin homology; PIPES, 1,4-piperazinediethanesulfonic acid; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; SH3, Src homology 3; TRITC, tetramethylrhodamine isothiocyanate.

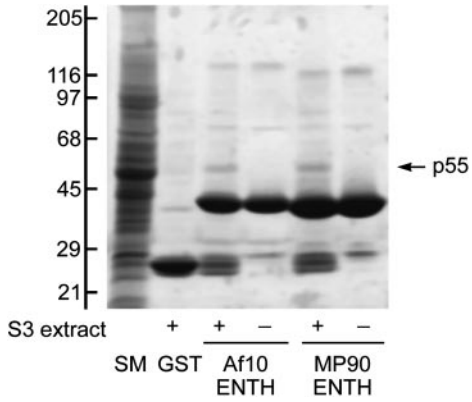

FIG. 1. **Identification of a 55-kDa protein from rat brain extracts that binds ENTH domains of Af10 and MP90.** Approximately 30 μg of GST alone or GST fused to the ENTH domains of Af10 or MP90 was pre-coupled to glutathione-Sepharose and then incubated with 2 mg of a soluble rat brain (*S3*) extract (+) or with buffer alone (−). The samples were washed extensively, and the proteins bound to the beads were resolved by SDS-PAGE along with an aliquot of the soluble starting material (*SM*) and detected by Coomassie Brilliant Blue staining. The *arrow* indicates the position of a 55-kDa affinity-selected protein (*p55*) that does not bind to GST alone and is not detected in GST fusion protein preparations (in the absence of brain extract).

FIG. 2. **ENTH domains bind tubulin from rat brain extracts.** Approximately 30 μg of GST alone, GST fused to the pentapeptide motif TNPFL (*GST-NPFL*), GST-epsin ENTH, GST-intersectin SH3A, GST-amphiphysin II SH3, GST-intersectin-l C2, GST-intersectin-l DH-PH, and GST-intersectin-l DH were pre-coupled to glutathione-Sepharose and used in binding assays with soluble rat brain (*S3*) extracts. Bound proteins were resolved by SDS-PAGE and processed for Western blot analysis with a monoclonal antibody against α-tubulin.

lipid-ligand pocket is coordinated by residues in α1, the α1–2 loop, α3, and α4 and is dependent on the formation and binding of α0, a helix that is not present or generated in the ANTH domain (12–14). The importance of helix α0 for epsin ENTH domain function is demonstrated by the fact that deletion or mutation of residues within this helix is sufficient to abrogate lipid interactions (12) and abolish the ability of mammalian epsin ENTH to induce curvature of clathrin-coated membrane lattices (14).

E/ANTH domains function in clathrin-mediated budding not only in higher order organisms but also in budding yeast. Genetic experiments demonstrate that the ENTH domains of the yeast epsin homologues Ent1p and Ent2p are essential for normal endocytic function and actin cytoskeletal structure, and expression of at least one ENTH domain is required to maintain viability in Ent1 and Ent2 double mutant strains (Ent1ΔEnt2Δ) (8, 9). However, the essential function of yeast epsin ENTH domains in Ent1pΔEnt2Δ mutants appears to be independent of their ability to bind lipids (26). Specifically, Aguilar *et al*. (26) have identified an ENTH domain mutant that fails to rescue Ent1ΔEnt2Δ cells despite the fact that this mutant continues to bind PtdIns(4,5)P_2. Based on these findings, the authors suggest that at least in yeast, another critical protein-binding partner(s) must exist to assist ENTH domain functions (26). In fact, previous studies into the function of this module have revealed that ENTH domains can mediate interactions with proteins; the ENTH domain of mammalian epsin 1 binds weakly to the vesicular coat protein coatomer and more robustly to human promyelocytic leukemia Zn^{2+} finger protein (PLZF), a transcriptional repressor (11). Together these studies suggest multiple roles for the ENTH domain as both a lipid- and protein-binding module.

As part of our functional analyses of the ENTH domain, we sought to identify new protein targets for this module. Through affinity selection assays, we identified tubulin as a binding partner for E/ANTH domains isolated from several different species. We propose that interaction with tubulin is an evolutionarily conserved property of E/ANTH domains.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies for epsins 1 and 2 were prepared by injecting two rabbits (denoted as 2345 and 2346) with 50 μg of GST fusion protein encoding the C-terminal 511 amino acids of mouse epsin 2 (formerly referred to as intersectin-binding protein 2) (27) as described (28). Sera were monitored for antibody production by Western blotting, and epsin-specific antibodies were affinity-purified as described (28). Polyclonal synaptojanin antibody was prepared as described (29); a monoclonal antibody (AC1M11) against α-adaptin (30) was the generous gift of Dr. Margaret Robinson (Cambridge University). Monoclonal α- and βIII-tubulin antibodies were purchased from Sigma.

