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Mutations in *Drosophila* Enabled and Rescue by Human Vasodilator-stimulated Phosphoprotein (VASP) Indicate Important Functional Roles for Ena/VASP Homology Domain 1 (EVH1) and EVH2 Domains

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Drosophila Enabled (Ena) was initially identified as a dominant genetic suppressor of mutations in the Abelson tyrosine kinase and, more recently, as a member of the Ena/human vasodilator-stimulated phosphoprotein (VASP) family of proteins. We have used genetic, biochemical, and cell biological approaches to demonstrate the functional relationship between Ena and human VASP. In addition, we have defined the roles of Ena domains identified as essential for its activity in vivo. We have demonstrated that VASP rescues the embryonic lethality associated with loss of Ena function in *Drosophila* and have shown that Ena, like VASP, is associated with actin filaments and focal adhesions when expressed in cultured cells. To define sequences that are central to Ena function, we have characterized the molecular lesions present in two lethal *ena* mutant alleles that affected the Ena/VASP homology domain 1 (EVH1) and EVH2. A missense mutation that resulted in an amino acid substitution in the EVH1 domain eliminated in vitro binding of Ena to the cytoskeletal protein zyxin, a previously reported binding partner of VASP. A nonsense mutation that resulted in a C-terminally truncated Ena protein lacking the EVH2 domain failed to form multimeric complexes and exhibited reduced binding to zyxin and the Abelson Src homology 3 domain. Our analysis demonstrates that Ena and VASP are functionally homologous and defines the conserved EVH1 and EVH2 domains as central to the physiological activity of Ena.

INTRODUCTION

Changes in cytoskeleton assembly and cell adhesion in response to extracellular cues are critical in regulating diverse cellular functions (Damsky, 1993; Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Montell, 1994). Translation of these extracellular signals into cytoskeletal changes are mediated by signal transduction pathways

(Tanaka and Sabry, 1995). In *Drosophila*, the Abelson (Abl) tyrosine kinase and its substrate Enabled (Ena) are thought to play a role in regulating cytoskeletal changes during axonogenesis in the CNS of the developing embryo (Gertler *et al.*, 1989, 1995; Bennett and Hoffmann, 1992; Hill *et al.*, 1995). This hypothesis has been bolstered by the recent identification of *Drosophila* Ena as a member of the cytoskeletal-associated Ena/human vasodilator-stimulated phosphoprotein (VASP) family of proteins (Gertler *et al.*, 1996). Mem-

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bers of this family in addition to *Drosophila* Ena include VASP, murine Ena (Mena), and murine Ena/VASP-like (EVL) protein. Mena and VASP have been localized to the actin cytoskeleton and are *in vitro* ligands for the focal adhesion-associated protein zyxin and the actin-binding protein profilin (Reinhard *et al.*, 1992, 1995a; Haffner *et al.*, 1995; Gertler *et al.*, 1996). *Drosophila* Ena also binds profilin *in vitro* (our unpublished results). Mena and VASP interact with Act A, a protein from *Listeria monocytogenes* required for the recruitment of host actin filaments around the intracellular bacteria, and VASP and Mena may serve as links between ActA and profilin. Consequently, these proteins may enhance Act A-mediated F-actin recruitment that is used as a means of intracellular movement by the bacteria (Chakraborty *et al.*, 1995; Pistor *et al.*, 1995; Pollard, 1995; Gertler *et al.*, 1996). Additional support for a direct role in cytoskeleton assembly comes from the observation that overexpression of neuronally enriched forms of Mena in cultured cells results in actin-rich membrane projections (Gertler *et al.*, 1996) and that targeting of the Mena- and VASP-binding protein zyxin to the plasma membrane causes the elaboration of actin-rich projections (Golsteyn *et al.*, 1997).

The Ena/VASP family of proteins is characterized by a common overall structural domain organization consisting of conserved N- and C-terminal domains separated by a less-conserved central proline-rich region (Gertler *et al.*, 1996). This N-terminal 113-amino-acid domain, or the Ena/VASP homology domain 1 (EVH1), is 58% identical between *Drosophila* Ena and human VASP. The EVH1 domain mediates VASP and Mena binding to the focal adhesion-associated protein zyxin as well as *Listeria* Act A (Gertler *et al.*, 1996). The EVH1 domain is also similar to the WP1 domain found in Wiskott–Aldrich syndrome protein (Symons *et al.*, 1996). Wiskott–Aldrich syndrome is characterized by cytoskeletal abnormalities in T cells and platelets (Derry *et al.*, 1995; Kolluri *et al.*, 1995; Kwan *et al.*, 1995; Villa *et al.*, 1995; Wengler *et al.*, 1995; Zhu *et al.*, 1995). The central proline-rich regions of the Ena, Mena, and VASP proteins, which vary greatly in length, are important for binding to both Src homology 3 (SH3) domains and the actin-binding protein profilin (Gertler *et al.*, 1995, 1996; Reinhard *et al.*, 1995a) (our unpublished results). The C-terminal 35 amino acids of these proteins, or the EVH2 domain, is 31% identical between *Drosophila* Ena and human VASP and consists of a series of conserved charged repeats with a spacing predicted to form an extended highly charged α helix (Haffner *et al.*, 1995). Homology to this region is also seen in human expressed sequence tag r74407 and the mouse cDNA NDPP-1 (Sazuka *et al.*, 1992). Several functions have been proposed for the EVH2 domain, including subcellular localization and mediation of multimer formation

(Haffner *et al.*, 1995; Gertler *et al.*, 1996), although conclusive evidence for these proposed functions has not been reported.

In addition to a role in cytoskeleton assembly, Ena/VASP proteins are constituents of signal transduction pathways. Ena has been linked genetically to the Abl tyrosine kinase signaling pathway by identification of *ena* mutations that act as dominant genetic suppressors of a loss of Abl tyrosine kinase function (Gertler *et al.*, 1990). Heterozygous loss of function mutations in *ena* rescue the lethality and defects in axonal architecture in embryos with mutations in Abl and other components of Abl-mediated signaling (Gertler *et al.*, 1989, 1993; Hill *et al.*, 1995). *Drosophila* that lack Ena function die during embryogenesis and also display defects in the axonal architecture of the nervous system. These include a reduction in the integrity of axon bundles and some axon misrouting consistent with a role for Ena in regulating the cytoskeleton (Gertler *et al.*, 1990, 1995). Ena is a specific substrate for Abl and also specifically interacts with the Abl SH3 domain (Gertler *et al.*, 1995).

Ena's homology to a family of proteins clearly implicated in cytoskeletal dynamics led us to investigate possible conserved functions between Ena and VASP. We report that VASP can partially substitute for Ena function in *Drosophila*. In addition, we show that Ena and VASP are colocalized at focal adhesions and with actin filaments. We have characterized two lethal mutations and show that they result in defects in the EVH1 and EVH2 domains of *Drosophila* Ena. Furthermore, we demonstrate that these mutations disrupt conserved functions of Ena/VASP proteins, namely *in vitro* zyxin binding and multimerization. We further demonstrate that Ena and VASP are colocalized to focal adhesions with zyxin, providing indirect evidence that these proteins may interact in the intact cell. The evidence for conservation of function between *Drosophila* Ena and human VASP suggests that Ena may provide a regulated link between Abl-mediated signal transduction and cytoskeletal dynamics in the developing *Drosophila* CNS.

MATERIALS AND METHODS

Transfections and Western Blot Analysis

DNA encoding six histidine residues, the FLAG tag, or the hemagglutinin (Ha) tag was added to the 3' end of the coding sequence of the *ena* cDNA by PCR. *Drosophila* S2 cells were transiently transfected with VASP, Abl, Ena, or tagged Ena cDNAs in the pPac-PL expression vector. Cells were harvested after 60 h and lysed in immunoprecipitation buffer (0.5% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN), 1 μ g/ml each pepstatin, leupeptin, and aprotinin) or His buffer (0.5% Triton X-100, 20 mM NaPO₄, pH 7.8, 500 mM NaCl, 1 mM Na₃VO₄, 1 mM Pefabloc, 1 μ g/ml each pepstatin, leupeptin, and aprotinin). After lysis, cell debris was pelleted at 12,000 \times g for 20 min. Immunoprecipitations were carried out as previously described using anti-Ena (Gertler *et*

al., 1995), anti-VASP (Halbrugge *et al.*, 1990), anti-FLAG M2, or anti-Ha 12CA5 antibodies. His-tagged Ena was purified by incubating lysates with 35 μ l of Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen, Chatsworth, CA) for 1 h at 4°C. Nonspecifically bound proteins were eluted with His buffer plus 100 mM imidazole. Proteins were resolved on 7.5% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and blotted with anti-Ena, anti-VASP, anti-Ha 12CA5, anti-FLAG M2 (Molecular Probes, Eugene, OR), or anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) antibodies.

