

Targeted wild-type and jerker espins reveal a novel, WH2-domain-dependent way to make actin bundles in cells

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Summary

The espin actin-bundling proteins, which are the target of deafness mutations, are present in the parallel actin bundles of stereocilia and microvilli and appear to increase their steady-state length. Here, we report a new activity of the espins, one that depends on their enigmatic WH2 domain: the ability to assemble a large actin bundle when targeted to a specific subcellular location. This activity was observed for wild-type espins targeted to the centrosome in transfected neuronal cells and for jerker espins targeted to the nucleolus in a wide variety of transfected cells as a result of the frameshifted peptide introduced into the espin C-terminus by the jerker deafness mutation. This activity,

which appears specific to espins, requires two espin F-actin-binding sites and the actin-monomer-binding activity of the espin WH2 domain, but can be mimicked by adding a WH2 domain to an unrelated actin-bundling protein, villin. Espins do not activate the Arp2/3 complex *in vitro*, and bundle assembly is not indicative of *in-vitro* nucleation activity. Our results suggest a novel way to build actin bundles at specific sites in cells.

Key words: Microtubule, WASP, Neuron, Nucleus, Hearing, RS domain

Introduction

The organization and dynamics of the actin cytoskeleton are controlled by a variety of actin-binding proteins. Because cellular shape, function and behavior depend on the proper positioning of specific types of actin cytoskeletal elements, considerable attention has been focused on the mechanisms by which actin-binding proteins regulate actin polymerization and filament organization locally. One of the best-understood mechanisms involves actin filament branching via the Arp2/3 complex, which is promoted by local activation of WASP family proteins: WASP, N-WASP and the Scar/WAVE proteins (Welch and Mullins, 2002; Millard et al., 2004). WASP family proteins contain homologous C-terminal peptides that include an actin-monomer-binding WH2 (WASP homology 2) domain followed by the C (central) and A (acidic) peptides, which bind the Arp2/3 complex. Upon activation, the actin monomer bound to the WH2 domain is believed to contribute to a stable actin filament 'nucleus' that is elongated. Accordingly, the WH2 domain of WASP family proteins is necessary for their nucleation-promoting activity (Machesky et al., 1999). The WH2 domain appears to be a versatile motif for binding actin monomer and has been recognized in a variety of other proteins, including verprolin/WASP-interacting protein, Srv2/cyclase-associated protein, the β -thymosins, ciboulot, the missing in metastasis proteins and Spir (Paunola et al., 2002;

Mattila et al., 2003; Woodings et al., 2003; Quinlan et al., 2005).

Recently, we discovered a WH2 domain in the espins, a class of actin-bundling protein found in microvillus-type parallel actin bundles (PABs) and Purkinje cell dendritic spines (Loomis et al., 2003; Sekerková et al., 2003; Sekerková et al., 2004). Espins are abundant in the microvillus-like projections of chemosensory and mechanosensory cells, including the stereocilia of hair cells in the inner ear (Sekerková et al., 2004), and are the target of mutations that cause deafness and vestibular dysfunction in mice and humans (Zheng et al., 2000; Naz et al., 2004; Donaudy et al., 2006). Encoded by a single gene, all espin isoforms contain the WH2 domain and a 116-aa C-terminal peptide, the actin-bundling module, which is necessary and sufficient for their potent actin-bundling activity *in vitro*. However, espin isoforms contain different N-terminal peptides that can include multiple ankyrin-like repeats, an additional F-actin-binding site and proline-rich peptides, which can bind profilins or the IRSp53 SH3 domain (Chen et al., 1999; Sekerková et al., 2003; Sekerková et al., 2004; Sekerková et al., 2006).

When expressed in transfected cells, espins exert profound effects on the organization of the actin cytoskeleton, ranging from the formation of coarse stress-fiber-like actin bundles in fibroblastic cells (Bartles et al., 1998; Chen et al., 1999) to the

dramatic barbed-end elongation of microvillar PABs in epithelial cells (Loomis et al., 2003). Here, we report that the targeting of espin constructs to centrosomes or nucleoli in transfected cells causes the de novo assembly of large actin bundles at these locations and that this activity requires two espin F-actin-binding sites and the actin-monomer-binding activity of the espin WH2 domain. Moreover, we show that addition of a WH2 domain confers this activity on a different protein with two F-actin-binding sites, thereby revealing a novel way to make a large actin bundle at a specific subcellular location.

Results

Formation of an actin bundle at the centrosome in neuronal cells

When expressed in rat PC12 pheochromocytoma cells under the control of the cytomegalovirus promoter, the GFP-espins elicited the formation of a large cytoplasmic F-actin bundle in a perinuclear location (Fig. 1A-C). This F-actin bundle was especially noticeable, because in the absence of espin these cells contained relatively low levels of F-actin, as revealed by staining with fluorescent phalloidin (see untransfected control cell in upper right corner of Fig. 1C). The GFP-espins were distributed uniformly throughout the large actin bundle. Lower amounts of GFP-espins and levels of F-actin higher than those

in control cells were also detected at the periphery of the transfected cells, in structures resembling filopodia (Fig. 1A-C). Identical results were obtained when espins were expressed without a GFP tag and localized by espin antibody. The formation of a perinuclear actin bundle in response to the expression of GFP-espins was not observed in fibroblastic (BHK, NRK, 3T3) or epithelial (LLC-PK1) lines, even though the GFP-espins were expressed at comparable levels. The perinuclear actin bundle was, however, observed in a second neuronal cell line, the mouse Neuro-2a neuroblastoma line, and in a subset (~10%) of transfected cells in primary rat hippocampal neuron cultures (Fig. 1D,E). Although observed occasionally in fully polarized hippocampal neurons, the large bundle was more commonly found in neuronal cells that were not fully polarized (Fig. 1D,E).

To infer how the espin-induced perinuclear actin bundle formed, we examined PC12 cells at relatively early times (4-8 hours) after transfection. Because GFP-espins and F-actin were colocalized throughout bundle formation, only GFP-espins localizations are shown in Fig. 1F-H to chronicle key stages. Relatively low levels of GFP-espins and F-actin first accumulated in a starburst-like collection of smaller bundles disposed in radial fashion about the centrosome (Fig. 1F), which was revealed by labeling the microtubule-organizing center with γ -tubulin antibody (red dots in Fig. 1F-H). These smaller bundles then collapsed and became consolidated into a larger bundle (Fig. 1G,H). One end of the bundle remained in close proximity to the centrosome (Fig. 1H).

When examined by transmission EM, the centrosomal actin bundle (CAB) consisted of tightly packed microfilaments and resembled espin crosslinked actin bundles formed *in vitro* (Bartles et al., 1998; Chen et al., 1999) in that neither definitive cross-bridges nor extensive regions showing the cross-striations indicative of paracrystalline order could be recognized (Fig. 2A). Myosin S1 decoration revealed that the actin filaments in CABs were oriented in a parallel fashion throughout a given section (Fig. 2B-D).