Generation of Recombinant Constructs—GST fusion protein constructs encoding the C-terminal pentapeptide sequence TNPFL of epsin 2 (GST-NPFL) and intersectin SH3A (27), the ENTH domain of enthoprotin (4), amphiphysin II SH3 (31), intersectin C2, DH-PH, and DH (32) domains were generated as described. GST-ENTH MP90 (amino acids 1–137) (33) and GST-ENTH Af10 (amino acids 1–153) (3) were amplified by PCR using full-length cDNA templates and cloned between the *Eco*RI and *Bam*HI sites of pGEX-2TK (Amersham Biosciences). GST-HIP1-ANTH (amino acids 1–125) and GST-HIP12-ANTH (amino acids 1–150) expression constructs were generated by PCR amplification from their respective full-length cDNAs (20, 34), followed by cloning into the pGEX-6P vector (Amersham Biosciences). The 3′-end of each HIP construct was tagged with codons for three glycines followed by six histidine residues and a stop codon. cDNAs encoding wild-type rat epsin 1, the K76A mutant (12), and human AP180 (KIAA0656, identified by the Kazusa DNA Research Institute) (35) were used as templates for PCR amplification of all epsin and AP180 constructs. PCR products were digested with *Eco*RI and *Xho*I and subcloned into either the corresponding sites of pGEX-4T1 to generate GST-Epsin ENTH (amino acids 1–146), and GST-AP180 ANTH (amino acids 2–288) or the *Eco*RI and *Sal*I sites of pEGFP-C2 for generation of GFP-Epsin (amino acids 1–553) GFP-Epsin ΔENTH (amino acids 147–553) and GFP-AP180 ANTH (amino acids 2–288) constructs. A GFP-Epsin ENTH (amino acids 1–208) construct was generated by PCR amplification and cloning into the *Eco*RI and *Bam*HI sites of pEGFP-C2. Rat epsin 1 α-helices α1–2 (amino acids 19–47), α2–3 (amino acids 37–65), α3–4 (amino acids 50–86), and α7 (amino acids 112–129) were generated by PCR amplification and cloning into *Bam*HI and *Eco*RI sites of pGEX-4T1 or into *Bgl*II and *Eco*RI sites of pEGFP-C1 to generate GST- and GFP-tagged constructs, respectively. AP180 α7 (amino acids 115–141) was generated by PCR amplification and cloning into *Bam*HI and *Xho*I sites of pGEX-4T1 or into *Bgl*II and *Sal*I sites of pEGFP-C2. All expression constructs were verified by DNA sequencing. GST fusion proteins were over-expressed in bacteria and purified according to the manufacturer's instructions (Amersham Biosciences).

Affinity Selection Assays—Rat brains were homogenized in buffer A (10 mM HEPES-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin) and centrifuged at 750 × *g* for 5 min. The supernatant was centrifuged a second time at 205,000 × *g* for 30 min to recover the soluble fraction. After adding Triton X-100 (1% final concentration) to the supernatant, aliquots (2 mg protein) were incubated overnight at 4 °C with glutathione-Sepharose bound to ~25 μg of the GST fusion protein constructs. Beads were extensively washed in buffer A with 1% Triton X-100, and bound proteins were resolved by SDS-PAGE and detected by Coomassie Brilliant Blue staining or by Western blotting with monoclonal antibodies to α- or βIII-tubulin. In other experiments, purified tubulin, made free of microtubule-associated proteins (MAPs) by phosphocellulose chromatography (ICN Biomedicals), was diluted to 200 nM in 4 °C buffer B (0.1 M MES, pH 6.4, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP,

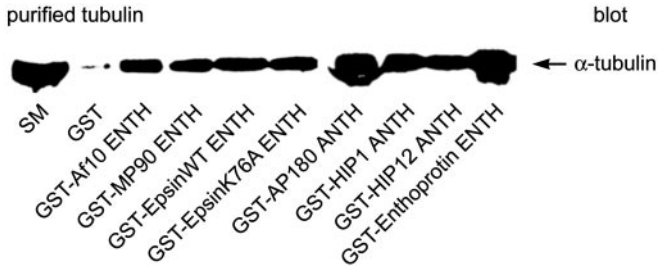

FIG. 3. **E/ANTH domains display conserved binding to purified tubulin.** GST-ENTH domains from Af10, MP90, wild-type rat epsin 1 (*GST-EpsinWT ENTH*), rat epsin 1 encoding the point mutation K76A (*GST-EpsinK76A ENTH*), and enthoprotin, and GST-ANTH domains from AP180, HIP1, and HIP12, as well as GST alone, were pre-coupled to glutathione-Sepharose and incubated with 200 nM purified tubulin. The samples were washed extensively, and the proteins bound to the beads were resolved by SDS-PAGE, along with an aliquot of the soluble starting material (*SM*). The proteins were transferred to nitrocellulose and blotted with an antibody against α-tubulin.

FIG. 4. **Specific E/ANTH domain helical segments mediate tubulin binding.** Approximately 30 μg of GST alone or GST fused to peptides encoding the helical regions α1–2, α2–3, α3–4, and α7 of rat epsin 1 and α7 of AP180 was pre-coupled to glutathione-Sepharose and used in binding assays with purified tubulin. Bound proteins were resolved by SDS-PAGE and processed for Western blot with an antibody against α-tubulin.