Sequencing of Mutant *ena* Alleles

Total RNA was prepared from *Drosophila* pupae carrying heterozygous wild-type and mutant *ena210* or *ena23* alleles using Tri reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription PCR was carried out by priming first-strand synthesis with an Ena oligonucleotide that hybridizes to the last 20 nucleotides in the Ena open reading frame. The Ena coding sequences were subsequently amplified with three sets of primers that amplified the 5' 600 nucleotides, middle 600 nucleotides, and 3' 700 nucleotides, respectively. PCR products were subcloned into the TA cloning vector system (Invitrogen, San Diego, CA), and at least eight independent subcloned PCR products were sequenced for each of the PCR products. As expected, half of the subcloned PCR products carried the *ena210* or *ena23* mutation, and half were wild type.

Site-directed Mutagenesis

DNA was purified by standard techniques (Maniatis *et al.*, 1982). Site-directed mutagenesis of Ena was performed by the method of Deng and Nickoloff (1992). Oligonucleotides containing single-base changes that changed Ala-98 to Val and Lys-636 to a stop codon were incorporated into a full-length Ena cDNA. The entire mutagenized DNA fragment was sequenced to confirm the absence of any other mutations that may have been inadvertently generated during the procedure.

Preparation and Purification of Recombinant Ena and Fusion Proteins and In Vitro Synthesis of Proteins

The His-tagged Ena construct was subcloned into the baculovirus vector pVL1393 (Invitrogen). *Spodoptera frugiperda* SF9 cells were cotransfected with 2.5 μ g of pVL1393-Ena plus 200 ng of Baculogold viral DNA (Pharming, San Diego, CA) to recover recombinant virus. High-titer virus stocks were generated and used to infect 2×10^8 SF9 cells. At 48 h after infection, cells were harvested and lysed in 20 ml of 0.5% Triton X-100, 20 mM NaPO₄ (pH 7.8), 500 mM NaCl, 1 mM Na₃VO₄, 1 mM Pefabloc, and 1 μ g/ml each pepstatin, leupeptin, and aprotinin. After lysis, cell debris was pelleted at 12,000 \times g for 20 min, and the lysate was incubated with 2 ml of NTA-agarose (Qiagen) for 1 h at 4°C. Nonspecifically bound proteins were eluted with His buffer plus 100 mM imidazole and Ena protein was eluted with 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 500 mM NaCl, and 300 mM imidazole and stored at 4°C.

A PCR fragment encoding the *Drosophila* Abl SH3 domain (amino acids 82–325) was subcloned into the *Bam*HI site of the pGEX2TK vector (Pharmacia, Piscataway, NJ). The chicken zyxin translated region encoding amino acids 1–542 was subcloned into the *Eco*RI site of a modified pGEX expression vector. The chicken zyxin LIM domain (amino acids 349–542) and the chicken zyxin proline-rich region (amino acids 1–348) were also subcloned into the *Eco*RI site of a modified pGEX vector (Schmeichel and Beckerle, 1994). GST fusion proteins were expressed in *Escherichia coli* DH5 α and prepared as previously described (Gertler *et al.*, 1995). Ena protein and Ena A97V mutant protein were produced in vitro using the Single Tube Protein System (Novagen, Madison, WI) after subcloning into

the pCITE vectors. All proteins were quantified by Coomassie blue staining or Western blot.

Solution- and Filter-binding Assays

For solution-binding assays, transfected S2 cell lysates were incubated with equivalent amounts of GST-SH3 or GST-Zyxin fusion proteins immobilized on glutathione-Sepharose (Pharmacia) for 1 h at 4°C. Beads were washed once with immunoprecipitation buffer and boiled in SDS sample buffer. Bound proteins were analyzed by SDS-PAGE, followed by Western blotting with anti-Ena or anti-VASP antibodies. Ena and VASP overlays were carried out essentially as previously described (Reinhard *et al.*, 1995a). Fifty micrograms of total protein from human platelets and 100 ng of purified porcine platelet zyxin were separated by SDS-PAGE and transferred to nitrocellulose. The blot was overlaid with 1 μ g/ml Ena purified from baculovirus-infected cells, and bound Ena was detected by the anti-Ena antibody followed by ¹²⁵I-protein A.

Yeast Two-Hybrid System Screen

A cDNA encoding the C-terminal 243 amino acids of Ena was fused to the sequence encoding the GAL4 DNA binding domain in the pAS1-CYH2 vector to create pAS-EnaC. The yeast strain *Saccharomyces cerevisiae* Y190, which contains the reporter genes HIS3 and LacZ, was cotransformed with pAS-EnaC and the *Drosophila* larval library pAct (a gift of Dr. Stephen J. Ellidge, Baylor College of Medicine, Houston, TX) in which cDNAs are fused to the sequence encoding the GAL4 activation domain. Transformants (2.05×10^7) were screened for activation of the reporter genes by spreading the cells on medium lacking histidine but supplemented with 30 mM 3-aminotriazole. Colonies that grew in the absence of histidine appeared within 7 d after plating and were assayed for β -galactosidase production by a membrane transfer assay. Plasmid DNA from the positive clones was isolated and sequenced.

Genetic Stocks and Germ Line Transformation

Drosophila stocks containing *ena* point mutations and chromosomal deletions removing the *ena* gene have been previously described (Gertler *et al.*, 1990, 1995). Flies were cultured on a *Drosophila* cornmeal-yeast extract medium. To construct the transposons used in this study, Ena or VASP coding sequences were subcloned into the pUAST vector, which contains a minimal promoter preceded by five GAL4 binding sites (Brand and Perrimon, 1993). The plasmid DNA was injected into *white* mutant *Drosophila* embryos, and P element transposase was supplied by coinjection of plasmid pPi25.7wc. Transformants were identified by pigmentation in the eyes provided by the *white* gene in the transposon, and the chromosomal location of the transposons was determined genetically. Stocks were generated containing both the transgene and heterozygous *enaGC5* mutations.

ena Mutant Rescue Crosses

Chromosomes containing inversions that eliminate the *ena* gene product (*enaGC1* and *enaGC5*) were used in this analysis (Gertler *et al.*, 1990, 1995). Ubiquitous expression of the Ena transgenes was driven by the GAL4-e22c enhancer trap (generously provided by N. Perrimon, Harvard University, Boston, MA). For rescue experiments, *enaGC5/Cyo*, UAS-Ena, or UAS-VASP virgin females were crossed with *enaGC1*, GAL4-e22c/Cyo males. Progeny were scored for the presence of rescued Cy+ *enaGC1/enaGC5* flies. Animals containing two *ena* mutant chromosomes were identified as Cy+. For each cross, the percentage of adult survival was calculated by dividing the number of Cy+ adult flies recovered by the expected number of Cy+ animals. The expected number of Cy+ animals was assumed to be equal to one-half the number of CyO siblings scored. The entire progeny were scored from each cross. Rescue with the other transgenes was normalized with respect to the rescue seen

with the wild-type Ena transposon. The values presented are averages from two or three independent crosses. The absolute amount of rescue seen with the Ena transposons varies from cross to cross depending on temperature and how crowded the crosses were.

Immunofluorescence Microscopy of Transfected Cells

Ptk2 cells (CCL56; American Type Culture Collection, Manassas, VA) were grown on coverslips in MEM (Life Technologies, Gaithersburg, MD) supplemented with 1% glutamine, 1% nonessential amino acids, and 10% FCS. Cells were transfected by the calcium phosphate method with pCMV/Ena which consists of the Ena cDNA in the pRc/cytomegalovirus (CMV) expression vector (Invitrogen), or pVSV-VASP which consists of human VASP N-terminally tagged with an epitope of VSV glycoprotein G in the expression vector pcDNA3 (Invitrogen). Sixteen hours after addition of the precipitate, the cells were washed once in PBS, and fresh medium was added. Human fibroblasts were grown on coverslips in DMEM (Life Technologies)/10%. Cells were transfected by Fugene 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN) with pCMV/Ena or the respective constructs into which the Ena23 or Ena210 mutations had been introduced.

After 42–53 h, cells were washed with PBS, fixed in 3.7% formaldehyde, permeabilized by 0.2% Triton X-100, and incubated with rabbit antiserum raised against amino acids 55–235 of Ena (diluted 1:6 in PBS, 11 μ g/ml) and, for the detection of VSV-G-tagged VASP, with mAb P5D4 (Sigma, St. Louis, MO), diluted 1:5000 in PBS. Zyxin was detected with the mAb 164D4 raised against human zyxin. In Ptk2 cells, primary antibodies were detected by TRITC-labeled donkey anti-rabbit antibodies (Ena staining) and DTAF-labeled goat anti-mouse antibodies (Dianova, Hamburg, Germany). Actin was detected with fluorescein-labelled phalloidin (Molecular Probes, Eugene, OR). In human fibroblasts, primary antibodies were detected by Oregon Green-labeled goat anti-rabbit antibodies (Ena staining) and Cy3-labeled donkey anti-mouse antibodies (zyxin staining). The specimens were examined with a Leitz (Wetzlar, Germany) Aristoplan microscope equipped with epifluorescence. Photographs were taken with Kodak Ektachrome Elite 400 film (Eastman Kodak, Rochester, NY).