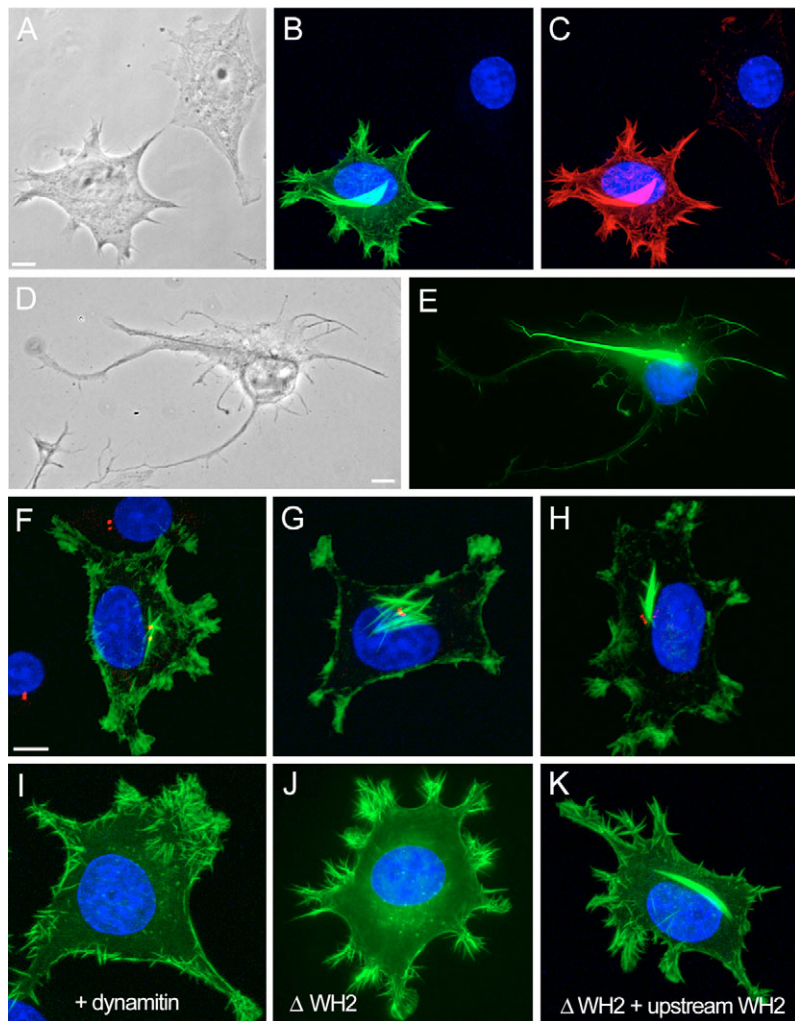


Fig. 1. Formation of a centrosomal actin bundle (CAB) in neuronal cells transfected with GFP-espins (green). (A-C) Colocalization of GFP-espins (B) and F-actin labeled with Texas-Red-phalloidin (C, red) in a large perinuclear actin bundle in a transfected PC12 cell (A, phase). (D,E) GFP-espins (E) in a large perinuclear actin bundle in a transfected primary hippocampal neuron (D, phase). (F-H) GFP-espins in bundle assembly intermediates observed in PC12 cells 4-8 hours after transfection (in order of appearance) highlighting relationship to microtubule-organizing center labeled with γ -tubulin antibody (red dots). (I) Inhibition of CAB formation upon cotransfection of PC12 cell with GFP-espins and myc-dynamitin constructs. (Dynamitin expression was confirmed with myc antibody; not shown.) (J) Absence of CAB formation in a PC12 cell transfected with GFP-espins WH2-domain-deletion construct. (K) Restoration of CAB formation in a PC12 cell transfected with the GFP-espins WH2-domain-deletion construct containing an upstream WH2 domain. Blue, nuclei labeled with DAPI. Bars, 5 μ m.

This parallel alignment was maintained for up to $\sim 10 \mu\text{m}$ in single sections; little or no branching was observed, although the S1-decorated CABs often appeared to be composed of interwoven sub-bundles that passed in and out of the section plane (Fig. 2B-D). To infer CAB directionality, we examined sections that included $>4 \mu\text{m}$ longitudinal stretches of S1-decorated CAB running unambiguously between the perinuclear region and plasma membrane. In nine out of nine cells analyzed, the barbed end of the S1 faced the plasma membrane, suggesting a barbed-end-out polarity for the espin-induced CABs and their constituent filaments (Fig. 2B-D).

The spatial relationship to the centrosome (Fig. 1F-H), and especially the star-like actin bundle array observed at early times (Fig. 1F), suggested that the location of the CAB might be determined in part by minus-end-directed transport of the espins along microtubules. Consistent with this hypothesis,

overexpression of the dynactin p50 subunit dynamitin, which interferes with dynein-dynactin motor-complex-mediated transport along microtubules (Burkhardt et al., 1997), blocked CAB formation and resulted instead in an accumulation of GFP-espin and F-actin in filopodia-like projections at the cell periphery and dorsal surface (Fig. 1I). A similar result was obtained when cells were treated with the microtubule-depolymerizing drug nocodazole ($1 \mu\text{M}$) after transfection, implying a requirement for an intact microtubule network. The inhibitory effect of nocodazole was reversible; a CAB formed 1-2 hours after washing out the drug. In our EM analysis, we encountered examples of microtubules running alongside CABs (Fig. 2A,C, arrowheads), however no regular spatial relationship was noted between CABs and microtubules by immunofluorescence.

To examine the effect of espin concentration on CAB formation, we transfected PC12 and Neuro-2a cells with a series of GFP-espin constructs that contained deletion mutations in the cytomegalovirus promoter to give GFP-espin expression levels that were estimated previously on western blots to be 2, 10 and 40% of those attained with the full-strength (wild-type) promoter (Loomis et al., 2003). A CAB still formed when the espin was expressed at the 40% and 10% levels, but at the 10% level CAB frequency and size were reduced. This was especially the case for the PC12 cells, which expressed each construct at a lower level than Neuro-2a cells. CABs were not observed in either cell line when the GFP-espin was expressed at the 2% level. Under these conditions, the GFP fluorescence was no longer visible by fluorescence microscopy, but transfected cells could still be recognized by immunofluorescence using antibody to GFP.

The formation of a CAB was observed in response to all espin isoforms tested: rat espins 1, 2A, 2B, 2D, 3A, 3B, and 4 and human espin 3A. Different transcriptional start sites distinguish four major espin isoform size classes, designated 1-4 (Fig. 3A), and splice variants are further specified alphabetically (Sekerková et al., 2004). [Espin 2D is the revised name for Purkinje cell espin 1+ (Sekerková et al., 2003).] Even though different isoforms made CABs, the larger isoforms, such as 1 and 2B, tended to make CABs that appeared somewhat larger and more consolidated than those made by the smaller isoforms 3A, 3B and 4. This difference could reflect the presence of an additional F-actin-binding site in the larger isoforms (Fig. 3A) (Chen et al., 1999). The