10% glycerol, 1% Triton X-100, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin) and centrifuged for 1 h at 4 °C at 100,000 × *g* to remove insoluble aggregates. Aliquots (1 ml) of the supernatant were incubated for 4 h at 4 °C with ~25 μg of GST fusion proteins (pre-coupled to glutathione-Sepharose), the samples were washed in buffer B, and material bound to the beads was resolved by SDS-PAGE and processed for Western blot analysis.

Tubulin Binding Affinity Measurements—Purified tubulin was diluted to 10 μM in binding buffer (80 mM PIPES, pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM GTP, 10% glycerol) and incubated for 30 min on ice. The sample was centrifuged for 10 min at 10,000 × *g* to remove insoluble aggregates. Aliquots of the supernatant, diluted in binding buffer to final concentrations ranging from 0.015 to 4 μM, were added to 10 μg of GST fusion protein constructs pre-coupled to glutathione-Sepharose in a final reaction volume of 250 μl for 4 h at 4 °C. The samples were washed in binding buffer, and material bound to the beads was resolved by SDS-PAGE and processed for Western blot analysis along with a standard curve of purified tubulin. Following Western blot with α-tubulin or βIII-tubulin antibodies, the amount of either tubulin retained by the beads was determined by scanning the blots and comparing the optical density to that of the standard curve using NIH Image computer software.

ENTH Domain Binding to Taxol-stabilized Microtubules—The binding of ENTH domain to microtubules was assessed using a microtubule-associated protein spin-down assay kit (Cytoskeleton Inc.) according to the manufacturer's instructions. In brief, highly purified tubulin (50 μM) in PEM buffer (80 mM PIPES, pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$) was incubated for 20 min at 35 °C in the presence of 1 mM GTP and 2% sucrose to allow for microtubule formation. The sample was then diluted 1:10 in PEM buffer with 1 mM GTP and 40 μM taxol. Aliquots of the taxol-stabilized microtubules (20 μl) were added to purified MAP-2, bovine serum albumin (both supplied by the manufacturer), or purified GST fusion protein constructs (pre-spun for 40 min at 100,000 × *g* to remove insoluble aggregates), each diluted to 30 μl in PEM buffer with 1 mM GTP and 40 μM taxol. Triton X-100 was added to each 50 μl sample to 1% final, and the samples were then incubated for 20 min at

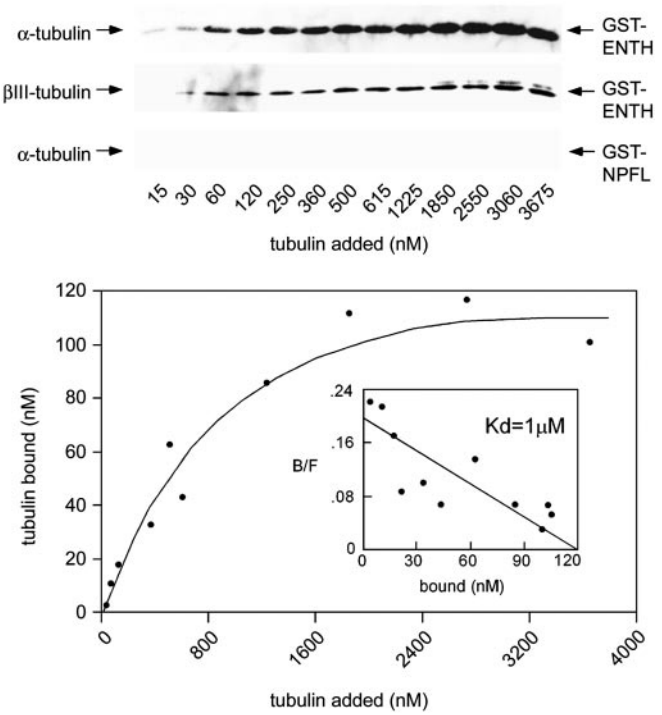

FIG. 5. **Saturation binding analysis of ENTH domain/tubulin interactions.** Increasing concentrations of purified tubulin were added to GST-MP90 ENTH (*GST-ENTH*) or GST-NPFL fusion proteins pre-coupled to glutathione-Sepharose, and the tubulin specifically bound to the beads was detected with antibodies against α-tubulin or βIII-tubulin. The amount of α-tubulin bound to the beads was quantified as described under "Experimental Procedures"; saturation binding curves and Scatchard plots were derived from the data.

room temperature before being loaded onto 50 μl of cushion buffer (PEM, 20% sucrose, 20 μM taxol). The samples were spun at 25 °C for 40 min 100,000 × *g*, the upper 50 μl sample was retained, the cushion was carefully removed, and the pellet was resuspended in SDS-PAGE sample buffer.