RESULTS

VASP Can Functionally Substitute for a Complete Loss of Ena Protein in *Drosophila*

Ena and VASP share sequence identity in two regions of the protein thought to be important for function (Figure 1A). We wondered whether these similarities could be sufficient to permit VASP to partially compensate for a lack of Ena protein during *Drosophila* development. To test this, we generated stable transgenic *Drosophila* expressing human VASP or *Drosophila* Ena. Five VASP and three Ena transgenic lines that were independently derived were tested for their ability to rescue the lethality of *ena* null mutants. When expressed ubiquitously via the UAS/GAL4 binary expression system (Brand and Perrimon, 1993), VASP partially rescued *ena* mutant lethality, allowing 25–85% of the *ena* mutant progeny to survive to adulthood compared with 79–100% rescue by the Ena transgene (Table 1). As expected, no *ena* mutant flies from these crosses eclosed in the absence of expression of an Ena or VASP transgene. All the flies rescued with the VASP transgene were visibly normal and had comparable fertility and survival 4 wk after eclosion to

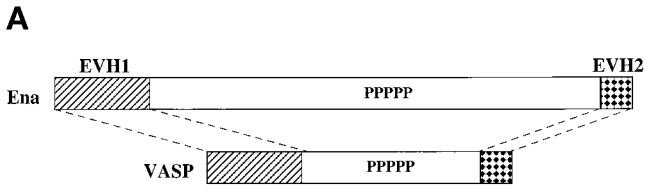
flies that were rescued with a *Drosophila* Ena transgene. The range in rescue seen with the different transgenes is likely due to differences in expression levels or patterns as a result of the different insertion sites of the transgenes. This result suggests that *Drosophila* Ena and human VASP have overlapping *in vivo* functions and affect similar cellular processes.

Ena and VASP Are Colocalized to Focal Adhesions and Actin Stress Fibers

VASP is localized to focal adhesions, stress fibers, and cell–cell contacts (Reinhard *et al.*, 1992). The Ena protein is localized to the axonal tracts of the developing *Drosophila* embryonic CNS (Gertler *et al.*, 1995), but the small size of these cells makes higher-resolution localization difficult. Because Ena and VASP have some conserved function *in vivo*, we compared their subcellular distribution when expressed in mammalian cells. Both Ena and VASP were detected at actin filaments and focal adhesion contacts. Coexpression of Ena and VASP resulted in an essentially identical pattern of expression (Figure 2). These results indicate that both proteins have domains sufficient to permit binding *in vivo* to proteins in the actin cytoskeleton and focal adhesions.

Identification of Lethal Mutations in the EVH1 and EVH2 Domains of *Drosophila* Enabled

Mutant *ena* alleles that were generated by mutagenesis with ethyl-nitroso-urea during the genetic screen that identified *ena* as a dominant genetic suppressor of *abl* mutations result in 100% lethality when expressed over an *ena* deficiency (Table 2). Because ethyl-nitroso-urea normally introduces single-base changes, we tested for the expression of Ena protein from these mutant alleles by Western blot analysis of single embryos from stocks of flies heterozygous for the *ena* mutation of interest. A chromosomal inversion (*enaGC8*) known to be null for Ena protein was used as a control. Because the parents were heterozygous for the *ena* mutant allele, 25% of the embryos picked were expected to be homozygous for the *ena* mutations, 25% for the wild-type *ena* allele, and 50% heterozygous for both the mutant and wild-type alleles. As expected, 25% of the embryos from the *enaGC8*/wild-type parents did not express the Ena protein (Figure 3, top panel). Half of the embryos from the *ena23*/wild-type parents expressed two sizes of Ena protein, and 25% had only the slower-migrating species, identical to the wild-type protein. The other 25% of the embryos were presumably homozygous *ena23* mutant embryos that expressed a faster-migrating form consistent with the presence of a smaller protein (Figure 3, middle panel). Embryos from the *ena210*/wild-type parents all expressed a full-length protein (Figure 3, bottom panel).



B

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ENA 1 {MTEQSIIGARASVMVYDDNQKKWVPSGSSS.GLSKVQIYHHQNNNTRFV
VASP 1 {MSETVICSSRATVMVLYDDGNKRWLPAGTGPQAFSRVQIYHNPTANSFRV

ENA 50 GRKLQ.DHEVVVINCILKGLKYNQATATPHQWRDSKFVYGLNFSSQND
VASP 51 GRKMQPDQVQVINCAIVRGVKNQATPNFPHQWRDARQVWGLNFGSKEDAA

ENA 99 NFARAMMHALEVLG)RVANNPGGPPPTNGNGYEEDMGYRMTTSEDAAILRQ
VASP 101 QFAAGMASALEALEG).....

ENA 149 NNSIGGHVTPSAQPTSTQTNQNNIPQSPPTPOGHRTSSAPPAPQPPQQQ
VASP .....

ENA 199 QQQQAAQMGQPGSHYPTGNGPTSNGLPQQVNSQIPFAPQQPQQQFQQ
VASP .....

ENA 249 QQQQQYQMVQAGYAPSQQYQPHYVLSNSNPNTLVHQYPTQQAQQQPP
VASP .....

ENA 299 QAPQPPLQNGGMVVGSHLPSASANSVVYASQQQMLPQAHFQAPQAPT
VASP .....

ENA 349 MPGPGYGGPPVPPPQQQAENPYGQVPMPPMNPSSQQQPGVPLNRMSQ
VASP 116 .....GGPPPPALPTWSVPNGPSPPEEVEQQKRQQPGPSEHIERRVNSA

ENA 399 GPGGPPAPAPPPPPPSFGGAAGGPPPPAPPQMFNGAPPFPPMGGGPP
VASP 160 GGPAPAPAGGPPPP.....GPPPP.....GPPPPGL..PPSG

ENA 449 APPAPPAMGGGPPAPGGGAPPPPPPPGLGGAPKKEDPQADLMGSLAS
VASP 193 VPAAAHGAGGGPPAPPLPAAQGGGGGAGAPG.....LAA

ENA 499 QLQFKLKKNKVTTTAPENSGSSTSSGGSGNYGTIGRSSNGMASMMDEMA
VASP 229 ATACAKLRK...VSKQEEASGGPTAPKAES.....GRSGGG..GLMEEMN

ENA 549 KTLARRRAQAEKKDPDPEAEVKKRPWEKSNLTPHLKSGGAGSGSAGSGHE
VASP 269 AMLARRRKATQVGEKTPKDE.....SANO

ENA 599 GANGNSGGAGSNTTNSGGESPRPMRKRFGSASEETILKVNVDGLSLASN
VASP 293 EEPEARVPAQSESVRPWEKNSSTLPRMKSSSVTSETQCTPSS.SSDY

ENA 649 GDLDTLKAETVREMRLKTKVNEIIDAIKSEFNRR 684
VASP 342 SDFORVKOELLBEVKKELOKVKEETIEAFVQELRKRKRS 380
    
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Figure 1. Ena and VASP have structural domain and sequence similarity, and the *ena* 210 and *ena* 23 mutations map to these regions of similarity. (A) Comparison of the overall structure and structural domain organization of Ena and VASP. The conserved EVH1 domain (hatched box) and EVH2 domain (checked box) are separated by a central domain of variable length, which contains a region rich in prolines. (B) Comparison of the deduced amino acid

To explain the embryonic lethality associated with *ena210* and *ena23*, we speculated that these mutant Ena proteins harbored lesions in critical functional domains. Identification of these mutations could be informative about the function of other Ena/VASP family members, because they have a high degree of functional conservation with Ena. To analyze the nature of these mutations, the coding regions of *ena210* and *ena23* were sequenced after reverse transcription PCR of total RNA from *Drosophila* pupae that were heterozygous for the *ena* mutation being examined. A single C→T mutation was identified in the EVH1 domain of *ena210* that results in an A97V change (Figure 1B). Examination of the EVH1 domains from previously cloned family members demonstrated complete conservation of this alanine in all reported Ena/VASP family members (Gertler *et al.*, 1996). Two changes were found in *ena23*. First was an A→G change in the proline rich-domain of Ena changing N379F. The second was an A→T mutation that introduced a stop codon 52 amino acids from the C terminus of Ena that deletes the EVH2 domain (Figure 1B) and encodes a protein consistent with the smaller protein seen in the single-embryo Western blots.

The N379F mutation identified in *ena23* involves an amino acid that is not conserved in any other Ena/VASP family member (Gertler *et al.*, 1996). However, the introduction of the stop codon (K636Stop) deletes the conserved EVH2 domain. Consequently, we focused on this mutation and tested whether the K636Stop described above contributed to the lethality of *ena23*. The K636Stop mutation was generated by site-directed mutagenesis in an Ena P element transposon, and transgenic flies were generated. The UAS/GAL4 binary expression system was used to ubiquitously express wild-type or mutant Ena K636Stop in Ena null *Drosophila* embryos. Two Ena K636Stop transgenic lines were tested and rescued from 0 to 8% of the expected homozygous *ena* mutant flies. Both lines expressed truncated Ena K636Stop protein as assessed by Western blots of lysates from transgenic flies induced by GAL4 to express the UAS-*ena23* transgene (our unpublished observations). This indicates that the removal of the EVH2 domain is in part responsible for the lethality seen in the *ena23* mutant flies. The

Figure 1 (cont). sequences and sequence motifs of *Drosophila* Ena and human VASP. The central regions of both Ena and VASP have multiple polyproline motifs. The C-terminal 35 amino acids contain a mixed charge cluster containing a charged residue at every fifth position (marked in bold). The conserved alanine that is altered in *ena* 210 mutants is in bold and marked with an asterisk. The EVH1 domains are enclosed in brackets, and the EVH2 domain is underlined. The location of the stop codon in *ena23* is marked by a plus sign. Sequence alignment was generated using the Genetics Computer Group (Madison, WI) BestFit program. Amino acid identity is indicated by vertical lines, and similarity is indicated by dots.