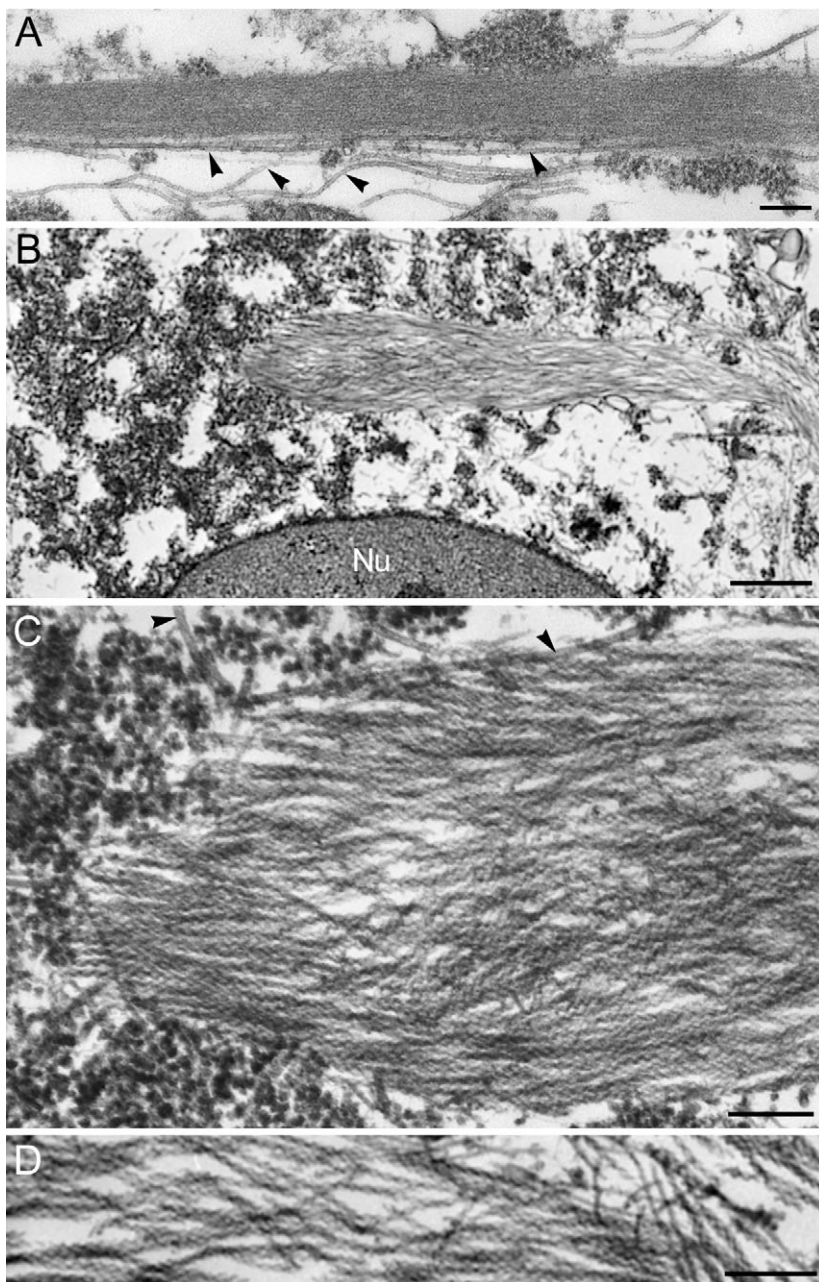


Fig. 2. EM analysis of CABs in transfected PC12 cells without (A) and with (B-D) S1 decoration showing that CABs are PABs. Nu, nucleus. (C,D) Left and right ends of bundle in B, respectively. S1 barbed ends face to the right. Arrowheads indicate microtubules. Bars, $0.2 \mu\text{m}$ (A,C,D); $1 \mu\text{m}$ (B).

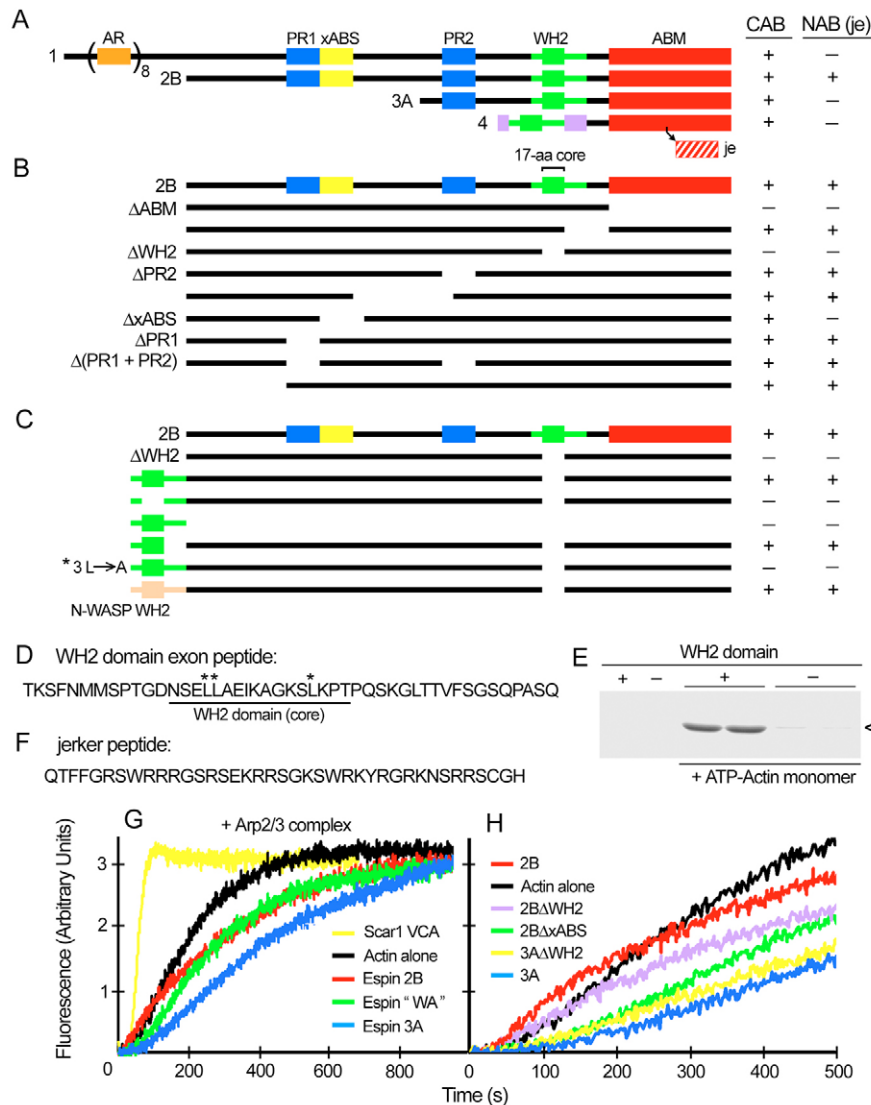


Fig. 3. Espin isoforms, domains and activities. (A-C) Isoform and domain dependence for forming the centrosomal actin bundle (CAB) by wild-type espins and the nuclear actin bundle (NAB) by jerker (je) espins.

(A) Representatives of the four major espin-isoform size classes. ABM, actin-bundling module; AR, ankyrin-like repeat; je, jerker peptide (red and white diagonal stripe; sequence shown in F), the frameshifted peptide that replaces the C-terminal part of the espin actin-bundling module at the site of the jerker deafness mutation (black arrow, illustrated for espin 4); PR, proline-rich peptide; xABS, 23-aa additional F-actin-binding site; purple colour indicates peptides unique to espin 4. (B) Effect of truncation or deletion mutations indicating a requirement for the actin-bundling module, WH2 domain and, in the case of jerker espin 2B, the 23-aa additional F-actin-binding site.

(C) Restoration of CAB or NAB forming activity by adding an upstream WH2 domain to the espin 2B WH2-domain-deletion construct. *3L→A, WH2 domain with its 3 leucine residues (asterisks in D) mutated to alanine. (D) Peptide encoded by espin WH2-domain exon with its 17-aa core underlined. *, leucines mutated to alanine in *3L→A construct (activities shown in C). (E) Pull-down assay showing WH2-domain-dependent binding of ATP-actin monomer to espin 2B. <, actin band. (F) Sequence of jerker peptide. (G,H) Pyrene-actin polymerization assay for the designated construct (0.4 μM) in the presence (G) or absence (H) of 10 nM Arp2/3 complex showing absence of nucleation-promoting and nucleation activity of espins in vitro. Espin "WA", espin C-terminal peptide that begins 12 aa upstream of the 17-aa core of the WH2 domain. s, second.

formation of a CAB was not observed when other actin-bundling proteins (mouse or chicken villin, human T-fimbrin, human fascin or 'constitutively active' S39A-human fascin) were expressed at comparable levels to GFP-espins in these cells using the same vector. However, GFP-tagged versions of these other actin-bundling proteins did associate with CABs when expressed with untagged espin in co-transfected cells.

To identify the espin domains required for CAB formation, we transfected PC12 cells with espin 2B constructs missing known structural or functional domains and scored for the presence or absence of a CAB in cells expressing levels of construct similar to those of wild-type espin 2B on the basis of GFP fluorescence intensity (Fig. 3B). Espin 2 isoforms have been detected in two types of neuron: cerebellar Purkinje cells and vomeronasal sensory neurons (Sekerková et al., 2003; Sekerková et al., 2004). Espin 2B, which corresponds to espin 1 missing its N-terminal ankyrin-like repeats (Fig. 3A), affords an optimal representation of other espin domains and has been characterized extensively in vitro and in vivo (Chen et al., 1999; Loomis et al., 2003; Sekerková et al., 2003; Sekerková et al., 2004). A number of domains could be eliminated without

inhibiting CAB formation, including the profilin-binding proline-rich peptides, singly or doubly, or the 23-aa additional F-actin-binding site adjacent to the N-terminal proline-rich peptide (Fig. 3B). Elimination of the espin C-terminal actin-bundling module, which is believed to contain two F-actin-binding sites positioned roughly at each end (Bartles et al., 1998), blocked formation of the CAB (Fig. 3B) and resulted instead in the GFP-espin fluorescence being distributed diffusely throughout the cytoplasm.

