

# Spectrin functions upstream of ankyrin in a spectrin cytoskeleton assembly pathway

Amlan Das, Christine Base, Srilakshmi Dhulipala, and Ronald R. Dubreuil

Program in Cell & Developmental Biology, Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL 60607

**P**revailing models place spectrin downstream of ankyrin in a pathway of assembly and function in polarized cells. We used a transgene rescue strategy in *Drosophila melanogaster* to test contributions of four specific functional sites in  $\beta$  spectrin to its assembly and function. (1) Removal of the pleckstrin homology domain blocked polarized spectrin assembly in midgut epithelial cells and was usually lethal. (2) A point mutation in the tetramer formation site, modeled after a hereditary elliptocytosis mutation in human erythrocyte spectrin,

had no detectable effect on function. (3) Replacement of repetitive segments 4–11 of  $\beta$  spectrin with repeats 2–9 of  $\alpha$  spectrin abolished function but did not prevent polarized assembly. (4) Removal of the putative ankyrin-binding site had an unexpectedly mild phenotype with no detectable effect on spectrin targeting to the plasma membrane. The results suggest an alternate pathway in which spectrin directs ankyrin assembly and in which some important functions of spectrin are independent of ankyrin.

## Introduction

The protein spectrin is the defining element of a nearly ubiquitous submembrane protein network in animal cells. The spectrin supergene family includes a diverse set of proteins that share two main structural features: the spectrin repeat and a calponin-homology actin-binding domain (for review see Roper et al., 2002). The “immediate family” of the founding member spectrin includes a group of closely related gene products that assemble as tetramers of  $\alpha$  and  $\beta$  subunits, that associate with specific subdomains of the plasma membrane in many cells, and that share the ability to form cross-linked arrays with actin. Most, but not all, of these family members are linked to plasma membrane proteins via the adaptor ankyrin (for review see Bennett and Baines, 2001).

The functions of several spectrins and ankyrins have been tested in gene knockout experiments. One consistently observed effect is that loss of spectrin or ankyrin leads to a failure of interacting membrane proteins to accumulate at the appropriate site. In the most extreme case, RNAi knockdown of ankyrin-G in cultured bronchial epithelial cells led to the loss of an entire domain of the plasma membrane (Kizhatil and Bennett, 2004). However, in other knockout studies, loss of spectrin or ankyrin led to more subtle effects in which specific interacting mem-

brane proteins were lost from the domain normally occupied by the spectrin cytoskeleton. For example, loss of  $\beta$  spectrin in *Drosophila melanogaster* led to loss of Na,K ATPase from the basolateral domain of epithelial cells (Dubreuil et al., 2000); loss of  $\beta$ IV spectrin in mouse brain led to loss of voltage-dependent sodium channels from axon initial segments and the node of Ranvier (Komada and Soriano, 2002); knockouts of ankyrin-G and -B in mouse brain led to reduced levels of voltage-dependent sodium channels and L1 family cell adhesion molecules (Scotland et al., 1998; Zhou et al., 1998; Jenkins and Bennett, 2001).

Recent studies also indicate an important role for the spectrin cytoskeleton in muscle (for review see Mohler and Bennett, 2005). Defects in ankyrin-B are associated with human cardiac arrhythmia and sudden cardiac death (Mohler et al., 2003). Loss of ankyrin-B leads to a concomitant loss of Na,K ATPase, Na,Ca exchangers and inositol triphosphate receptors and perhaps other proteins from their normal cellular sites (Mohler et al., 2004b). A similar cardiac defect in humans results from a mutation of the voltage-dependent sodium channel in its ankyrin-G-binding site (Mohler et al., 2004a).

Although the consequences of loss of function are becoming clear, the cues that trigger assembly of the spectrin cytoskeleton within specialized membrane domains remain unknown. Evidence placing ankyrin upstream of spectrin in the assembly pathway has come from studies of cultured cardiac myocytes from ankyrin-B knockout mice. An engineered ankyrin-B

Correspondence to Ronald R. Dubreuil: ron@uic.edu

Abbreviations used in this paper: HS, heat shock; PH, pleckstrin homology; PtdIns, phosphatidylinositol.