Table 1. Rescue of *ena* mutant flies with *ena* and *VASP* transgenes

Transgene	Experiment	Expected no. of <i>ena</i> mutant flies ^a	Observed no. of rescued <i>ena</i> mutant flies	Rescue (%) ^b	Average rescue per line (%)
wt1A	1	140	107	76	79
	2	82	63	77	
	3	135	112	83	
wt1B	1	118	116	98	100
	2	96	102	106	
	3	131	128	98	
wt10A	1	115	93	81	87
	2	87	76	87	
	3	78	72	92	
VASP.A	1	116	94	81	85
	2	90	82	91	
	3	123	101	82	
VASP.3	1	65	32	49	40
	2	61	18	30	
VASP.K	1	65	16	25	24
	2	104	25	24	
	3	87	20	23	
VASP.C	1	116	33	28	24
	2	117	23	20	
	3	102	24	24	
VASP.F	1	118	39	33	26
	2	115	32	27	
	3	92	16	17	

^a Expected number of *ena* mutants was calculated as one-half of the observed *ena* heterozygotes.

^b Percent rescue was determined by dividing the observed number of Cy+ *ena* homozygotes by the expected number of *ena* mutants.

identification of lethal mutations in the EVH1 and EVH2 domains of Ena is strong evidence that these domains are critical for *in vivo* Ena function and by analogy for *in vivo* functions of the Ena/VASP family of proteins.

Drosophila Enabled Binding to Zyxin Is Disrupted In *ena210*

Previous studies with VASP and Mena have demonstrated that the EVH1 domain is involved in binding the focal adhesion-associated protein zyxin (Reinhard *et al.*, 1995a; Gertler *et al.*, 1996). Based on these observations, we speculated that the EVH1 domain from *Drosophila* Ena would also bind zyxin. We examined the zyxin binding properties of Ena with a filter binding assay and found that Ena, like VASP and Mena, bound to the zyxin protein (Figure 4A). The zyxin protein consists of three highly conserved LIM domains and a proline-rich domain. We expressed full-length zyxin and the LIM and proline-rich domains of zyxin individually as GST fusion proteins and tested in solution-binding assays for binding to Ena or to human VASP. As expected, both proteins bound to the full-length zyxin protein. Both proteins also bound to the proline-rich region of zyxin but not to the LIM domains (Figure 4B), consistent with results obtained for VASP and Mena (Niebuhr *et al.*, 1997) and indicat-

ing that the ability to bind zyxin is conserved in *Drosophila* Ena and mediated through the same region of zyxin. To investigate whether Ena and zyxin can associate *in vivo*, we compared their subcellular distribution in mammalian cells transfected with Ena. Both Ena and zyxin are detected at focal adhesion contacts and on microfilaments (see Figure 9, 1A and 1B).

To determine whether the A97V change affected zyxin binding to the Ena EVH1 domain, we examined the zyxin binding properties of wild-type and A97V mutant Ena by generating this mutation in the Ena cDNA and cloning these cDNAs into the pCite expression vector. Wild-type or Ena A97V proteins were produced using a combined *in vitro* transcription-translation reaction and tested in solution-binding assays for their ability to bind GST-zyxin. Although equal amounts of the wild-type and mutant proteins were expressed, less mutant Ena A97V protein was pulled down by the GST-zyxin fusion protein when compared with the wild-type Ena protein (Figure 5C). In contrast, both proteins bound equally well to the GST Abl SH3 domain, demonstrating that the mutation is specific for an EVH1 domain function and does not disrupt the proline-rich central region of Ena (Figure 5B). This suggests that the mutation does not cause an overall disruption of the Ena protein structure. To further confirm that the A97V change in Ena affects

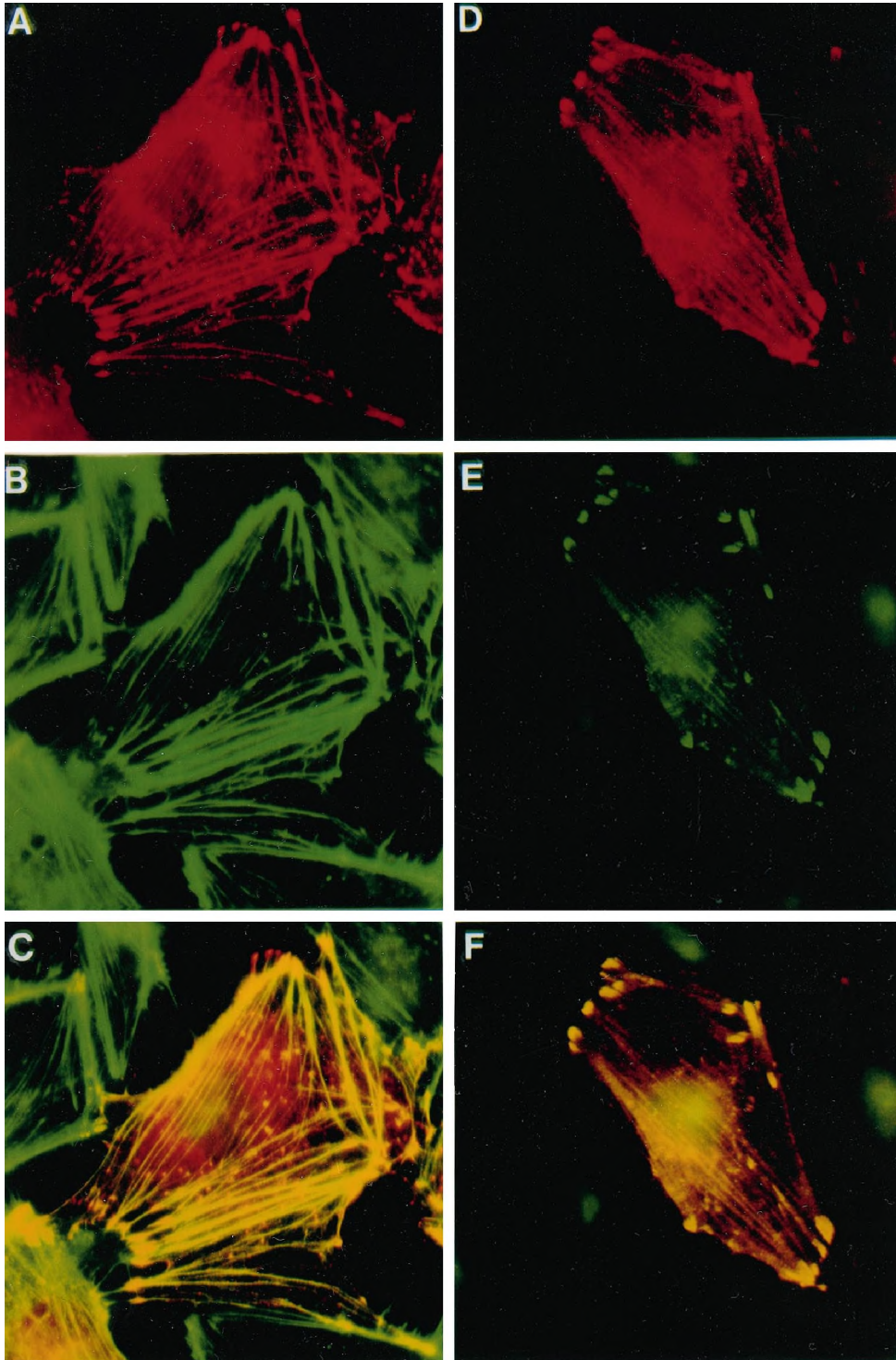


Figure 2. Colocalization of Ena and VASP proteins to stress fibers and focal contacts in transfected PtK₂ cells. PtK₂ cells were transiently transfected with pCMV/ena (A–C) or pCMV/ena and pVSV-VASP (D–F) and processed for double-label immunofluorescence microscopy. Ena was detected with rabbit polyclonal antibodies (A and D), VASP with a monoclonal mouse anti-VSV antibody (E), and F-actin by fluorescein-labeled phalloidin (B). A merge of A and B is shown in C, and a merge of D and E is shown in F. Fluorescent staining was by TRITC for Ena and DTAF for VASP. Ena expressed alone is localized either in focal contacts and stress fibers or is also variably found in spot-like structures, which also stain for F-actin (A–C). Coexpression of Ena and human VASP results in an identical localization of the two proteins in focal contacts and stress fibers (D–F). Controls show no cross-reactivity of the anti-Ena antibodies with VASP. Nuclear fluorescence is nonspecific background because of the secondary antibody.