Through the analysis of additional deletion constructs, we identified a requirement for the espin WH2 domain (Fig. 3B). This domain is highly conserved among espins. The first 30 amino acids of the peptide encoded by the WH2 domain (Fig. 3D), including the 17-aa core of the WH2 domain, are identical in espins from *Fugu* to human, making this domain even more conserved than the actin-bundling module. WH2 domains, which occur in a variety of modular proteins involved in actin cytoskeletal regulation, are thought to be versatile adapters for binding ATP-actin monomers (Paunola et al., 2002). Accordingly, deletion of the 17-aa core of the espin WH2 domain caused a dramatic reduction in the ability of

espin 2B to bind ATP-actin *in vitro* in pull-down assays carried out under conditions intended to maintain the actin in monomeric form (Fig. 3E). A similar result was obtained previously for espin 3A and espin 3B (Sekerková et al., 2004). Moreover, deletion of the 17-aa core also reduced the rapid recovery of photobleached GFP- β -actin observed throughout the length of the long microvilli of espin-expressing epithelial cells, suggesting that the WH2 domain of PAB-associated espins can also bind actin monomer *in vivo* (Loomis et al., 2003). Beyond its amino acid sequence and ability to bind actin monomer *in vitro* and *in vivo*, this peptide in the espins shares additional attributes with well-characterized WH2 domains (see below). Deletion of the 17-aa core of the espin WH2 domain eliminated CAB formation and resulted instead in the accumulation of GFP-espin and F-actin in filopodia-like structures at the cell periphery (Fig. 1J, Fig. 3B). The segment required for CAB formation was narrowly centered on the 17-aa core of the WH2 domain, because deletion of a 41-aa peptide positioned only 7-aa downstream of the WH2 core did not inhibit formation of the CAB (Fig. 3B).

The requirement for the espin WH2 domain in CAB formation precluded an analysis of CAB actin treadmilling dynamics by fluorescence recovery after photobleaching. PABs containing espins show a rapid, WH2-domain-dependent, post-bleach recovery of GFP- β -actin fluorescence along their entire length that masks their actin treadmilling (Loomis et al., 2003). Because the incorporation of espins into pre-existing microvillar PABs does not require the WH2 domain, we were able to delete the WH2 domain to reduce this rapid recovery and reveal actin treadmilling in the espin-containing microvillar PABs of transfected epithelial cells (Loomis et al., 2003). However, elimination of the WH2 domain was not an option for CABs, because the WH2 domain was required for CAB formation. We were, however, able to infer actin treadmilling in CABs by using latrunculin A, a cell-permeable inhibitor of actin polymerization (Coue et al., 1987). Treatment of CAB-containing PC12 cells for 1.5 hours with 1 μ M latrunculin A resulted in a dramatic shrinkage or disappearance of CABs, which then reformed within 1 hour after washing out the drug.

Formation of an actin bundle at the nucleolus by jerker espins

The analysis of jerker espins afforded a second illustration of how the targeting of espins can result in actin bundle formation at a specific subcellular site. The espin gene of the jerker mouse has a frameshift mutation (2426delG) that causes recessive deafness and vestibular dysfunction, accompanied by abnormally short and degenerated hair cell stereocilia (Zheng et al., 2000). Espins bearing the jerker mutation have the C-terminal 63 amino acids of their actin-bundling module replaced with a novel 39-aa peptide – the jerker peptide – which is rich in positively charged amino acids (Fig. 3A,F). Jerker espin proteins are unstable and/or synthesized inefficiently *in situ*, because homozygous jerker mice are deficient in espin proteins, yet maintain normal levels of the mutated espin mRNAs (Zheng et al., 2000). Even though regulatory mechanisms must limit the accumulation of jerker espin proteins in the tissues of jerker mice, we were able to express jerker espin proteins in transiently transfected mammalian cell lines. Our results were similar whether

examining fibroblastic (BHK, NRK), neuronal (PC12, Neuro-2a) or epithelial (LLC-PK1) cell lines and whether using GFP-tagged or untagged jerker espins. Therefore, only results obtained examining GFP-jerker espins in subconfluent LLC-PK1 cells are shown.

When expressed in transfected mammalian cells, espin 2B bearing the jerker mutation (jerker espin 2B) became efficiently concentrated in the nucleus and resulted in the formation of a large nuclear actin bundle (NAB; Fig. 4A-C). Although, at first glance, the outcome resembled CAB formation by wild-type espins (Fig. 1A-C), confocal microscopy confirmed that the bundle formed by the GFP-jerker espin was internal to the nuclear lamina, as revealed by lamin antibody (red in Fig. 4D). The NAB exhibited a variety of shapes, ranging from a tightly packed donut-like bundle (Fig. 4D) to a curved or wavy linear bundle, often with frayed ends (Fig. 4A-C). The GFP-espin and F-actin were colocalized throughout the NAB (Fig. 4B,C). We attempted to examine NABs by EM, but found the actin filaments difficult to resolve from other electron-dense nuclear material, even with deoxyribonuclease treatment and additional detergent extraction.

Time-course experiments revealed an unexpected pathway of assembly for the NAB – one involving the nucleolus (Fig. 4E-T). At early times after transfection, the GFP-jerker espin 2B accumulated in relatively large phase-dense subnuclear compartments (Fig. 4E-H), typically one to three in number. In other experiments (see below), these structures were identified as nucleoli by labeling with antibodies to nucleolar proteins, such as nucleolin or fibrillarin. Initially, the GFP-jerker espin-containing nucleoli showed no accumulation of F-actin that could be revealed by fluorescent phalloidin (Fig. 4G). Next, small actin bundles, detected as narrow strands containing GFP-jerker espin and F-actin, were observed as a thin rim at the nucleolar periphery and as small arms that radiated outward from the rim into the surrounding nucleoplasm (Fig. 4I-L). These small bundle strands grew longer and thicker, curved to form spirals (Fig. 4M-P) and eventually became consolidated into a mature NAB (Fig. 4Q-T). This assembly pathway could account for the varied shape of the NAB: donut-shaped bundles would result when there was extensive overlap of curved sub-bundles to form a closed circle, whereas linear bundles would result when there was insufficient overlap or separation of sub-bundles.

The earliest steps in the NAB assembly pathway (Fig. 4E-L) implicated GFP-jerker espin protein that was targeted to the nucleolus. This nucleolar targeting proved to be attributable to the novel 39-aa peptide (Fig. 3F) located at the distal C-terminus of the proteins (Fig. 3A). The jerker peptide was sufficient to cause efficient targeting of a GFP-jerker peptide fusion construct to the nucleolus (Fig. 5A-D), suggesting that it contained a nucleolar localization sequence. However, unlike GFP-jerker espin 2B (Fig. 5E,F), the GFP-jerker peptide did not cause NABs or noticeable accumulation of F-actin in the nucleus even when expressed at comparable levels (Fig. 5G,H). Conversely, GFP-jerker espins lacking the jerker peptide did not accumulate in the nucleus or assemble NABs. Thus, the 39-aa jerker peptide, which was necessary and sufficient for the nucleolar targeting, was necessary but insufficient to form a NAB.