Immunofluorescence Analysis—Cortical neurons dissected from embryonic day (E) 15–16 mice were titurated, plated on poly-L-lysine-coated coverslips, and maintained in Neurobasal medium supplemented with 1× B27 (Invitrogen), 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin as described (36). PC12 cells plated on rat tail collagen-coated coverslips were transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions and maintained In Dulbecco's modified Eagle's medium containing 10% bovine calf serum, 10% horse serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Prior to fixation with 3% paraformaldehyde, cells were rinsed twice with room temperature phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4). In the case of cortical neurons, cells were fixed after 3 days of *in vitro* culture, whereas PC12 cells were fixed 5 days following transfection; in both cases cells were maintained in their particular medium throughout their respective culturing periods. Fixed cells were processed for indirect immunofluorescence with various antibodies and for direct immunofluorescence with TRITC-conjugated phalloidin as described (37). Images of cortical neurons and PC12 cells were captured using a Zeiss 510 laser scanning confocal microscope. Transfected PC12 cells bearing neurites were quantified in four experiments as the ratio of transfected cells with processes greater than one cell body relative to the total population of transfected cells

Immunoprecipitation Analysis—A soluble rat brain extract, prepared as described above, was pre-cleared by incubation with protein A-Sepharose (Sigma) for 1 h at 4 °C and then was incubated with rabbit anti-epsin 2 antisera (2345 and 2346) coupled to protein A-Sepharose overnight at 4 °C. Beads were washed with buffer A containing 1% Triton X-100, and proteins were resolved by SDS-PAGE and processed for Western blot analysis.

RESULTS

Identification of Tubulin as an ENTH Domain-interacting Protein—In an effort to identify proteins that interact with the

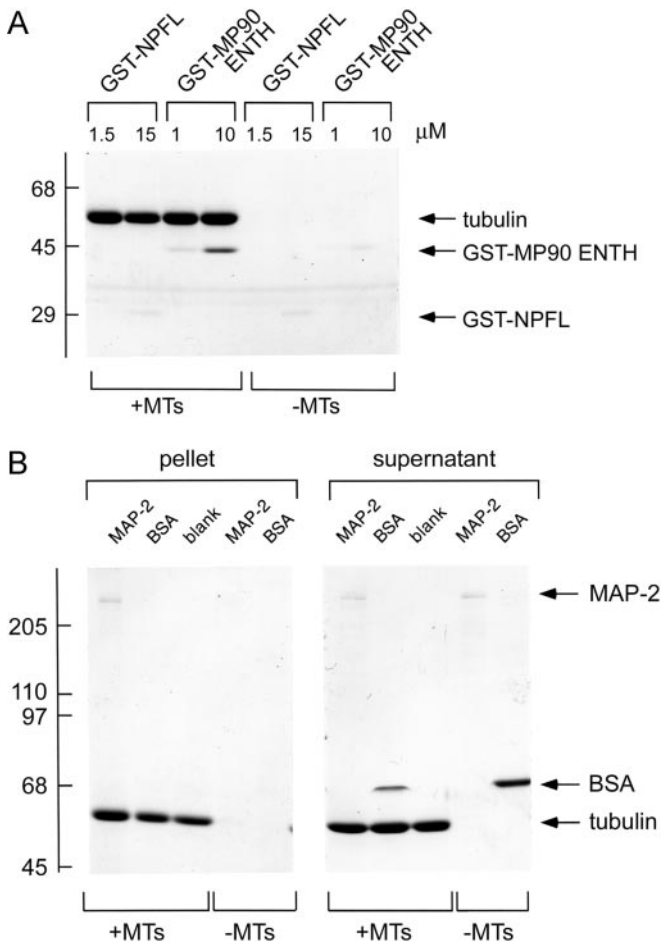

FIG. 6. **ENTH domain binds assembled microtubules.** *A*, GST-NPFL (1.5 or 15 μM) or GST-MP90 ENTH (1 or 10 μM) was incubated with assembled, taxol-stabilized microtubules (*+MTs*) or with buffer alone (*−MTs*). After incubation at 30 °C, proteins were centrifuged, and the pellet fraction was resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. *Arrows* on the *right* indicate the mobilities of the tubulin subunits and the two GST fusion proteins. *B*, a sample of assembled microtubules stabilized with taxol or of buffer lacking microtubules was incubated with MAP-2, bovine serum albumin (*BSA*), or buffer alone (*blank*) as indicated. Following incubation at 30 °C, the samples were centrifuged, and the proteins in the pellets (*left panel*) and supernatants (*right panel*) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. *Arrows* on the *right* indicate the mobilities of the tubulin subunits as well as MAP-2 and bovine serum albumin.

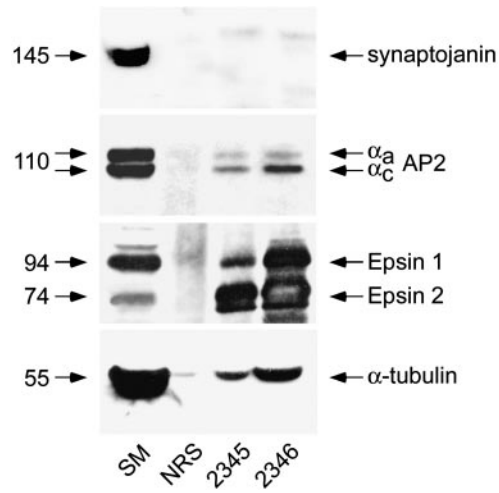

FIG. 7. **Co-immunoprecipitation of tubulin with epsins 1 and 2.** Epsin antisera 2345 and 2346 (epsin antibody) along with pre-immune sera 2345 (*NRS,* normal rabbit serum) were pre-coupled to protein A-Sepharose and incubated with 1 mg of a soluble rat brain extract overnight at 4 °C. The beads were washed extensively, and the material specifically bound to the beads was recovered and processed for Western blot analyses with antibodies against the α_a and α_c subunits of AP2 against synaptojanin and affinity-purified epsin antibody 2345 as indicated. *SM*, starting material.