Table 2. Viability of *ena210* and *ena23* mutant flies

Genotypes	Experiment	Expected no. of Ena mutant flies ^a	Observed no. of Ena mutant flies
<i>enaGC1/enaGC5</i>	1	120	0
	2	115	0
<i>ena23/enaGC1</i>	1	120	0
	2	117	0
<i>ena210/enaGC1</i>	1	126	0
	2	101	0

^a The entire progeny was scored for each cross. The expected number of Ena mutant flies was calculated as one-half the number of the balanced, heterozygous siblings.

zyxin binding, we examined the ability of this mutant protein to colocalize with zyxin in transfected mammalian cells. In contrast to wild-type Ena and zyxin, which are clearly present in the focal contacts, the

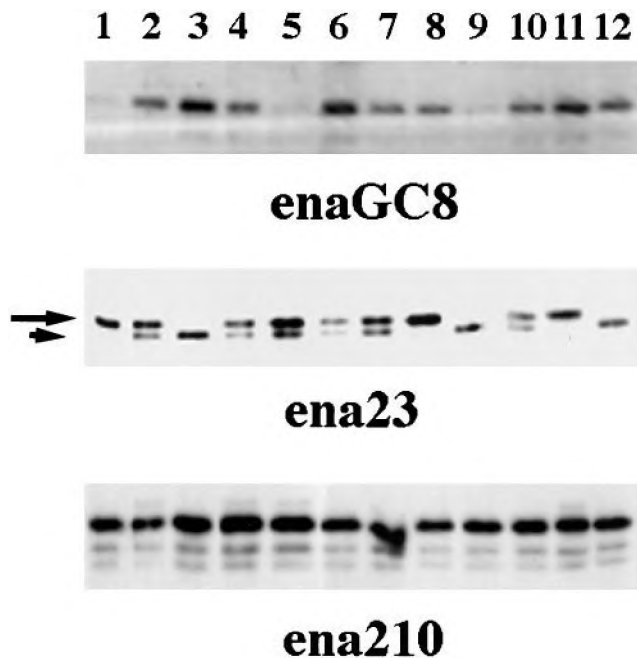


Figure 3. Analysis of *ena* mutant alleles. Single-embryo Western blot analysis of heterozygous *ena* mutant stocks. Embryos were collected for 2 h and then aged 5 h. Single embryos were picked and lysed in sample buffer and Western blotted. For the *enaGC8* chromosomal inversion, 25% of the embryos were null for Ena protein (top panel, lanes 1, 5, and 9). This blot was stripped and reprobed with an anti-fax antibody to demonstrate the presence of embryonic proteins in all 12 lanes. The *ena23* allele appears to encode an Ena protein with a small truncation, because 25% of the embryos encode only a smaller protein (small arrow) of ~80 kDa (middle panel, lanes 3, 9, and 12), and 50% of the embryos encode both full-length Ena (large arrow) and the truncated protein. The *ena210* allele appears to encode a full-length protein, because all embryos picked express full-length Ena.

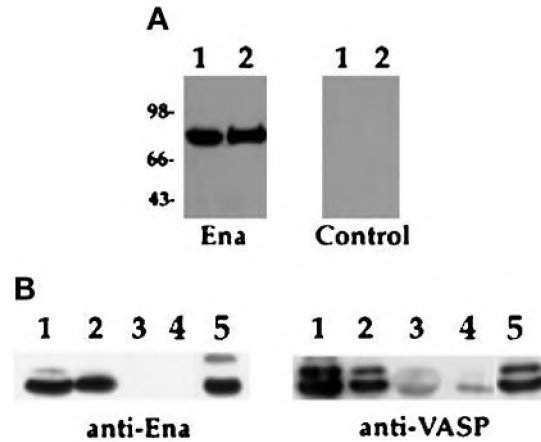


Figure 4. Binding of Ena and VASP proteins to zyxin and mapping of the binding domain in zyxin. (A) Ena and VASP overlays were carried out essentially as previously described (Reinhard *et al.*, 1995a). Fifty micrograms of total protein from human platelets (lane 1) and 100 ng of purified porcine platelet zyxin (lane 2) were separated by SDS-PAGE and transferred to nitrocellulose. The blot was overlaid with Ena purified from baculovirus-infected cells, and bound Ena was detected by the anti-Ena antibody followed by ¹²⁵I-protein A (left panel). The control (right panel) was treated identically, except that the overlay solution contained no Ena protein. Ena protein bound to an 83-kDa protein present in platelet lysates (lane 1), which comigrates with full-length zyxin (lane 2), whereas the control panel gave no detectable signal (right panel). (B) S2 cells were transfected with Ena or VASP, and proteins were bound to GST fusion proteins as follows: full-length zyxin (lane 1), zyxin proline domain (lane 2), zyxin LIM domains (lane 3) (Schmeichel and Beckerle, 1994), and GST alone (lane 4). Retained proteins were detected with anti-Ena N-terminal (left panel) or anti-VASP (right panel) polyclonal antibodies. Both proteins bound to full-length zyxin and to the zyxin proline domain (lanes 1 and 2). Neither protein bound to the zyxin LIM domain (lane 3). The faint signal seen in lanes 3 and 4 in the anti-VASP blot is due to background binding between VASP and the GST fusion proteins. Aliquots of transfected cell lysates were analyzed for expression of Ena or VASP protein (lanes 5). These results are representative of three independent experiments.

A97V mutant Ena exhibits diffuse staining and is absent from the focal contacts (see Figure 9, 2A and 2B). We conclude that EVH1 binding domain function is required for Ena's function in *Drosophila*.

The EVH2 Domain of *Drosophila Enabled* Mediates Multimerization

Truncation of the EVH2 domain by the mutation identified in Ena K636Stop results in embryonic lethality, suggesting that this region of Ena may mediate a critical function of the Ena protein. Initial clues to this function came by the isolation of the EVH2 domain of Ena in a yeast two-hybrid screen for *Drosophila* proteins that interact with the C-terminal 243 amino acids of Ena. The C-terminal 243 amino acids of Ena were fused to the DNA binding domain of the yeast transcription factor Gal4 and

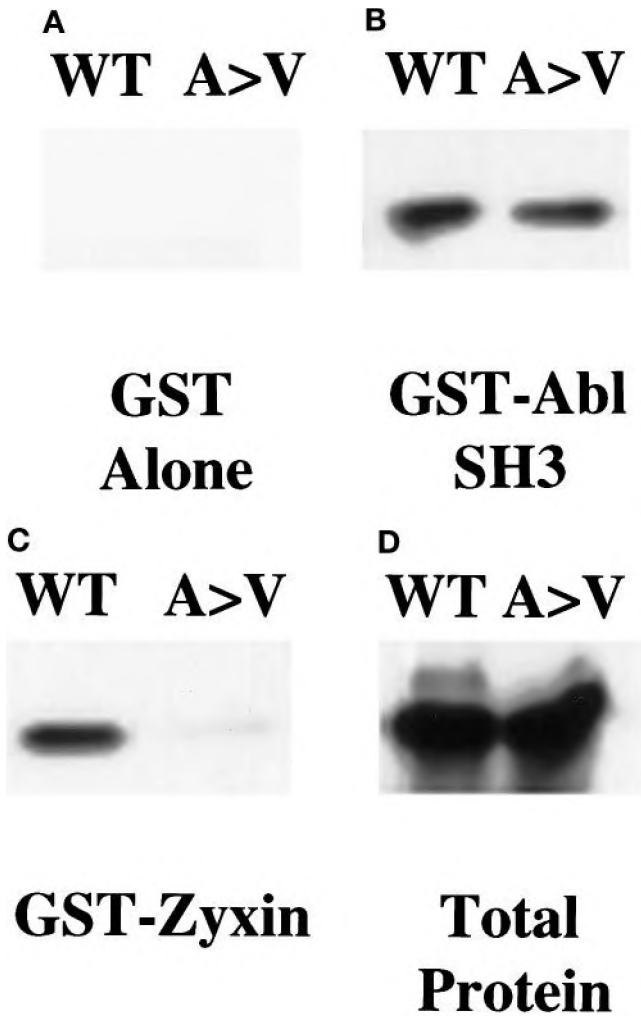


Figure 5. *ena* 210 mutation disrupts zyxin binding. Full-length wild-type Ena (WT) or *ena210* (A97V) proteins were prepared using an *in vitro* transcription-translation reaction and bound to GST alone (A), GST-AblSH3 (B), or GST-zyxin (C). Retained proteins were detected with anti-Ena N-terminal polyclonal antibody. Both proteins were expressed equally well (D). As expected, the GST negative control did not bind detectably to either protein (A). Both proteins bound equally well to the GST-AblSH3 domain (B). The wild-type Ena protein bound to the GST-zyxin protein, whereas binding to the A97V mutant protein was reduced. (C) These results are representative of three independent experiments.

used to screen a third instar larval library whose inserts were fused to the activation domain of Gal4. The separately expressed domains are unable to activate transcription of the reporter genes *HIS3* and *LacZ* unless a protein-protein interaction takes place (Chien *et al.*, 1991). Clones (20.5 million) were screened, 9 of which interacted with Ena as assessed by expression of both the *HIS* and *LacZ* reporter genes. Two of these clones were partial Ena cDNAs encoding Ena amino acids 628–684 and 634–684, respectively. The interaction was specific, because a