Expression of jerker espin 2B using the vectors with the

mutated cytomegalovirus promoter revealed a concentration dependence for NAB formation similar to that noted above for wild-type espin 2B in CAB formation. NAB size and frequency were reduced when jerker espin 2B was expressed at the 40% and 10% levels. NABs were not observed when GFP-jerker espin 2B was expressed at the 2% level, but the GFP-jerker espin 2B was still targeted to the nucleolus, as revealed using antibody to GFP. As we observed for CABs, latrunculin A treatment caused NABs to shrink or disappear, however higher concentrations of drug (5 μ M) and longer incubation times (3 hours) were required.

To identify the espin domains required for NAB formation, we transfected LLC-PK1 cells with GFP-jerker espin 2B constructs lacking different domains and scored for the presence or absence of a NAB in cells expressing levels of construct comparable with those giving a strong NAB-forming response by full-length GFP-jerker espin 2B. The domain requirements for NAB formation showed many similarities to those for CAB formation, but there were some differences (Fig. 3B). The proline-rich peptides could be eliminated, singly or doubly, without reducing NAB formation (Fig. 3B). Moreover, NAB formation required what remained of the actin-bundling module upstream of the jerker peptide (Fig. 3B). This remaining part of the actin-bundling module is believed to

contain one of two F-actin-binding sites that contribute to the actin-bundling module (Bartles et al., 1998). The espin WH2 domain also proved to be required for NAB formation (Fig. 3B). Deletion of the 17-aa core of the espin WH2 domain resulted in the accumulation of the jerker espin in nucleoli and a variable number of smaller nuclear foci, but no detectable nuclear or nucleolar F-actin (Fig. 5I,J). Deletion of the 41-aa peptide positioned only 7 aa downstream of the WH2 core did not inhibit NAB formation (Fig. 3B).

Unlike the situation for CAB formation, the 23-aa additional F-actin-binding site (Fig. 3A) (Chen et al., 1999) was required for NAB formation (Fig. 3B). GFP-espins 2B lacking the 23-aa additional F-actin-binding site was efficiently targeted to nucleoli and smaller nuclear foci, but it did not form NABs or cause significant accumulation of F-actin (Fig. 3B; similar to Fig. 5I,J). This suggested that NAB formation by jerker espin 2B required two F-actin-binding sites and that the 23-aa additional F-actin-binding site could substitute for the loss of the distal F-actin-binding site from the actin-bundling module as a result of the jerker mutation (Fig. 3A). Accordingly, jerker espin 3 and jerker espin 4, which do not contain this 23-aa additional F-actin-binding site (Fig. 3A), did not form NABs or cause an accumulation of nuclear F-actin (Fig. 3A). Although these proteins were efficiently targeted to nucleoli,

they gave results similar to those obtained with the GFP-jerker peptide alone (Fig. 5G,H). Unexpectedly, jerker espin 1 – which, like the espin 2 isoforms, contains the 23-aa additional F-actin-binding site (Fig. 3A) – also failed to form NABs or cause accumulation of nuclear F-actin (Fig. 3A). This suggested that the eight ankyrin-like repeats at the N-terminus of espin 1 (Fig. 3A) suppressed the activity or availability of the additional F-actin-binding site. Thus, in addition to the jerker peptide, which was required for nucleolar targeting, NAB formation required two espin F-actin-binding sites and the espin WH2 domain.

Further characterization of the WH2-domain requirement

We reasoned that the potent actin-bundling (Bartles et al., 1998) and barbed-end PAB-elongating activities of the espins (Loomis et al., 2003), both of which stem from the actin-

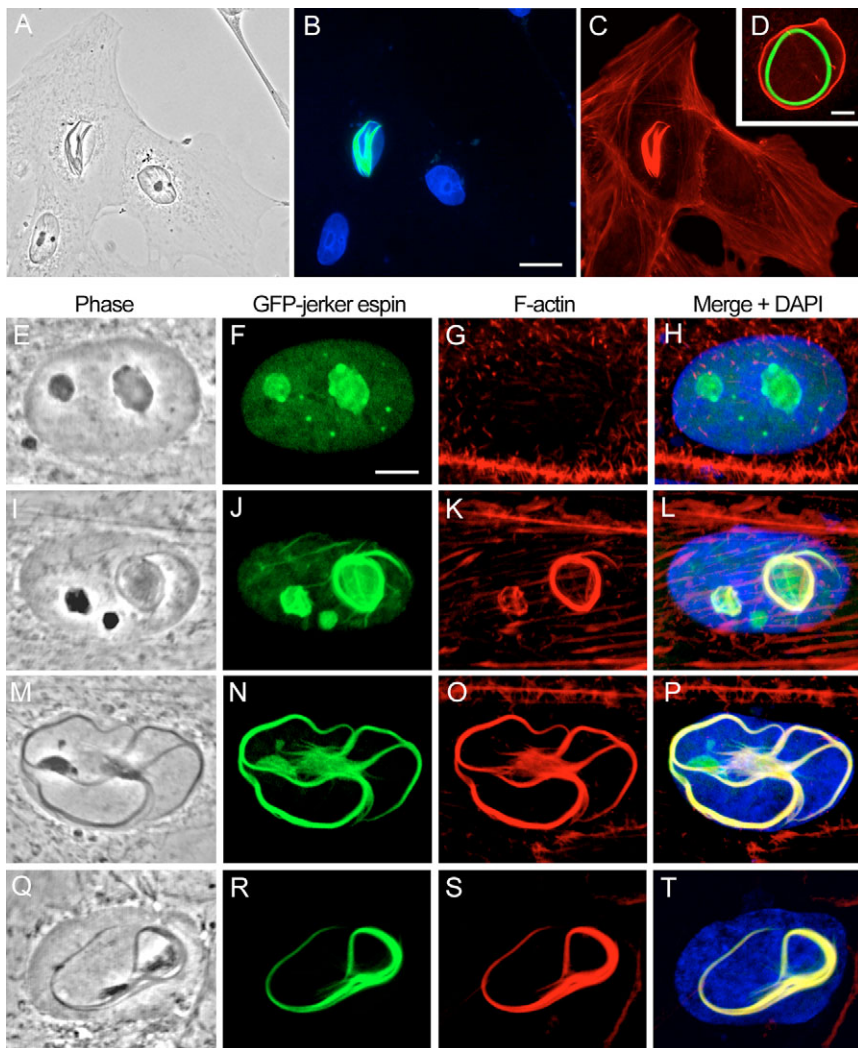


Fig. 4. Formation of nuclear actin bundle (NAB) in LLC-PK1 cells transfected with GFP-jerker espin 2B (green). (A-C) Colocalization of GFP-jerker espin (B) and F-actin labeled with Texas-Red-phalloidin (C, red) in a large actin bundle in a transfected cells (A, phase; blue in B, DAPI). (D) Confocal image showing that the GFP-jerker espin-labeled actin bundle is internal to the nuclear lamina revealed by lamin antibody (red). (E-T) Four rows of NAB assembly intermediates observed 4-8 hours after transfection (in order of appearance) highlighting the relationship to the nucleolus (phase dense) at early stages. Bars, 15 μ m (B); 5 μ m (D,F).

bundling module, could help to assemble a large actin bundle. However, because neither activity requires the espin WH2 domain, we examined further the basis for the WH2 requirement in the formation of CABs and NABs.

Although espins do not contain an obvious WASP-like C peptide, they do resemble WASP family proteins in linear domain organization: basic region (required for binding phosphatidylinositol 4,5-bisphosphate), proline-rich peptide, WH2 domain, and C-terminal peptide containing clusters of acidic amino acids (Sekerková et al., 2004). Therefore, we tested whether espins could act as nucleation-promoting factors for Arp2/3-complex-mediated actin polymerization. Espin 3A failed to activate the Arp2/3 complex *in vitro*, even at relatively high concentrations (400 nM in Fig. 3G). For comparison, at this same concentration, the Scar1 VCA peptide (V, alternative abbreviation for the WH2 domain) caused pronounced activation (Fig. 3G), even though it is a relatively weak nucleation-promoting factor (Zalevsky et al., 2001). To address the possibility of autoinhibition (Kim et al., 2000), we also tested an espin C-terminal construct that began just 12 aa upstream of the 17-aa core of the WH2 domain and, hence, was missing an N-terminal peptide. This espin 'WA' fragment also failed to activate the Arp2/3 complex (Fig. 3G). A slight, but reproducible shortening of the polymerization lag time was noted with espin 2B at concentrations greater than 250 nM (400 nM shown in Fig. 3G), but this effect was also observed in the absence of the Arp2/3 complex (Fig. 3H). In fact, the polymerization curves obtained with the different espins constructs were similar in the presence and absence of Arp2/3

complex. Thus, espins did not appear to activate the Arp2/3 complex directly. Accordingly, the acidic aa clusters in the espin C-terminal peptide lack the conserved tryptophan that is found in most Arp2/3 activators (Marchand et al., 2001), and when examined by EM (Fig. 2) CABs showed no evidence of the filament branching commonly associated with Arp2/3 complex involvement (Millard et al., 2004).