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transgene lacking spectrin-binding activity appeared to function identically to the wild-type transgene, except that it failed to recruit  $\beta 2$  spectrin in its normal striated pattern (Mohler et al., 2004c). From these results, the authors concluded that ankyrin was upstream of spectrin in the spectrin cytoskeleton assembly pathway and that its function was independent of spectrin. However, a study of  $\beta IV$  spectrin knockout mice revealed that  $\beta IV$  spectrin and ankyrin-G are dependent on one another for assembly at the neuronal plasma membrane (Komada and Soriano, 2002).

There are also direct interactions between  $\beta$  spectrin and the plasma membrane. Two sites, one near the N terminus of  $\beta$  spectrin and one near the C terminus, were identified in binding studies with NaOH-stripped membranes from rat brain (Steiner and Bennett, 1988; Davis and Bennett, 1994; Lombardo et al., 1994). The discovery of these sites led to the hypotheses that (1) spectrin targeting could be achieved by ankyrin-independent interactions with the plasma membrane and (2) adaptor proteins such as ankyrin could have a secondary role in recruiting other interacting proteins to sites of spectrin assembly to form a unique membrane domain (Lombardo et al., 1994). Although the aforementioned studies focused on the interactions between the membrane association domains of  $\beta$  spectrin and putative protein receptors, the presence of a pleckstrin homology (PH) domain in membrane association domain 2 raised the possibility of an interaction between spectrin and phosphoinositides. The PH domain of mammalian  $\beta$  spectrins binds to inositol lipids and inositol triphosphate (Hyvonen et al., 1995; Wang and Shaw, 1995), and a reporter consisting of the PH domain from mouse  $\beta I$  spectrin fused to GFP was efficiently targeted to the plasma membrane in transfected COS cells (Wang et al., 1996). However, the relative importance of these ankyrin-independent binding sites to the initial assembly of spectrin has never been tested in vivo.

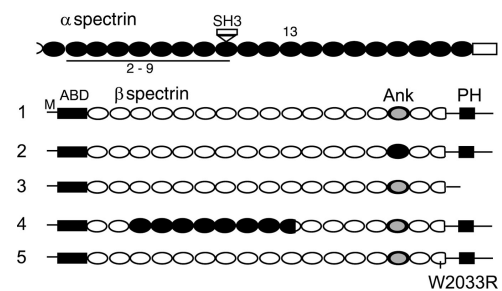
Here, we used a genetic approach to examine the relative contributions of specific sites in the  $\beta$  spectrin molecule to its targeting and function in polarized epithelial cells. All of the major functional sites in the mammalian spectrin molecule are conserved in *D. melanogaster*, making it a valid model system in which to address basic questions of spectrin's biological function and mechanism of action. One important advantage of the system is that there are only two spectrin isoforms in *D. melanogaster* and their functions do not appear to overlap (Dubreuil and Grushko, 1998; Thomas, 2001). There are only two ankyrin genes in *D. melanogaster*, and one of them (Dank2) appears to be expressed primarily in the nervous system (Bouley et al., 2000). Genetic studies in mammalian systems are complicated by the presence of five different  $\beta$  spectrin genes, two different  $\alpha$  spectrin genes, three different ankyrin genes, and further variation by alternative splicing (Bennett and Baines, 2001). Previous genetic studies in *D. melanogaster* have demonstrated that mutations in the  $\beta$  and  $\alpha$  spectrin genes are lethal in the late embryo and early larva, respectively (Lee et al., 1993; Dubreuil et al., 2000). The lethal phenotypes were fully rescued by recombinant transgenes in which the coding sequences for  $\alpha$  and  $\beta$  spectrin were expressed under control of the *D. melanogaster* ubiquitin promoter.

We tested modified transgene products in which specific functional sites were altered or deleted for their ability to rescue the lethal phenotype of  $\beta$  spectrin mutations and for their ability to be targeted to the basolateral domain of the plasma membrane in copper cells. These cells share several properties with gastric parietal cells in mammalian stomach, including the secretion of stomach acid (Dubreuil, 2004). Copper cells from spectrin mutants are amenable to immunohistochemistry, and we previously demonstrated that the basolateral distribution of the Na,K ATPase in these cells was dependent on  $\beta$  spectrin function (Dubreuil et al., 2000). We also characterized the effects of transgene modification on the behavior of spectrin in the salivary gland epithelium. The results support a model in which spectrin targeting occurs independently of ankyrin.