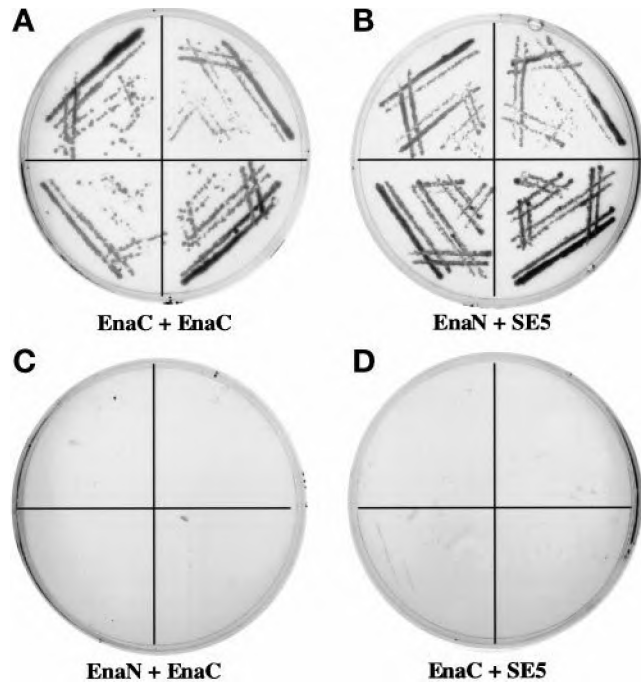


Figure 6. Interaction of the Ena EVH2 domain with the C-terminal 243 amino acids of Ena in the yeast two-hybrid system. The first 235 amino acids of Ena (GAL4DB-Ena_{1–235}) and last 243 amino acids of Ena (GAL4DB-Ena_{441–684}) were fused to the sequence encoding the GAL4 DNA-binding domain in the pAS1-CYH2 vector. These two constructs were cotransformed with GAL4AD-Ena_{628–684}, a clone in which amino acids 628–684 of Ena are fused to the sequence encoding the GAL4 activation domain and that was isolated in a yeast two-hybrid screen using the C terminal 243 amino acids of Ena as bait. Four independent colonies from each of the cotransformations were spread on medium lacking histidine and analyzed for growth. GAL4DB-Ena_{441–684} containing the C-terminal region of the Ena protein can interact with GAL4AD-Ena_{628–684} (A, EnaC + EnaC), whereas GAL4DB-Ena_{1–235} cannot (C, EnaN + EnaC). As a control, GAL4DB-Ena_{1–235} and GAL4DB-Ena_{401–684} were also tested for their ability to interact with GAL4AD-SE5, a clone identified from the same larval library as GAL4AD-Ena_{628–684} by its ability to interact with GAL4DB-Ena_{1–235}. As expected, GAL4DB-Ena_{1–235} can interact with GAL4AD-SE5 (B, EnaN + SE5), whereas GAL4DB-Ena_{401–684} cannot (D, EnaC + SE5).

construct with the Ena N-terminal domain fused to the DNA binding domain of Gal4 did not interact with these same isolated clones (Figure 6). Seven additional unique sequences were also identified that will be described in a later report. We thus speculated that the EVH2 domain may be involved in mediating multimerization of Ena/VASP proteins.

To assess the ability of Ena to multimerize, Ena expression vectors were generated in which full-length Ena was tagged at the C terminus with a 6-histidine tag for purification on Ni-NTA resin. Hemagglutinin or FLAG epitopes were then added to the C terminus of either full-length Ena or Ena

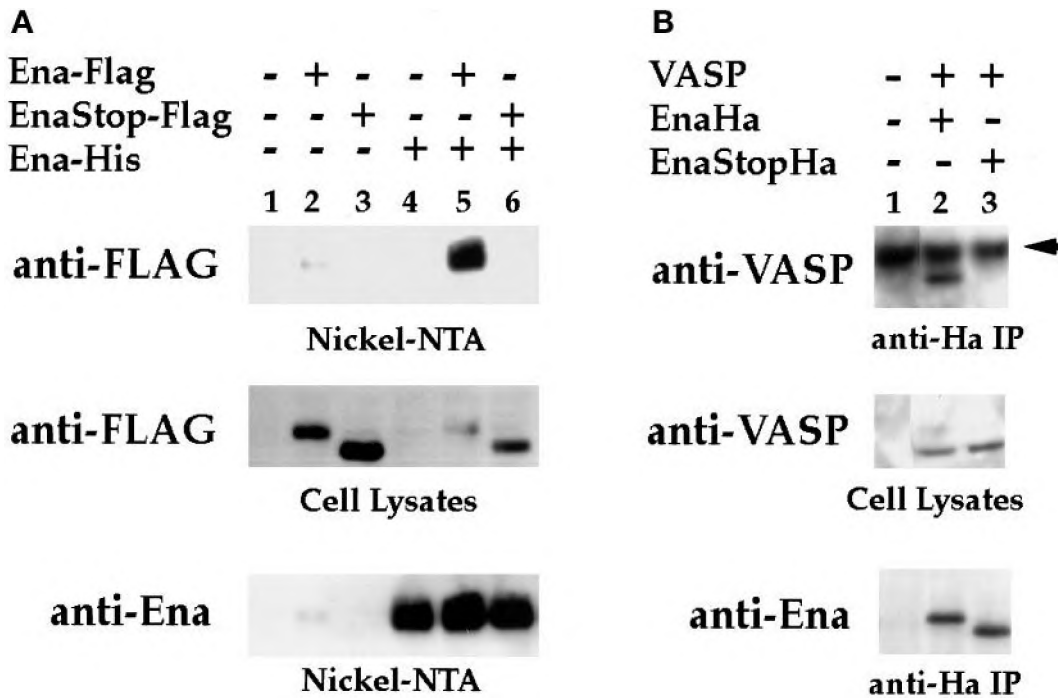


Figure 7. The EVH2 domain mediates multimerization. (A) S2 cells were transfected with the following expression constructs: no DNA (lane 1), Ena-FLAG (lane 2), Ena K636Stop-FLAG (EnaStopFlag; lane 3), Ena-His (lane 4), Ena-FLAG + Ena-His (lane 5), and Ena K636Stop-FLAG (EnaStopFlag) + Ena-His (lane 6). Cells were lysed, and extracted proteins were purified on Ni-NTA agarose and blotted with anti-FLAG (top panel) or anti-Ena (bottom panel) antibodies. Aliquots of cell lysates representing <2% of the total protein used in the purifications were analyzed with the anti-FLAG antibody for expression of the FLAG-tagged proteins, and both the Ena-FLAG and EnaK636Stop-FLAG proteins were expressed in the presence of Ena-His (middle panel). However, only full-length Ena-FLAG copurified with the Ena-His on the nickel NTA resin (top panel, compare lanes 5 and 6). A small amount of contaminating Ena-FLAG was purified with the Nickel-NTA resin (top panel, lane 2). (B) S2 cells were transfected with the following expression constructs: no DNA (lane 1), VASP + Ena-Ha (lane 2), and VASP + Ena K636Stop-Ha (EnaStopHa; lane 3). Transfected cell lysates were immunoprecipitated with 5 μ g of anti-Ha antibody. Complexes were subsequently analyzed by Western Blot with an anti-VASP antibody (top panel) or anti-Ena antibody (bottom panel). Aliquots of cell lysates representing <2% of the total protein used for the immunoprecipitations were analyzed for expression of the VASP protein with an anti-VASP antibody (middle panel). VASP was expressed in the presence of Ena-Ha and Ena K636Stop-Ha (middle panel, lanes 2 and 3), but VASP only copurified with full-length Ena-Ha in the anti-Ha IPs (compare lanes 2 and 3, top panel). The dark band present in all lanes in the top panel is the heavy-chain antibody from the anti-Ha IPs cross-reacting with the secondary antibody of the Western blot (arrow).

K636Stop. The tagged Ena and Ena K636Stop proteins were recognized by commercially available mAbs directed against FLAG (M2) or Ha (12CA5) epitopes. S2 cells were transfected with the FLAG-tagged Ena or Ena K636Stop expression vectors in the presence or absence of the His-tagged Ena expression vector. Transfected cell lysates were purified on Ni-NTA resin, and isolated complexes were analyzed by Western blot using the anti-FLAG mAb. When the His-tagged Ena was expressed in the presence of the full-length FLAG-tagged Ena, a strong FLAG signal was seen, suggesting that the two differentially tagged proteins formed a complex in cultured cells (Figure 7A, lane 5). However, there was no evidence of complex formation between the His-tagged Ena and the FLAG-tagged Ena K636Stop protein, indicating that this interaction required the Ena EVH2 domain (Figure 7A, lane 6).