Espin 3A inhibited actin polymerization *in vitro* by extending the lag time and decelerating polymerization relative to actin alone (Fig. 3G,H). However, as expected at this 1:10 molar ratio of espin to actin, deletion of the 17-aa core of the WH2 domain from espin 3A had little effect (Fig. 3H). This suggested that the deceleration stemmed primarily from a reduced availability of filament ends because of filament bundling (Murray et al., 1996). Espins are unusually potent actin-bundling proteins (Bartles et al., 1998; Chen et al., 1999). Some deceleration was also evident with espin 2B, but it appeared to be counteracted by the slight shortening of lag time (Fig. 3G,H). This combination of lag shortening and deceleration by espin 2B was reminiscent of the effects noted previously for elongation factor 1 α , which also displays actin-bundling activity (Murray et al., 1996).

The weak lag-shortening effect observed for espin 2B required all three of its actin-binding regions. Deletion of the 23-aa additional F-actin-binding site from espin 2B brought about a large increment in lag time, causing the early part of the polymerization curve to approach that for espin 3A (Fig. 3H), which does not contain the 23-aa additional F-actin-binding site (Fig. 3A). By comparison, deletion of the 17-aa core of the WH2 domain caused the early part of the curve to be more similar to that for actin alone, with deceleration being observed at later times (Fig. 3H). Deletion of the actin-bundling module caused the curve to be highly similar to that of actin alone throughout the

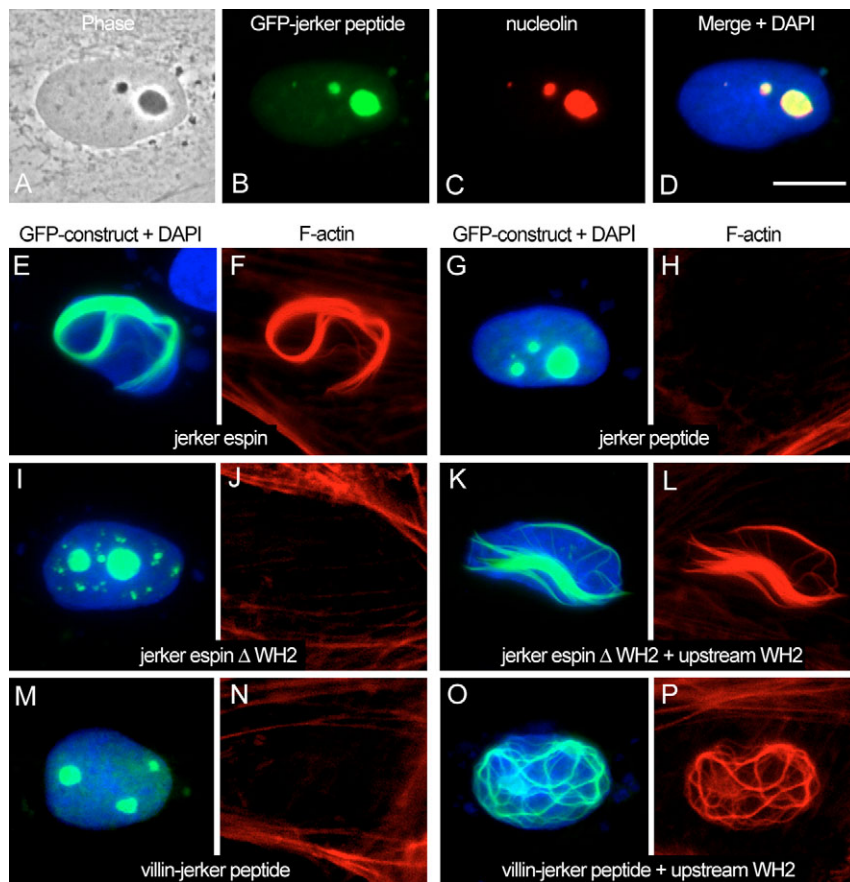


Fig. 5. Domain dependence of NAB formation. (A-D) Targeting of the GFP-jerker peptide construct (B) to nucleoli, as revealed by labeling with nucleolin antibody (C). (E,F) Colocalization of GFP-jerker espin (E) and F-actin labeled with Texas-Red-phalloidin (F) in a NAB. (G,H) Targeting of the GFP-jerker peptide construct to the nucleolus (G) does not cause F-actin accumulation (H). (I,J) The GFP-jerker espin WH2-domain-deletion construct is targeted to nucleoli and to small foci in the nucleoplasm (I), but fails to accumulate F-actin in the nucleus (J). (K,L) Reintroduction of an upstream WH2 domain into GFP-jerker espin WH2-domain-deletion construct results in restoration of NAB-forming activity, causing the colocalization of GFP construct (K) and F-actin (L) in a large NAB. (M,N) The GFP-villin-jerker peptide is targeted to nucleoli (M), but does not cause accumulation of nuclear F-actin (N). (O,P) Introduction of the espin WH2 domain upstream of the villin-jerker peptide construct confers NAB forming activity and causes colocalization of the GFP-WH2-domain-villin-jerker peptide construct (O) with F-actin (P) in fine NABs. Blue, nuclei labeled with DAPI. Bar, 10 μ m.

entire time course (data not shown), reinforcing the conclusion that the deceleration resulted from filament bundling. Note that this latter construct, which contains both the 23-aa additional F-actin-binding site and the WH2 domain and is free from the decelerating effect of the actin-bundling module, failed to shorten the lag time. In summary, although weak lag-shortening activity was observed for one wild-type espin isoform, espin 2B, the activity was observed only at relatively high protein concentrations *in vitro* and did not correlate with the isoform/domain requirements of CAB formation. Notably, the lag-shortening effect was not observed for espin 3A, which made CABs, and the 23-aa additional F-actin-binding site, which was required for the lag-shortening effect, was not required for CAB formation (Fig. 3A,B,H). Thus, CAB formation was not indicative of *in-vitro* nucleation activity on the part of the espins. We did not examine the effects of jerker espins on actin polymerization *in vitro* because of an inability to express and purify the mutated recombinant proteins.

We returned to cell transfection experiments to examine further the basis for the WH2-domain requirement. To investigate the importance of WH2-domain context, we reintroduced the WH2 domain into the espin 2B WH2-domain-deletion constructs, but in a different location. The 144-nt espin WH2-domain-encoding exon was positioned in-frame upstream of the cDNA coding sequence of the espin 2B WH2-domain-deletion constructs to produce wild-type and jerker espin 2B constructs that contained the espin WH2 domain ~400 aa N-terminal to its normal location (Fig. 3C). Remarkably, these constructs were just as active as intact wild-type espin 2B and jerker espin 2B at forming a CAB (Fig. 1K, Fig. 3C) and a NAB (Fig. 5K,L, Fig. 3C), respectively. Control constructs with the peptide encoded by the WH2-domain exon minus the 17-aa WH2-domain core reintroduced at the same upstream location remained inactive (Fig. 3C). Consistent with the requirement for espin F-actin-binding sites, targeting of the 48-aa peptide encoded by the espin WH2-domain exon to the nucleolus as a GFP-WH2-domain-jerker-peptide construct, without the other parts of espin, also failed to elicit NABs or to cause nucleolar/nuclear accumulation of F-actin (Fig. 3C). Similarly, the corresponding GFP-WH2-domain construct, without the jerker peptide attached, failed to target the centrosome (data not shown) or cause noticeable F-actin accumulation in transfected neuronal cells (Fig. 3C). These results suggested that, in the absence of the other parts of espin, the WH2 domain is not sufficient to cause F-actin accumulation or localization to the centrosome. Accordingly, when the peptide encoded by the espin WH2-domain exon was placed upstream of another actin-bundling protein, chicken villin, the resulting GFP-WH2-domain-villin construct also did not localize to the centrosome or cause CAB formation.