ENTH domain (1) we performed affinity chromatography on rat brain extracts using GST fusion constructs encoding the ENTH domains of Af10 and MP90. Af10, isolated from *Avena fatua*, is a protein of unknown function (3), and MP90, originally identified as a mitotic phosphoprotein present in *Xenopus laevis* embryos (33), is a member of the epsin family of proteins. Pull-down experiments with these two fusion proteins, followed by detection of bound proteins with Coomassie Blue staining, revealed the selective purification of a 55-kDa protein species (Fig. 1). Based on the size and abundance of the affinity-selected protein, we predicted that it might be tubulin. To explore this hypothesis, we performed pull-down experiments with GST fusions to the ENTH domain of rat epsin 1, a variety of modular domains including Src homology 3 (SH3), Dbl homology (DH), pleckstrin homology (PH), and C2 domains, and a protein fragment encoding the pentapeptide TNPFL. Affinity-selected proteins were resolved by SDS-PAGE and Western blotted with a monoclonal antibody that recognizes the α-isoform of $\alpha\beta$-tubulin heterodimers. Only the ENTH domain-containing GST fusion protein bound α-tubulin (Fig. 2). These data confirm that α-tubulin binds the ENTH domain of epsin and demonstrate the specificity of the interaction compared with other modular domains and peptides. The GST-Af10-ENTH domain also selectively recovered α-tubulin from soluble extracts prepared from rat testis, liver, and heart and from COS-7 and A431 cell lines (data not shown), demonstrating that ENTH domains can interact with α-tubulin present in a broad range of tissues.

Tubulin Binding Is Direct and Conserved among E/ANTH Domains—As the soluble brain extracts used in Figs. 1 and 2 include microtubule-associated proteins, there was the possibility that the observed ENTH/tubulin interactions are indirect. To address this issue, GST fusion proteins encoding ANTH domains isolated from AP180, HIP1, and HIP12 and ENTH domains from Af10, MP90, enthoprotin, and epsin were immobilized on glutathione-Sepharose and incubated with MAP-free, purified $\alpha\beta$-tubulin heterodimers. Purified tubulin bound to each of the E/ANTH domain constructs but not to the control GST fusion protein, as determined by Western blot analyses with antibodies specific for either α-tubulin (Fig. 3) or βIII-tubulin (data not shown). Therefore, we conclude that E/ANTH domains are capable of direct interactions with soluble tubulin. Importantly, tubulin bound equally well to wild-type epsin ENTH and a mutated form of the epsin ENTH domain (K76A) that abrogates lipid binding (12) (Fig. 3), indicating that lipid interactions are not required to mediate the observed E/ANTH binding to tubulin.

Localization of Tubulin Binding to Specific E/ANTH Domain Helices—In AP180, the lipid binding pocket of the ANTH domain comprises multiple residues within α1, α2, and the loop between them, whereas the epsin ENTH lipid pocket is contributed to by residues in α0, α1, α1–2 loop, α3, and α4 (12–14). To determine whether the elements within the E/ANTH domain responsible for tubulin binding are distinguishable from lipid-binding residues, we generated GST fusion proteins encoding various helical segments from epsin ENTH and AP180 ANTH (not all of the GST fusion proteins encoding isolated helices could be tested for tubulin binding due to insolubility). Soluble GST fusion proteins encoding helical segments α1–2,