Ena and the Ena/VASP family of proteins share similarity in the EVH2 domain. To determine whether this sequence conservation translated to functional conservation, we looked for complex formation between VASP and Ena or Ena K636Stop. S2 cells were transfected with VASP and Ha-tagged Ena or Ena K636Stop expression vectors. Transfected cell lysates were immunoprecipitated with 5 μ g of anti-Ha antibody, and complexes were analyzed by Western blot using an anti-VASP antibody. As seen with Ena, VASP associated with full-length Ena (Figure 7B, lane 2), whereas no evidence of complex formation was detected with Ena K636Stop, which lacks the EVH2 domain (Figure 7B, lane 3). Taken together, the coprecipitation and yeast two-hybrid data suggest that the EVH2 domain is necessary and sufficient to mediate Ena multimerization with both Ena and VASP. There-

fore, this EVH2 function appears to be conserved within the Ena/VASP family and may be essential for Ena's *in vivo* effects.

The EVH2 Domain Is Important for Ena's Ability to Bind the Abl-SH3 Domain and Zyxin

Because the ability to multimerize may be an important property of the Ena protein, we speculated that it may be required for other known functions of Ena. Studies in other laboratories have indicated that multimerization of proteins can alter their ligand-binding capacity (Oh *et al.*, 1997; Seiffert, 1997). We thus decided to test the effect of removing the Ena EVH2 domain on its affinity for the Abl-SH3 domains and zyxin. S2 cells were transfected with Ena or Ena K636Stop expression vectors. Full-length zyxin and the Abl SH3 domain were expressed as GST fusion proteins and bound in solution to the transfected cell lysates, and bound complexes were analyzed by Western blot analysis using the anti-Ena antibody. Although the mutant and wild-type Ena proteins were expressed equally well, less of the Ena K636Stop protein was pulled down by both the Abl SH3 domain (Figure 8C, lane 3) and zyxin (Figure 8D, lane 3) when compared with the full-length Ena protein (Figure 8, C and D, lanes 2). To further confirm that the Ena K636Stop change in Ena affects its ability to bind zyxin, we examined the ability of this mutant protein to colocalize with zyxin in transfected mammalian cells. In contrast to wild-type Ena and zyxin, which are clearly present in the focal contacts, K636Stop mutant Ena exhibits more diffuse staining with reduced staining in the focal contacts (Figure 9, 3A and 3B).

DISCUSSION

In this report, we have taken advantage of the *Drosophila* system to demonstrate *in vivo* that there is a striking degree of functional conservation between *Drosophila* Ena and human VASP, both members of the recently described Ena/VASP family of proteins. Specifically, we have demonstrated that human VASP can partially substitute for a loss of Ena in the developing *Drosophila* embryo. This is particularly striking in light of the limited sequence identity that these two proteins share, primarily in the EVH1 and EVH2 domains. In addition, two lethal Ena mutations that map to the EVH1 and EVH2 domains of *Drosophila* Ena have been described and characterized. Identification of functional mutations in the EVH1 and EVH2 domains of Ena are of particular interest because of the high degree of similarity in both sequence and function between different Ena/VASP family members, which, by analogy, implicates these protein domains as critical functional domains in other Ena/VASP family members.

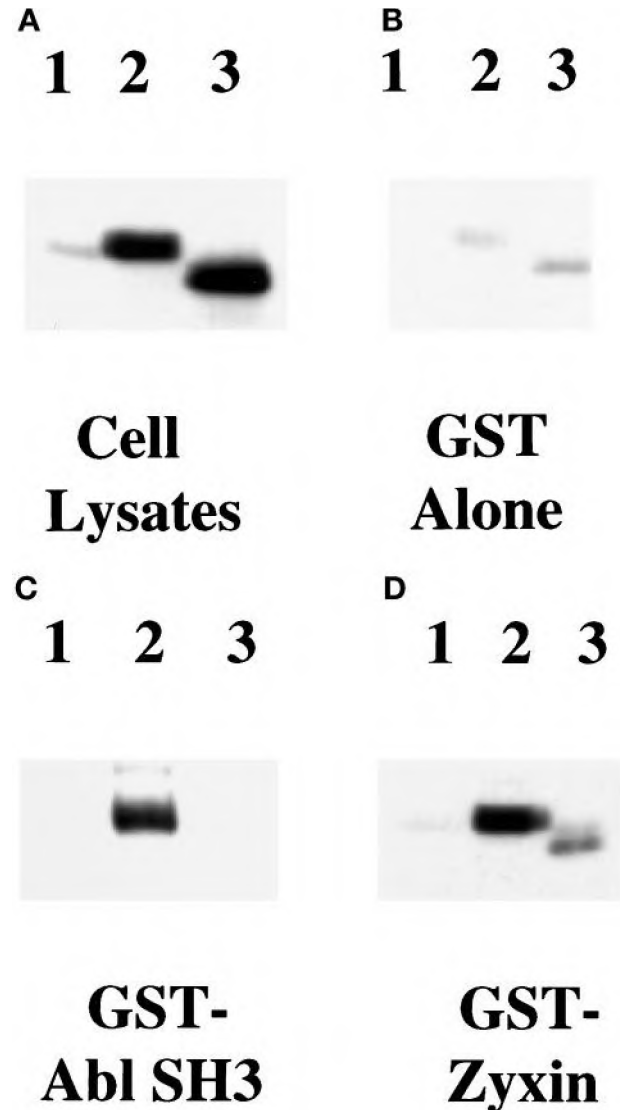


Figure 8. Ena K636Stop is impaired in its ability to bind zyxin and the Abl-SH3 domain. S2 cells were transfected with no DNA (lanes 1), full-length Ena (lanes 2), or Ena K636Stop (lanes 3) expression vectors. Transfected cell lysates were incubated with GST-Zyxin (D) or Abl SH3 (C) domain fusion proteins or with GST alone as a negative control (B). Cells were lysed, and aliquots of the cell lysates were compared to confirm that equal amounts of the full-length and truncated Ena proteins were expressed (A). Lysates were incubated with the GST fusion proteins, and retained proteins were detected with anti-Ena N-terminal polyclonal antibody. Little protein bound to the GST negative control (B). A decrease in binding of the truncated Ena K636Stop protein was seen with both zyxin and Abl SH3 when compared with the full-length Ena protein (C and D, compare lanes 2 and 3). These results are representative of three independent experiments.

One of the shared properties of Ena and VASP that may account for the observed rescue of Ena mutant flies by expression of human VASP is the ability to bind *in vitro* to the focal adhesion protein zyxin. Pre-