The amino acid sequence immediately C-terminal to the WH2 core domain can be of great importance in determining the functions of WH2-domain-containing proteins (Herzog et al., 2004; Irobi et al., 2004; Quinlan et al., 2005). This sequence in espins (Fig. 3D), which bears no obvious similarity to those in other WH2-domain-containing proteins, seemed unimportant for forming CABs and NABS; an upstream WH2 domain truncated only 3 aa C-terminal to the 17-aa core of the espin WH2 domain (Fig. 3D) also efficiently restored bundle-forming activity to the espin 2B WH2-domain-deletion constructs (Fig. 3C). Quinlan et al. (Quinlan et al., 2005)

recently reported that mutation of the three leucine residues in the WH2-domain core to alanine eliminated actin-monomer binding. Restoration of bundle-forming activity to the espin 2B WH2-domain-deletion constructs was not achieved when the upstream WH2 domain had its three key leucine residues mutated to alanine (Fig. 3C), underscoring the requirement for actin-monomer binding by the espin WH2 domain to build CABs and NABS. Moreover, substitution of the upstream espin WH2-domain peptide with another actin-monomer-binding WH2 domain (Yamaguchi et al., 2000), the second WH2 domain (aa residues 417-466) of rat N-WASP, also restored actin bundle-forming ability to the espin 2B WH2-domain-deletion constructs (Fig. 3C). This ability of the N-WASP WH2 domain to efficiently substitute for the espin WH2 domain suggested that these two WH2 domains have a comparable affinity for binding ATP-actin monomers. Taken together, these results suggested that, in its requirement to support large actin bundle formation at the centrosome or nucleolus, the espin WH2 domain was serving as an actin-monomer-binding module that could function even when placed at a markedly different location in the espin molecule.

Finally, to determine whether the espin WH2 domain was capable of conferring actin-bundle-forming ability on a different targeted protein with two F-actin-binding sites, we examined the effect of introducing the WH2 domain at the N-terminus of chicken villin, an actin-bundling protein that contains no obvious WH2 domain or homology to the espins. In these experiments, the cDNA encoding the 39-aa C-terminal peptide of the jerker espins was placed in frame at the 3' end of the villin coding sequence to target the constructs to the nucleolus (Fig. 5M). The GFP-villin-jerker peptide construct was targeted to the nucleolus and, to a lesser extent, the nucleoplasm (Fig. 5M), but there was no evidence of F-actin accumulation in the nucleolus or nucleus (Fig. 5N). However, when the peptide encoded by the espin WH2-domain exon was placed upstream of the villin-jerker peptide construct, NABs formed (Fig. 5O,P). The NABs appeared finer and less consolidated than those formed by jerker espins (Fig. 5O,P). These differences could reflect the fact that villin is less potent than espin at causing the elongation of PABs (Loomis et al., 2003) or that these targeted GFP-villin constructs appeared to be expressed at somewhat lower levels than the GFP-jerker espin constructs. Like the NABs formed by jerker espins, the GFP-WH2-domain-villin-jerker peptide construct was uniformly distributed throughout bundles (Fig. 5O,P). No NABs or nuclear F-actin accumulation were observed in response to the corresponding control construct missing the 17-aa core of the espin WH2 domain, which was nonetheless efficiently targeted to nucleoli and expressed at comparable levels. Thus, when a WH2 domain is joined to a peptide that can bundle actin filaments – a situation that occurs naturally in the case of the espins – the resultant protein can build large actin bundles when targeted to a specific cellular location.

Discussion

When targeted to a specific location in transfected cells, espins promote the local assembly of a large actin bundle. This activity, which appears to distinguish espins from other actin-bundling proteins, depends on the ability of espins to interact with actin monomers via their WH2 domain and reveals a new way to build an actin bundle at specific sites in cells.

A remarkable aspect of the WH2-domain-dependent actin-bundle-building activity described here is the flexibility we observed regarding the placement and source of the WH2 domain. Positioning the WH2 domain in a vastly different location in espin, substituting it with a WH2 domain from N-WASP or adding the WH2 domain to a different actin-bundling protein, villin, each yielded bundle-building activity. This flexibility strongly suggests that the role of the espin WH2 domain is to increase the local concentration of polymerizable actin monomers. In this capacity, the espin WH2 domain would deliver the ATP-actin monomers needed to fuel local nucleation or elongation reactions. The multiple F-actin-binding sites of espin (or villin) would then crosslink the product and stimulate further elongation via the same mechanism by which such crosslinks cause the barbed-end elongation of preformed microvillar PABs (Loomis et al., 2003). Our mutagenesis results are entirely consistent with an actin-monomer-binding function for the espin WH2 domain and substantiate the existence of multiple parallels between the espin WH2 domain and the WH2 domains of other proteins (Yamaguchi et al., 2000; Quinlan et al., 2005). The espin WH2 domain also contributes to the rapid post-bleach recovery of GFP- β -actin seen along the length of microvilli in espin-expressing epithelial cells (Loomis et al., 2003). The latter, which masks the ~ 0.25 $\mu\text{m}/\text{minute}$ treadmilling in the PAB at the microvillar core, suggests that the WH2 domain retains the ability to bind actin monomer even when the espin is part of a microvillar PAB (Loomis et al., 2003).

Relatively little is known about the availability of polymerizable actin monomers at the centrosome and in the nucleus. Although actin has been implicated in a variety of important nuclear processes (Bettinger et al., 2004), nuclear actin levels appear to be kept low by exportin-6-mediated transport of profilin-actin complexes (Stüven et al., 2003). The efficiency of this pathway may explain why the proline-rich peptides of espins, which can bind profilins (Sekerková et al., 2004) and presumably also profilin-actin complexes, cannot substitute for the WH2 domain as a source of actin monomers for building NABs. Interfering with the exportin 6 pathway (Stüven et al., 2003) or treatments such as DMSO or heat shock (Fukui and Katsu-Maru, 1979; Iida et al., 1986), can lead to the formation of nuclear actin paracrystals. Peculiar nuclear F-actin coils were observed previously in cells transfected with an N-terminal fragment of supervillin that does not include its C-terminal villin/gelsolin-homology domain (Wulfkuhle et al., 1999).

Our results suggest that equipping an actin-bundling protein with a WH2 domain can give rise to some extraordinary properties. One intriguing possibility is that the espin WH2 domain works in concert with the multiple F-actin-binding sites of espin to form actin bundles in cells. Even though recombinant espins did not activate the Arp2/3 complex or exhibit significant nucleation activity *in vitro*, we cannot rule out the possibility that espins contribute to the nucleation of actin polymerization in cells. Unlike the situation for CAB and NAB formation, the espin WH2 domain is not required for the espin-mediated elongation of microvillar PABs in transfected epithelial cells (Loomis et al., 2003). This difference could reflect the fact that these microvillar PABs do not form in response to espins, but simply elongate (Loomis et al., 2003), or the possibility that actin monomers are not limiting in

microvilli. The combination of WH2 domain and actin-bundling protein appears unique to espins, but could potentially extend to the missing in metastasis (MIM)-B and ABBA-1 proteins, which contain a WH2 domain with an IRSp53/MIM homology domain that can bundle actin filaments when dimerized (Woodings et al., 2003; Mattila et al., 2003; Millard et al., 2005). Interestingly, the only candidate espin orthologs currently recognized outside of the vertebrates, the forked proteins (Bartles et al., 1998), which mediate the formation of PABs in the developing neurosensory bristles of *Drosophila* pupae (Tilney et al., 1998), do not appear to contain a consensus WH2 domain.