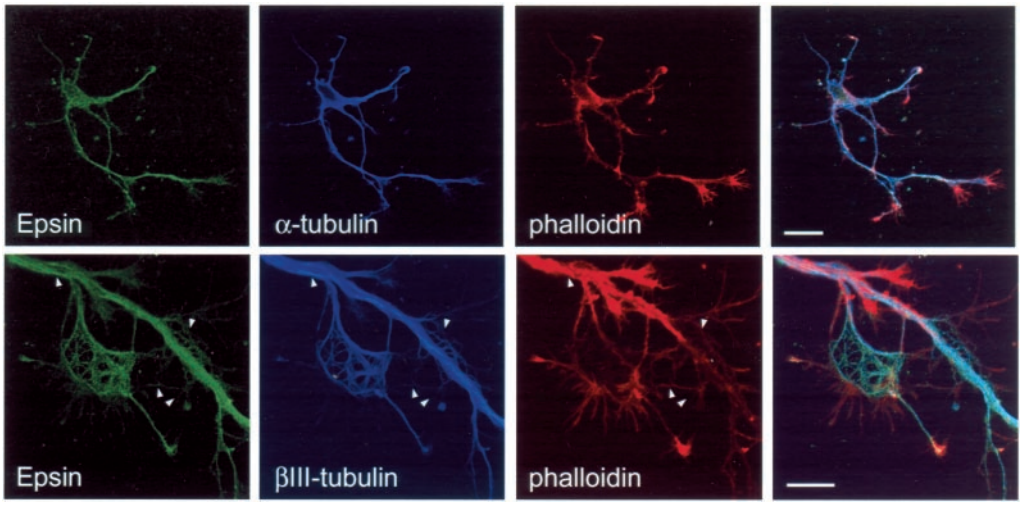

FIG. 8. **Endogenous epsin partially co-localizes with microtubules in cortical neurons.** Dissociated cortical neurons isolated from embryonic day 15–16 mice were cultured for 3 days *in vitro* and subsequently were processed for immunofluorescence using affinity-purified antibodies specific for epsins 1 and 2 (2345) (*green*), α-tubulin or βIII-tubulin monoclonal antibody (*blue*), and phalloidin staining (*red*). These neurons were analyzed by laser scanning confocal microscopy to determine the extent of co-localization between microtubules in images capturing an entire neuron (*top panels*) or focused on the growth cone (*bottom panels*). Punctate epsin staining co-localized with microtubules (*arrowheads* point to some of these regions) is seen upon superimposition of these micrographs (*upper right* and *lower right panels*). *Scale bars* represent 20 μm.

α2–3, α3–4, and α7 of epsin ENTH domain and α7 of AP180 ANTH domain were used in affinity chromatography assays with purified tubulin (Fig. 4). Western blot analysis with α-tubulin antibody revealed that the isolated helical regions of α1–2 and α7 of epsin ENTH and α7 of AP180 ANTH were sufficient to mediate this direct interaction *in vitro* (Fig. 4). Therefore, at least for epsin, multiple regions of the ENTH domain could be cooperatively involved in mediating tubulin interactions within the intact module. Although helix α7 in epsin ENTH or AP180 ANTH has not been implicated in lipid binding (12–14), this helix is predicted to mediate protein interactions based on a high degree of conservation of solvent accessible residues therein (11).

Characterization of the Affinity of the ENTH Domain for Tubulin—To measure the affinity of the tubulin/ENTH domain interaction, we performed saturation binding studies. As seen in Fig. 5, the addition of increasing concentrations of purified, soluble tubulin to a constant amount of GST-MP90 ENTH domain led to increasing binding that saturated at ~2.5 μM for α-tubulin. Western blot analyses with antibodies to the neuron-specific βIII-tubulin isoform revealed a similar saturation profile (Fig. 5). A comparison of the blot signals for the affinity-selected α-tubulin with known amounts of tubulin, resolved on adjacent lanes (data not shown), permitted a quantitative analysis of binding (Fig. 5). Scatchard plots revealed a dissociation constant of 1.05 ± 0.18 μM, $n = 3$ (Fig. 5 is a representative experiment). This value compares favorably with the dissociation constants of well established tubulin-binding proteins such as stathmin (38) and Tau (39).

ENTH Domains Bind Assembled Microtubules—Many of the specific functions of microtubules, including their roles in intracellular membrane trafficking, depend upon their dynamic ability to cycle between soluble αβ-tubulin heterodimers and insoluble microtubules (40). To determine whether ENTH domains interact with microtubules in addition to tubulin heterodimers, purified tubulin was assembled into microtubules that were stabilized with the addition of taxol. Upon incubation with microtubules followed by separation of soluble and insoluble fractions, a GST fusion protein to the ENTH domain of MP90 (GST-MP90 ENTH) sedimented only in the presence of microtubules, whereas an unrelated GST fusion protein (GST-NPFL) failed to co-sediment (Fig. 6*A*). In control experiments, MAP-2 sedimented only in the presence of microtubules, whereas bovine serum albumin did not sediment under any conditions (Fig. 6*B*). Thus, the ENTH domain can interact directly with both microtubules and soluble tubulin *in vitro*.

In Vivo Interaction of Epsin and Tubulin—To determine whether tubulin interacts with ENTH-domain-containing proteins *in vivo*, we performed co-immunoprecipitation assays for epsin from soluble rat brain extracts. Immunoprecipitation with epsin antibodies 2345 or 2346 revealed immunoreactive species at 94 and 74 kDa (Fig. 7) as determined by Western blotting with the 2345 antisera, in agreement with the reported molecular mobility of rat epsins 1 and 2, respectively (2, 18). In addition, the epsin immunoprecipitates contained the clathrin adaptor AP2, a previously described binding partner of epsin (2, 41, 42) and tubulin (Fig. 7). As control for specificity, we found that another component of the endocytic machinery, synaptojanin, was not recovered in the same immune complex (Fig. 7). Thus, based on co-immunoprecipitation data, epsins 1 and 2 may exist in protein complexes with tubulin and other endocytic proteins in rat brain extracts.