## Results

### Production and characterization of recombinant $\beta$ spectrin transgenes

We previously described a myc-tagged  $\beta$  spectrin transgene that rescued the lethal phenotype of mutations in the endogenous  $\beta$  spectrin gene (Dubreuil et al., 2000). Here, we produced modified transgenes in which selected functional domains were either altered or deleted. In one construct ( $\beta spec^{al3}$ ; Figs. 1 and 2), the putative ankyrin-binding segment 16 (repeat 15; Kennedy et al., 1991) was replaced with segment 13 from *D. melanogaster*  $\alpha$  spectrin. The rationale for this in-phase repeat replacement strategy was to eliminate ankyrin-binding activity without perturbing the overall structure of the modified protein. The transgene product (Fig. 2, lane 2) was identical in size to the  $\beta$  spectrin control transgene product (lane 1) in Western blots of total proteins from transgenic flies stained with anti-myc antibody. Another transgene was modified by in-phase replacement of repeats 4–11 of  $\beta$  spectrin with repeats 2–9 of  $\alpha$  spectrin



**Figure 1. Design of modified  $\beta$  spectrin constructs.** (top) Segments of  $\alpha$  spectrin include (from left to right) a partial spectrin repeat corresponding to helix C, 20 copies of the degenerate spectrin repeat (106 amino acids in a triple barrel  $\alpha$  helix), an SH3 motif within segment 9, and a C-terminal EF hand domain. (1) Segments of the wild-type  $\beta spec^{2A}$  transgene include (from left to right) an N-terminal myc tag (M), an  $\sim 250$ -amino-acid actin-binding domain (ABD), 16 spectrin repeats, including the ankyrin-binding segment 16, a partial spectrin repeat corresponding to helices A and B, and the C-terminal PH domain. (2)  $\beta spec^{al3}$ ; segment 16 of  $\beta$  spectrin was replaced by segment 13 of  $\alpha$  spectrin. (3)  $\beta spec^{\Delta PH}$ ; a stop codon was inserted at the beginning of the PH domain. (4)  $\beta spec^{w2-9}$ ; repeats 4–11 of  $\beta$  spectrin were replaced by repeats 2–9 of  $\alpha$  spectrin. (5)  $\beta spec^{W2033R}$ ; a missense mutation that affects tetramer formation in human  $\beta$  spectrin I was introduced into the corresponding conserved position of *D. melanogaster*  $\beta$  spectrin.

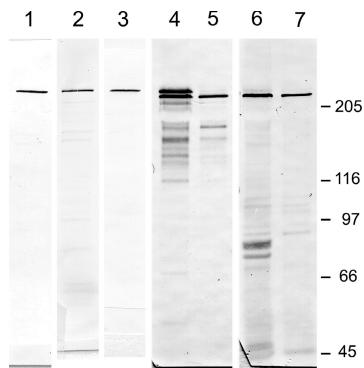


Figure 2. **Western blots.** Total adult *D. melanogaster* proteins were subjected to Western blotting with an antibody directed against the myc epitope tag (lanes 1–3 and 6–7) or a rabbit antibody against *D. melanogaster*  $\beta$  spectrin (lanes 4 and 5). Each lane represents 0.8 flies from lines expressing both the transgene and endogenous wild-type  $\beta$  spectrin, except where indicated. Lane 1,  $\beta$ spec<sup>3A</sup>; lane 2,  $\beta$ spec <sup>$\alpha$ 13</sup>; lane 3,  $\beta$ spec <sup>$\alpha$ 2-9</sup>; lanes 4 and 6,  $\beta$ spec <sup>$\Delta$ PH</sup>; and lanes 5 and 7,  $\beta$ spec <sup>$\Delta$ PH</sup> in a  $\beta$ spec<sup>em21</sup> mutant background in which endogenous wild-type  $\beta$  spectrin was absent. Molecular mass markers are shown to the right in kD.