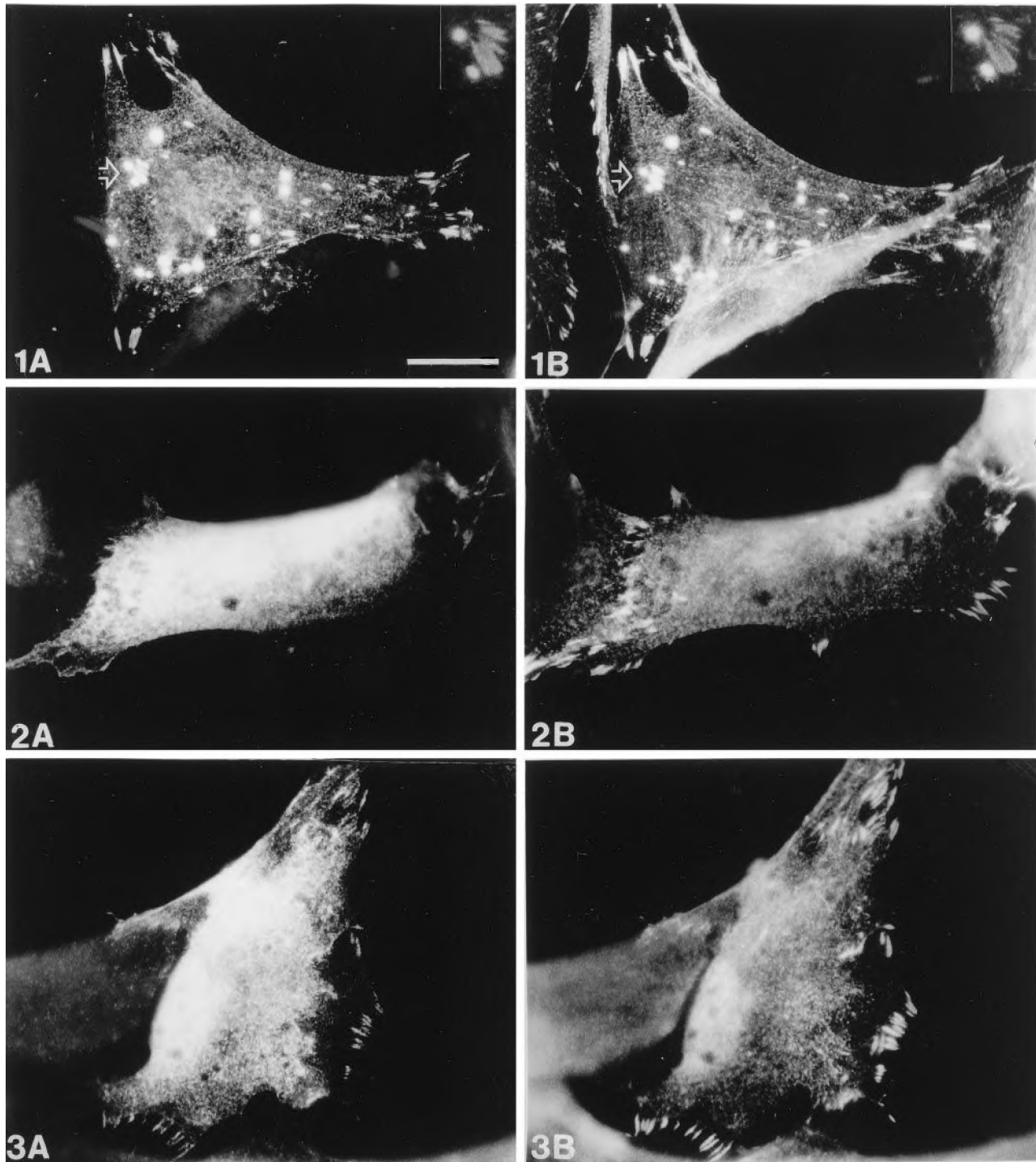


Figure 9. Comparison of the subcellular distribution of zyxin and wild-type or mutant Ena. Human fibroblasts were transiently transfected with pCMV/Ena (1), pCMV/EnaA97V (2), or pCMV/EnaK636Stop (3) and processed for double-label immunofluorescence microscopy. Ena staining is shown in A, staining of endogenous zyxin in B. After transfection of wild-type Ena (1A and 1B), colocalization of both Ena and zyxin is found in focal contacts, on microfilaments and in spot-like structures. A magnification of the area indicated by the arrow showing two “spots” and several focal contacts is seen at the top right (insets in 1A and 1B). In contrast, EnaA97V (2A and 2B) is diffusely distributed throughout the cytoplasm and totally absent from focal contacts, which are labeled by the zyxin antibody. EnaK636Stop (3A and 3B) is also diffusely distributed in the cytoplasm but shows some residual focal contact staining, although less pronounced than that of the wild-type Ena protein. Bar in 1A, 20 μm (valid for all panels).

vious studies demonstrated that the focal adhesion-associated protein zyxin binds VASP and Mena through the EVH1 domain (Reinhard *et al.*, 1995b; Gertler *et al.*, 1996). Here we demonstrated that this binding is conserved in Ena and is mediated by the zyxin proline-rich domain, consistent with recent peptide-mapping and competition studies of the Ena/VASP binding domain in zyxin (Niebuhr *et al.*, 1997). This would leave the zyxin LIM domains, which have been shown to mediate protein-protein interactions, available to link the zyxin-Ena/VASP complexes to other cellular proteins (Sadler *et al.*, 1992; Schmeichel and Beckerle, 1994; Arber and Caroni, 1996). Binding to zyxin may provide a specific mechanism for localization of Ena and VASP to focal adhesions that may permit Ena/VASP proteins to direct actin assembly to sites of contact between the cell and the extracellular environment. Consistent with this is our report that Ena, like VASP, is localized to focal adhesions and our observation that Ena colocalizes with endogenous zyxin when expressed in cultured mammalian cells.

We have also shown that zyxin binding is disrupted by the A97V mutation identified in the EVH1 domain of Ena in *ena210*. This alanine is conserved between *Drosophila* Ena and VASP, as well as in murine Ena, EVL, and *Drosophila* AE33 (Haffner *et al.*, 1995; DeMille *et al.*, 1996; Gertler *et al.*, 1996; Symons *et al.*, 1996). Taken together, these data suggest that this alanine is important for the overall structure of the EVH1 domain and that this domain is critical for Ena function. A *Drosophila* protein related to zyxin has been identified (our unpublished results). It will be interesting to determine whether this protein is a ligand for the Ena EVH1 domain, and further studies will be necessary to determine this. However, because this *Drosophila* protein has not yet been purified and antibodies directed against it are not available, it is not presently possible to test this possibility. It is also possible that Ena may bind a different *Drosophila* protein through this same highly conserved, critical binding domain. In fact, in addition to zyxin, VASP and Mena have also been shown to bind to both vinculin and bacterial Act A through the EVH1 domain, bolstering the argument that multiple proteins may interact with this domain to influence cytoskeletal assembly (Gertler *et al.*, 1996; Reinhard *et al.*, 1996). Efforts are currently under way to definitively identify all the potential binding partners for this critical domain of *Drosophila* Ena. Thus, although it is not yet clear that Ena/VASP proteins share the same binding specificity, our *in vivo* rescue data suggest that the EVH1 domains from these proteins recognize an overlapping set of proteins *in vivo*.

A second Ena mutation was identified that results in truncation of the Ena EVH2 domain. Although several potential functions have been proposed for this conserved protein domain, we provide compelling *in vitro* evidence that it may be involved in multimer

formation. This function of the EVH2 domain is conserved among family members, because Ena also forms multimers with VASP, and this multimerization requires the Ena EVH2 domain. Additionally, loss of the EVH2 domain reduces Ena's ability to bind to zyxin and to the Abl SH3 domain *in vitro*. Because the zyxin binding domain has already been mapped to the EVH1 domain (Gertler *et al.*, 1996; Niebuhr *et al.*, 1997) and the Abl-SH3 binding domain has been mapped to the Ena proline-rich sequences (Gertler *et al.*, 1995), we propose that this decrease in zyxin and Abl-SH3 binding may be due to a loss of ability to multimerize.

It is possible that removal of the Ena EVH2 domain disrupts the overall structure of the Ena protein, thus resulting in the reduction of zyxin and SH3 binding. This seems unlikely, however, in light of several observations. First, truncated forms of Ena lacking the EVH2 domain are expressed stably in both S2 cells and in *Drosophila* and are efficiently phosphorylated by the Abl tyrosine kinase (Gertler *et al.*, 1995). Second, Gertler *et al.* (1996) have shown that an isolated EVH1 domain is able to localize to focal adhesions in cultured cells when injected as a GST fusion protein, suggesting that removal of the Mena C terminus does not grossly alter the structure of the EVH1 domain (Gertler *et al.*, 1996). Because GST domains are reported to form multimers (Reinemer *et al.*, 1991), it is possible that GST-mediated multimerization may have compensated for the lack of EVH2 domain in the GST-EVH1 fusion protein in these experiments (Gertler *et al.*, 1996). Thus, we propose that loss of the EVH2 domain may reduce binding to Ena ligands through a loss of ability to multimerize. This would be similar to the effect of multimerization on the ligand-binding properties of both vitronectin and syndecan-4 (Oh *et al.*, 1997; Seiffert, 1997).

Zyxin binding is thought to be a mechanism for localizing Ena/VASP proteins to focal adhesions. Thus, multimerization of Ena/VASP proteins may be necessary for proper subcellular localization of these proteins to focal adhesions and the actin cytoskeleton. This would be consistent with results demonstrating that removal of the C-terminal 100 amino acids of VASP, which includes the EVH2 domain, results in an absence of VASP in the focal adhesions (Haffner, *et al.*, 1995). Interestingly, our results indicate that both the EnaA97V and the EnaK636Stop mutants show a rather diffuse intracellular localization and were clearly absent from focal contacts when expressed in mammalian cells.

Localization of the Ena/VASP family of proteins at focal adhesions places them at cellular structures where bidirectional signal transduction takes place (Machesky, 1997). Thus, it is of particular interest that in addition to being cytoskeletal proteins, this family of proteins represents docking sites and substrates for signal transduction molecules. Several kinases phos-

phorylate Ena, Mena, and VASP, indicating that multiple signaling pathways act on these proteins to regulate cytoskeleton assembly (Halbrugge, *et al.*, 1990; Gertler *et al.*, 1996; Comer *et al.*, 1997). A biological function has been demonstrated for the Abl-mediated phosphorylation of *Drosophila* Ena, because mutation of the Ena tyrosine phosphorylation sites to phenylalanine partially impairs the ability of Ena to restore viability to Ena mutant animals (Comer *et al.*, 1997). It is interesting to note that this unphosphorylatable form of Ena is only partially impaired in function and does retain some ability to rescue Ena mutants in light of the fact that VASP, a protein that is not a substrate of Abl, can also partially rescue a loss of ena function in *Drosophila*. Perhaps phosphorylation by Abl is only most important for fine tuning of Ena function. Thus, VASP, like the unphosphorylatable form of Ena, would substitute for Ena's basic functions, such as profilin and zyxin binding, ability to multimerize, and proper subcellular localization. However, it would be unresponsive to signals from Abl that more subtly regulate its activity and would thus not be able to rescue as well as a wild-type Ena protein.

Previous studies have demonstrated that two members of the growing Ena/VASP family of proteins, VASP and Mena, are linked to cytoskeletal assembly (Machesky, 1997). We now demonstrate the functional conservation of these proteins to *Drosophila* Ena by the ability of human VASP to partially compensate for a loss of *Drosophila* Ena *in vivo* and the identification of lethal Ena mutations in the most conserved domains of the Ena/VASP family. Despite their striking similarities, it is clear that Ena/VASP proteins regulate multiple distinct cellular processes and respond to different signal transduction pathways because VASP is involved in regulation of platelet adhesion and aggregation, and Ena and Mena are associated with neural development and function. We propose that these proteins could provide a bridge between signal transduction and the cytoskeleton. Further study of this new family of proteins promises to provide new insights into the regulation of cytoskeleton dynamics during development.

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