To further address the interaction between epsin and tubulin *in vivo*, we assessed the localization of endogenous epsins 1 and 2 relative to the microtubule cytoskeleton in dissociated cortical neurons. Consistent with the previously reported cellular distribution of rat epsins 1 and 2 to clathrin-coated pits (2, 18, 24), epsins displayed a punctate staining pattern (Fig. 8). Epsin-positive punctae were distributed throughout the cell body, in neuronal processes (Fig. 8, *upper panels*) and within growth cones (Fig. 8, *lower panels*). Although not exclusively associated with microtubule filaments, punctate staining of endogenous epsins 1 and 2 in neuronal processes was coincident with microtubules as revealed with α- and βIII-tubulin antibodies, and co-localization was particularly evident within growth cones (Fig. 8) where microtubules are splayed and distinct filaments are easily discernable (43, 44). Staining of the actin cytoskeleton in the same cell preparations revealed the full extent of the growth cone (Fig. 8). These data demonstrate that epsin can interact with tubulin *in vivo*, as illustrated by immunoprecipitation analyses, and that a significant component of epsin punctae are associated with the microtubule cytoskeleton in dissociated cortical neurons, indicative of a physiological interaction between the ENTH domain and microtubules.

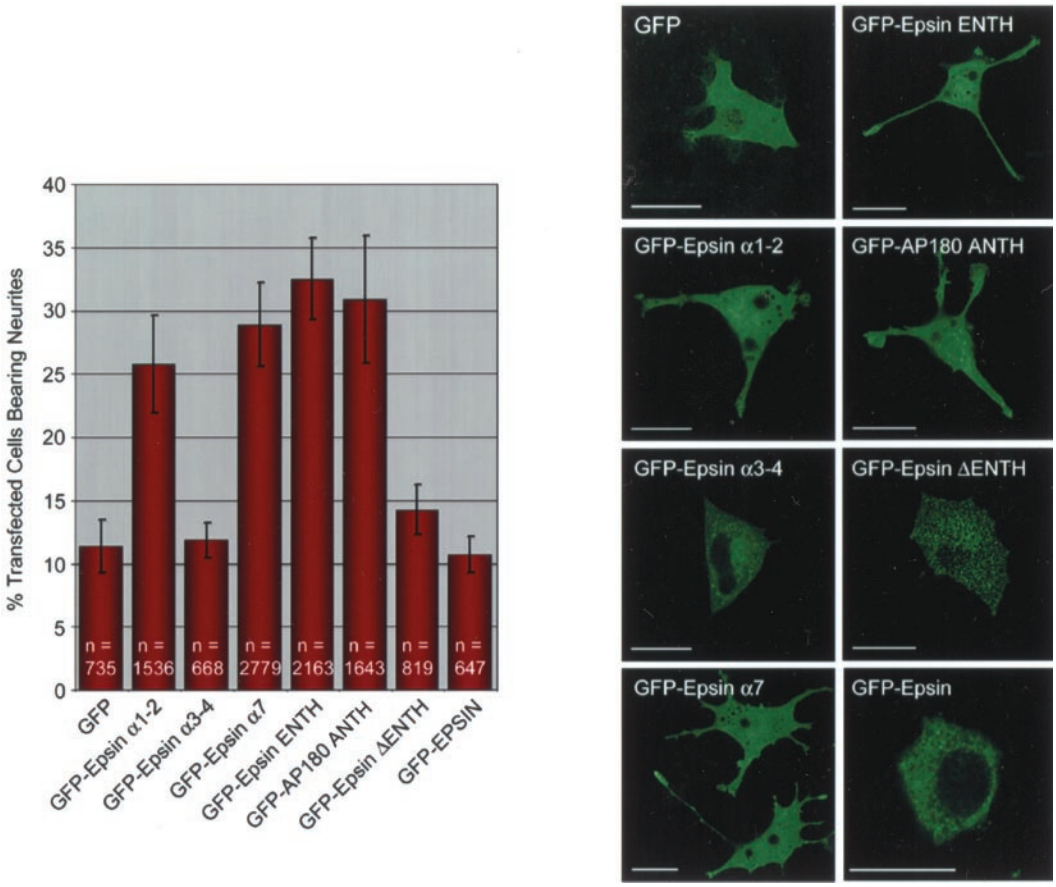

FIG. 9. **E/ANTH domains stimulate neurite outgrowth in PC12 cells.** PC12 cells transiently expressing GFP vector alone or GFP fusion proteins encoding epsin, epsin lacking the ENTH domain (Δ*ENTH*), epsin ENTH domain and AP180 ANTH domain, and the isolated α1–2, α3–4, and α7 segments of the epsin ENTH domain were cultured for 5 days, processed for immunofluorescence, and analyzed by laser scanning confocal microscopy. All transfected cells were quantified and analyzed for neurite outgrowth, defined as processes greater than one cell body. *Left*, graphical analyses of neurite outgrowth (four experiments were conducted, where n = the total number of transfected cells counted, as indicated in the bar graph for each construct). *Right*, representative cells for GFP, GFP-epsin, GFP-epsin ENTH, and GFP-AP180 ANTH. *Scale bars* represent 20 μm.