(Fig. 1, 4). This transgene encoded a myc-tagged product that was also similar in size and abundance to the recombinant  $\beta$  spectrin control (Fig. 2, lane 3). A third transgene was modified by replacing the first codon of the PH domain (2144) with a stop codon (Fig. 1, 3). Western blots stained with rabbit anti- $\beta$  spectrin antibody detected the authentic wild-type  $\beta$  spectrin (266 kD) as well as the expected truncated product (251 kD; Fig. 2, lane 4). Staining of an identical blot strip with the anti-myc antibody detected only the smaller, truncated product (lane 6). A fourth transgene was modified by replacing the tryptophan at codon 2033 with an arginine (Fig. 1, 5). The expected full-length product was also detected in blots of these flies stained with the anti-myc antibody (unpublished data).

The ability of these transgenes to rescue mutations in the endogenous X-linked  $\beta$  spectrin gene was tested using two different rescue strategies (depending on the site of transgene insertion; see Materials and methods for details). The expected progeny class ratios were different, but otherwise both strategies simply tested the ability of the rescue transgene to overcome the embryonic lethality of the mutant  $\beta$  spectrin gene. Representative cross results are shown in Table I. The control transgene (on chromosome III) was scored for its ability to replace the function of *Dp(1;3)Bar<sup>S3i</sup>D3*, a translocation of a wild-type  $\beta$  spectrin allele to chromosome III (Dubreuil et al., 2000). The observed 2:1 phenotypic ratio of sibling/transgene rescue adult males indicates a 100% rescue efficiency by the

Table I. **Efficiency of mutant rescue by modified  $\beta$  spectrin transgenes**

Transgene tested	Total females	Male siblings	Rescue males	Total
Wild type <sup>a</sup>	41	36	18 (100%)	95
$\Delta$ PH mutant <sup>a</sup>	95	64	0	159
$\alpha$ -13 mutant <sup>b</sup>	124	52	19 (17%)	195
$\alpha$ 2-9 mutant <sup>b</sup>	117	65	0	182

<sup>a</sup>Autosomal transgene in a C(1)Dx background.

<sup>b</sup>X-linked transgene on same chromosome as  $\beta$  spectrin mutation.