Although the targeting scenarios investigated here were exploited to reveal a novel activity of espins, they could have implications for espin biology. Centrosomal targeting of expressed espins is not evident in transfected fibroblastic or epithelial cell lines (Bartles et al., 1998; Chen et al., 1999; Loomis et al., 2003). One possibility is that expressed espins misfold or otherwise overwhelm the ubiquitin-proteasome pathway selectively in neuronal cells, which results in their delivery to aggregates near the centrosome. This process, which is known to involve transport along microtubule tracks via the dynein-dynactin motor complex (Kawaguchi et al., 2003; Burnett and Pittman, 2005), could explain the inhibition of CAB formation we observed with dynamitin overexpression or nocodazole treatment. Interestingly, centrosomal targeting was observed across the entire collection of structurally diverse espin isoforms, but not for other actin-bundling proteins expressed at comparably high levels. Moreover, the domain dependence noted for CAB formation suggests that many of the espin domains are functional and, therefore, folded properly. It should also be noted that espins are expressed endogenously in specific classes of neuron, cerebellar Purkinje cells and vomeronasal sensory neurons, and show a strict compartmentalization to dendritic spines and dendritic microvilli, respectively, which could conceivably result from motor-driven transport along dendritic microtubules (Sekerková et al., 2003; Sekerková et al., 2004). We have not yet noticed examples of CABs *in situ* in the neurons of adult animals. However, CAB formation may require a burst of espin expression at a particular stage of neuronal development, and, importantly, there may be ways to regulate both espin targeting and bundle-building activity *in situ*. Nevertheless, it remains possible that the type of espin-mediated, WH2-domain-dependent actin bundle assembly described here occurs on a smaller scale, locally in cells *in situ*, without resulting in the accumulation of such a massive actin bundle.

The targeting of jerker espins to nucleoli stems from the jerker peptide, which in our experiments behaved as a portable nucleolar localization sequence capable of specifying the nucleolar targeting of multiple heterologous proteins in a variety of cell types regardless of expression level. This 39-aa peptide, which is rich in arginine and serine residues, resembles peptides found in other nuclear proteins, including the protamines, the lamin B receptor, the Bcl2-associated transcription factor and the RS domains of SR protein splicing factors. As has been observed in other proteins targeted to nucleoli (e.g. Nagahama et al., 2004; Horke et al., 2004), the clusters of positively charged amino acids in the jerker peptide likely include signals for nuclear import and nucleolar retention. Even though the jerker espin mRNAs are maintained

at wild-type levels, jerker espin proteins do not accumulate in the tissues of jerker mice (Zheng et al., 2000). In view of the jerker espin concentration dependence we noted for NAB formation, this deficiency of jerker espin protein likely explains why we do not observe NABs in situ in the cells of jerker mice. Nevertheless, the highly efficient nucleolar targeting of jerker espins, which we observed even when expressed at low levels, could be the basis for the instability of these mutated proteins in situ.

In conclusion, our results suggest that there can be more to making a PAB in cells than filament/bundle stabilization through crosslinking and that under certain circumstances issues such as the local provision of actin monomers and actin-bundling protein targeting can be crucial. The robust, WH2-domain-dependent response to espins targeted to locations as diverse as the centrosome and nucleolus suggests additional roles for these proteins in actin-monomer delivery and bundle assembly as well as some possible applications in cellular engineering and nanotechnology.

Materials and Methods

Media and sera were from Invitrogen (Carlsbad, CA). PC12 cells were cultured in DMEM with 7% horse serum and 5% FBS, transfected by electroporation (Yoon et al., 2001) and plated onto coverslips coated with laminin (Roche, Indianapolis, IN). LLC-PK1 cells were cultured in MEM Alpha with 5% FBS, plated onto uncoated coverslips and transfected with Lipofectamine (Loomis et al., 2003). Cell lines were transfected with the designated cDNA constructs in a pEGFP-C (BD Biosciences Clontech, Palo Alto, CA) or pcDNA3 (Invitrogen) vector and examined 18–24 hours later, unless specified otherwise. In some experiments, cells were transfected with pEGFP-C vector constructs that contained deletion mutations in the cytomegalovirus promoter to give reduced levels of expression (Loomis et al., 2003). In other experiments, cells were incubated with 1 μ M nocodazole (Sigma, St. Louis, MO) or 1–5 μ M latrunculin A (Calbiochem, La Jolla, CA) or were cotransfected with a myc-tagged dynamitin construct (Burkhardt et al., 1997) (gift from Richard Vallee, Columbia University, New York, NY). Neuronal cultures were prepared from the hippocampi of embryonic day 18 rat embryos, transfected using a Nucleofector apparatus (Amaxa, Gaithersburg, MD) and plated on poly-L-lysine-coated coverslips in MEM with 10% horse serum and transferred to dishes containing an astroglial monolayer (Paganoni and Ferreira, 2005). The villin, fimbrin and fascin constructs and a majority of the espin constructs have been described (Loomis et al., 2003; Sekerková et al., 2004). Espin cDNA fragments containing the jerker mutation and N-WASP cDNAs were obtained by RT-PCR using RNA from jerker mouse kidney and rat cerebellum, respectively. Additional mutagenesis was carried out by PCR. Some constructs had a WH2-domain cDNA inserted between the *Bgl*III and *Eco*RI sites of the pEGFP-C2 vector, upstream of an espin or villin cDNA. All constructs were checked by DNA sequence analysis.

For light microscopy (Loomis et al., 2003), cells were fixed in 2–3.5% paraformaldehyde; briefly permeabilized with 0.1% Triton X-100; labeled with Texas-Red-phalloidin (Molecular Probes, Eugene, OR), DAPI (Sigma) or antibodies to human fibrillarin (Sigma), mouse nucleolin (gift from Lester Binder, Northwestern University, Chicago, IL), myc tag or lamin (gifts from Robert Goldman, Northwestern University, Chicago, IL) followed by Texas-Red-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA); mounted in 5% n-propylgallate in 90% glycerol/10% PBS; and examined at room temperature using a Zeiss Axioplan 2 Imaging microscope system (63 \times , 1.4 N.A. oil-immersion objective) equipped with an Axiocam digital camera or a Zeiss LSM 510 META confocal microscope (0.3–0.5 μ m z-sections; 100 \times , 1.4 N.A. oil-immersion objective). When labeling with γ -tubulin antibody (Sigma, T 3559), cells were fixed in methanol. Transient transfection inevitably results in a range of construct expression levels. Therefore, the ability of different constructs to form a large actin bundle at the centrosome or in the nucleus was scored after examination of at least 100 transfected cells on two to four coverslips with attention to matching expression levels visually on the basis of GFP fluorescence intensity. Actin bundles were labeled with rabbit skeletal muscle S1 (gift from Robert Goldman) as described (Svitkina and Borisy, 1998) and examined using a JEOL JEM-1200 EX electron microscope. Images were saved in TIF format, transferred to Photoshop (Adobe Systems) and assembled into composites with minor adjustments in brightness or contrast.

ATP-actin-monomer binding (Urano et al., 2001) was examined by pull-down assay using GST-espins constructs (Sekerková et al., 2004). Pyrene-actin polymerization assays were performed as described (Quinlan et al., 2005) using 4 μ M monomeric actin (labeled with 6% pyrene) in 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM HEPES, pH 7.0. The proteins assayed included espin constructs

with an N-terminal His tag (Sekerková et al., 2004) or a human Scar1 VCA fragment (aa residues 489–559) with a C-terminal His tag, with or without 10 nM Arp2/3 complex (Zalevsky et al., 2001).

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