E/ANTH Domains Stimulate Neurite Outgrowth—It was suggested recently that the tubulin-binding protein collapsin response mediator protein-2 stimulates neurite outgrowth in PC12 cells in conjunction with low doses of nerve growth factor, by drawing together tubulin heterodimers beyond a critical concentration required for polymerization (45, 46). This is consistent with the observation that PC12 cells require tubulin polymerization and reorganization of the microtubule cytoskeleton for neurite outgrowth to occur (47–51). To investigate whether ENTH domains might also be able to influence tubulin polymerization, we examined for alterations in neurite outgrowth in PC12 cells expressing ENTH domains. Expression of GFP-tagged fusion proteins encoding the individual E/ANTH domains of epsin or AP180 stimulated the outgrowth of processes defined as extensions greater than one cell body (Fig. 9). In contrast, expression of constructs encoding GFP, GFP-tagged full-length epsin, or GFP-epsin lacking the ENTH domain did not enhance neurite outgrowth (Fig. 9). Moreover, the isolated α1–2 and α-7 helices of the epsin ENTH domain, which bind tubulin *in vitro* (Fig. 4), stimulate outgrowth, whereas the α3–4 helices that do not bind tubulin (Fig. 4) have no effect (Fig. 9). Although the precise mechanisms by which E/ANTH domains stimulate neurite outgrowth remain to be elucidated, it appears that expression of the E/ANTH domain is self-sufficient to stimulate outgrowth within PC12 cells.

DISCUSSION

The E/ANTH domain is an evolutionarily conserved protein module composed of a super-helix of α-helices and located within the N terminus of a variety of proteins. Recently it was demonstrated that this module functions as a mediator of lipid interactions (12, 13). Previous studies have indicated that the domain also binds to proteins (11). In this study, we have characterized a novel interaction between tubulin and the E/ANTH domain isolated from several different species. Moreover, we have provided evidence that E/ANTH domain tubulin-binding elements are distinct from those implicated in lipid interactions. In this regard, the E/ANTH domain is reminiscent of other modular domains such as PH domains that bind to both inositol phospholipids and proteins (52). For example, the PH domain of the β-adrenergic receptor kinase binds to both PtdIns(4,5)P_2 and the G-protein β/γ subunit. In fact, simultaneous interaction with both ligands is necessary for the recruitment of the kinase to membranes (53). Moreover, PH domains from several proteins including Bruton's tyrosine kinase bind to both phosphoinositides and actin (54). C2 domains also interact with multiple ligands including Ca^{2+}, phospholipids, inositol polyphosphates, and proteins (55). Thus, like other modules, the E/ANTH domain appears to be multifunctional in terms of its ligand partners. Interestingly, this idea is consistent with recent studies demonstrating that as yet unidentified protein-binding partners are essential for E/ANTH domain function in yeast (26).

Based on our findings, we propose that the interaction between E/ANTH domains and tubulin/microtubules represents a general mechanism for linking functionally disparate E/ANTH domain-containing proteins to the cytoskeletal architecture. Of

the numerous proteins characterized and shown to harbor an E/ANTH domain, there is a propensity for these to function in clathrin-mediated membrane budding. It has long been demonstrated that tubulin is a component of clathrin-coated vesicles (56, 57). Thus, an interesting possibility is that tubulin interactions may help recruit E/ANTH proteins to endocytic vesicles. A functional link between endocytosis and the cytoskeleton has been examined in numerous studies. For example, in yeast, mutations in several genes, including the epsin genes, *ENT1* and *ENT2* (8), disrupt both endocytosis and the actin cytoskeleton. However, a study examining the dynamics of clathrin-coated pits in living cells indicates that while the actin cytoskeleton is involved in restricting pit mobility, actin is not entirely capable of determining where pits will form or of guiding vesicle movements (58). Thus, these authors have suggested that dynamic interactions between pits and other cytoskeletal structures, such as the microtubule network, are likely involved in spatial regulation of coated pit dynamics (58). Evidence supporting a direct role for microtubules in endocytosis consists of observations that endocytic vesicles can be captured by microtubules below the cell cortex and actively transported inward along microtubules (59, 60). Moreover, this movement is sensitive to various microtubule-destabilizing or -stabilizing drugs (61). Ostensibly, endocytic E/ANTH domain-containing proteins such as epsin and/or HIP1 and HIP12 could bridge the endocytic vesicles comprising these proteins directly to microtubules for microtubule-based transport through the cell cortex or subsequent trafficking through the endosomal pathway. Consistent with this notion, the endocytic protein Eps15, an *in vivo* interacting partner for epsin (2), has been demonstrated to move from the plasma membrane to various intracellular compartments of the endocytic pathway in a microtubule-dependent manner (62). Our observations could also provide a link between the actin- and tubulin-based cytoskeletal networks. For instance, the ANTH domain-containing protein HIP12 is a clear example of a molecule that physically links the actin cytoskeleton with components of clathrin-mediated endocytosis (21). With our demonstration that ANTH domains bind tubulin, HIP12 could additionally bridge these components to the microtubule cytoskeleton for coordinated regulation of endocytosis with actin networks. Elucidation of the precise role of E/ANTH domain interactions with tubulin in microtubule-based vesicular trafficking and/or in linking actin and microtubule networks awaits further investigation.

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Note Added in Proof—Rappoport *et al.* (Rappoport, J. Z., Taha, B. W., and Simon, S. M. (2003) *Traffic* **4,** 460–467) have recently reported the lateral movement of plasma membrane-associated dsRed-clathrin spots along microtubules parallel to the plasma membrane.

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