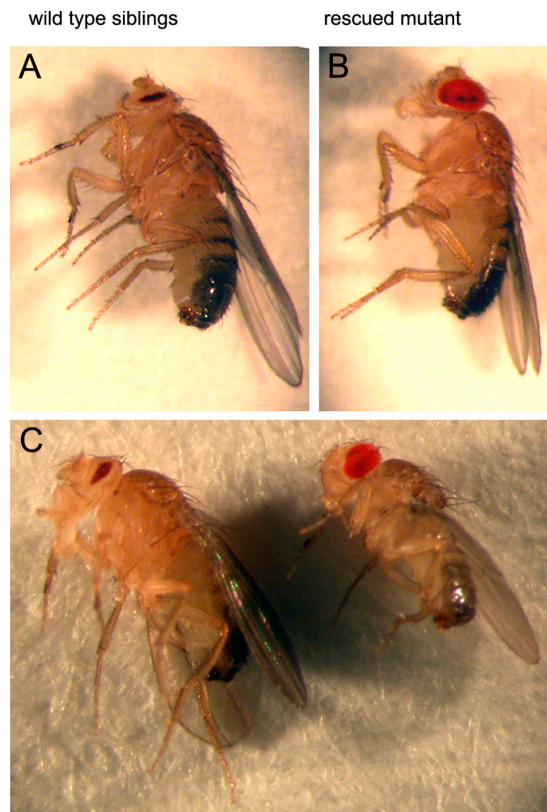


Figure 3. **A size defect in rescued male flies expressing the  $\beta$ spec <sup>$\Delta$ PH</sup> transgene.** In these crosses, all females carry the C(1)Dx chromosome and all males carry a lethal  $\beta$ spec<sup>em21</sup> allele on X. Males may survive to adulthood when they inherit either the *Dp(1;3)Bar<sup>S3i</sup>D2* translocation marked with a *Bar* eye phenotype (A) or an autosomal  $\beta$  spectrin transgene from the C(1)Dx parent. Progeny rescued by expression of the wild-type transgene  $\beta$ spec<sup>3A</sup> on chromosome 3 (B) appear identical in size to their siblings that carry a wild-type copy of the  $\beta$  spectrin gene on the translocation chromosome. Rescued flies are distinguished by the lack of the eye marker *Bar*. Rare rescue progeny expressing the  $\beta$ spec <sup>$\Delta$ PH</sup> transgene (Fig. 2, lane 5), also recognized by their non-*Bar* eye phenotype, are unusually small (C, right).

transgene. In contrast, the truncated transgene lacking the PH domain generally failed to rescue, although rare rescue flies were occasionally observed.

Interestingly, the  $\Delta$ PH rescue flies were unusually small in size (Fig. 3). Rescue males expressing the control transgene (non-*Bar*-eye; Fig. 3 B) were the same size as wild-type sibling males that carried the *Bar*-eye-marked translocation (Fig. 3 A). However, males rescued by the  $\Delta$ PH transgene were strikingly smaller in size compared with their *Bar*-eyed siblings (Fig. 3 C), and they were sterile. Western blots demonstrated that the  $\Delta$ PH rescue males expressed the myc-tagged truncated product (Fig. 2, lane 7). Only the truncated  $\Delta$ PH product was detected when blots were probed with the anti- $\beta$  spectrin antibody (lane 5), confirming the absence of wild-type  $\beta$  spectrin.

The function of the X-linked  $\beta$ spec <sup>$\alpha$ 2-9</sup> and the  $\beta$ spec <sup>$\alpha$ 13</sup> transgenes was tested after recombination with the  $\beta$  spectrin mutant chromosome ( $\beta$ spec<sup>em21</sup>). The  $\beta$ spec <sup>$\alpha$ 2-9</sup> transgene altogether lacked rescue activity (Table I), suggesting that replacement of repeats 4–11 of  $\beta$  spectrin removed an important functional site and/or compromised the structure of the molecule.



In contrast, the  $\beta\text{spec}^{\alpha 13}$  transgene exhibited rescue activity, although it was significantly less efficient than the wild-type transgene (17%; Table I). Many of the rescued progeny were fertile; however, some exhibited minor wing defects and many of them died during eclosion (unpublished data). This result suggested that either ankyrin-binding activity was not essential for spectrin function or ankyrin-binding activity occurred elsewhere in the  $\beta$  spectrin molecule.

#### Effects of $\beta$ spectrin mutations on targeting

Each of the  $\beta$  spectrin transgenes was tested for its effect on the targeting of spectrin, both in the presence and in the absence of wild-type  $\beta$  spectrin. Midguts from late embryos or larvae were dissected and stained with anti-myc antibody and rabbit anti-Scribble as a positive staining control. The myc-tagged control transgene product was previously shown (Dubreuil et al., 2000) to have the same distribution as endogenous  $\beta$  spectrin (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200602095/DC1>). The  $\beta\text{spec}^{\alpha 13}$  transgene modification was expected to perturb the association of spectrin with ankyrin and therefore to affect ankyrin-dependent targeting of spectrin to the plasma membrane. However, its distribution in copper cells was indistinguishable from the wild-type protein throughout the

septate junction, lateral, and basal domains (Fig. 4 A). Scribble staining marked the septate junction region (Fig. 4 B). The  $\beta\text{spec}^{\alpha 13}$  distribution was the same regardless of whether it was expressed in a wild-type background (Fig. 4 A) or in the absence of wild-type  $\beta$  spectrin (not depicted). Thus, segment 16 appeared to be dispensable for the targeting of spectrin to the plasma membrane.

In contrast, the  $\beta\text{spec}^{\Delta\text{PH}}$  product had an abnormal distribution regardless of whether it was expressed in a wild-type background or in a mutant background. Interestingly,  $\beta\text{spec}^{\Delta\text{PH}}$  was enriched in the septate junction region when wild-type  $\beta$  spectrin was present (Fig. 4 C). But in the  $\beta\text{spec}^{\text{em}6}$  mutant background, there was no apparent pattern of plasma membrane staining in copper cells (Fig. 4 E). Instead, staining was observed in a speckled pattern in the cytoplasm. The Scribble staining pattern was altered slightly in the  $\beta$  spectrin mutant gut, to the extent that spectrin mutations alter cell shape and tissue organization (Lee et al., 1993; Dubreuil et al., 2000). However, it was still possible to recognize the comma shapes that are characteristic of the septate junctions in copper cells (Fig. 4 F). Expression of the  $\beta\text{spec}^{\alpha 2-9}$  transgene failed to rescue the lethality of  $\beta$  spectrin mutations, but it delayed lethality until shortly after larval hatching. Staining of the  $\beta\text{spec}^{\alpha 2-9}$  product was reduced somewhat relative to controls but was still clearly

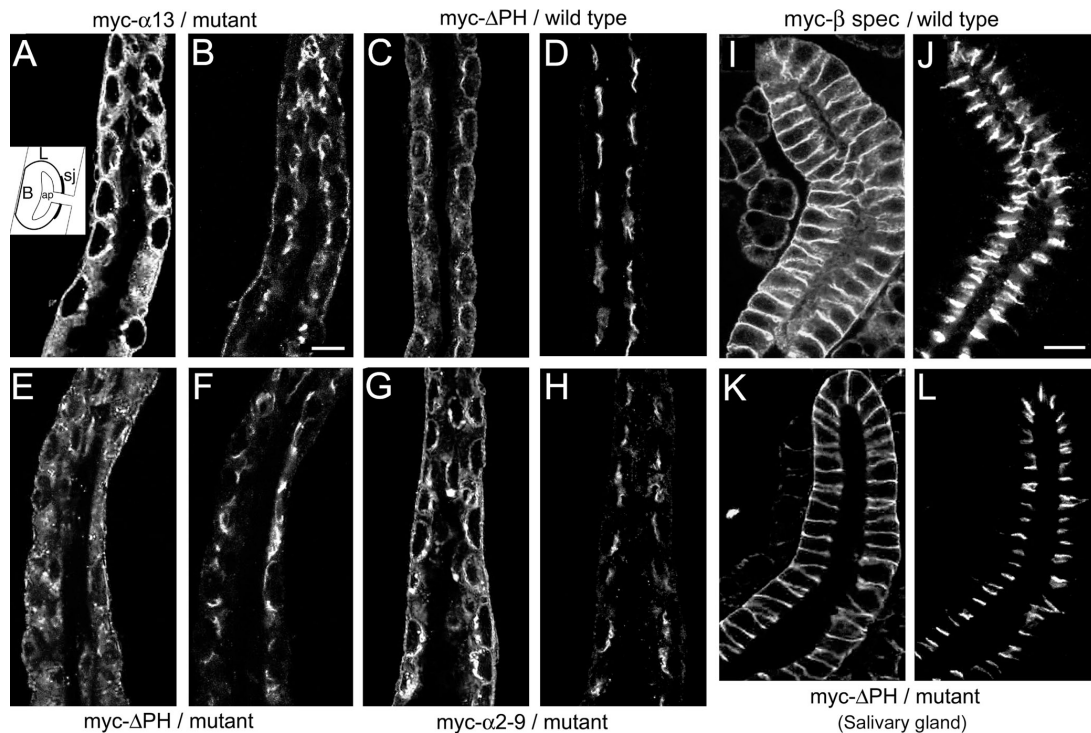


Figure 4. **Targeting of mutant  $\beta$  spectrin transgene products to the plasma membrane of copper cells.** Transgene products were detected with a mouse anti-myc antibody and TRITC-labeled secondary antibody (A, C, E, and G). A staining control, rabbit anti-Scribble antibody and FITC-labeled secondary antibody (B, D, F, and H), marked the position of septate junctions. The  $\beta\text{spec}^{\alpha 13}$  gene product was correctly targeted to the plasma membrane in a wild-type background (A) or in rescued mutants (not depicted). The  $\beta\text{spec}^{\Delta\text{PH}}$  transgene product was targeted primarily to the septate junction when expressed in a wild-type background (C), with relatively little staining of the lateral or basal regions.  $\beta\text{spec}^{\Delta\text{PH}}$  expressed in the absence of endogenous wild-type  $\beta$  spectrin was detected in a speckled pattern in the cytoplasm but was not localized to the plasma membrane (E). Staining of the  $\beta\text{spec}^{\alpha 2-9}$  transgene product in rescue larvae was weaker than wild-type controls but was still conspicuous at the septate junction and lateral and basal domains (G). The wild-type myc-tagged transgene product (I) and the  $\beta\text{spec}^{\Delta\text{PH}}$  product (K) were also evaluated in the salivary gland epithelium of wild-type larvae and  $\beta\text{spec}^{\text{em}6}$  mutant embryos, respectively. Scribble staining, as above, identified the lateral subdomain corresponding to the septate junction (J and L). Bars, 10  $\mu\text{m}$ .

detectable throughout the septate junction, the lateral domain, and the basal domain (Fig. 4 G). Thus, the region encompassing segments 4–11 of  $\beta$  spectrin was important for function but not for polarized assembly.

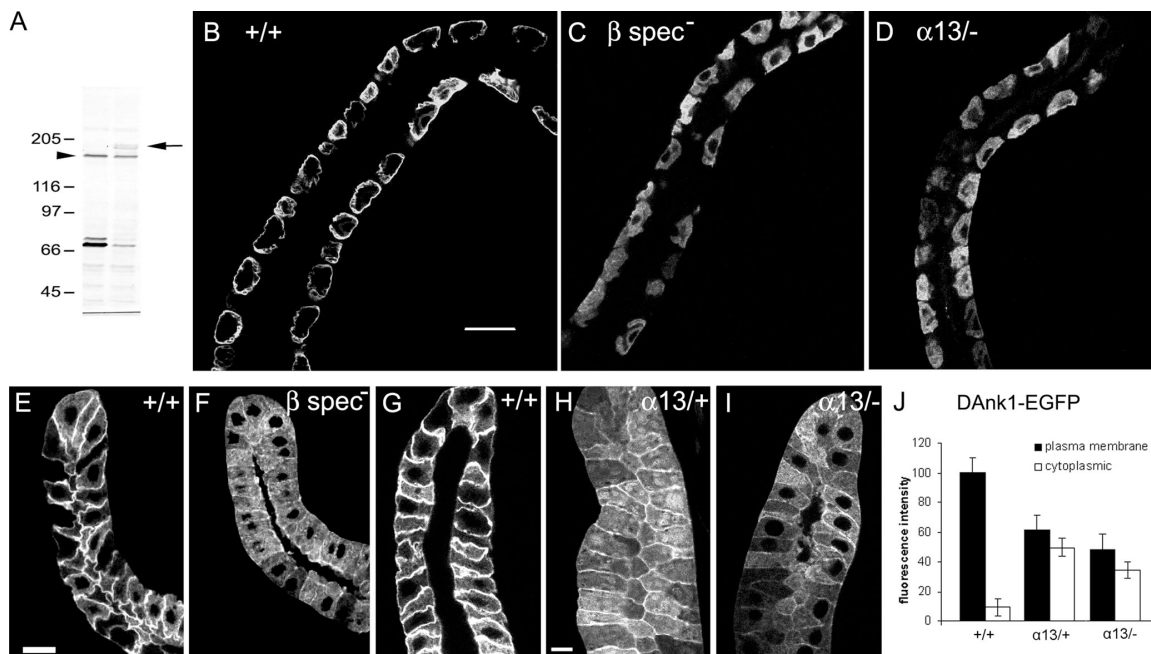
We also examined the behavior of modified  $\beta$  spectrin transgenes in the salivary gland epithelium. Both the endogenous wild-type  $\beta$  spectrin (not depicted) and the myc-tagged wild-type transgene product (Fig. 4 I) were present throughout the lateral membrane domain, including the septate junction (marked by Scribble staining; Fig. 4 J). All of the transgene products that we analyzed, including  $\beta$ spec <sup>$\Delta$ PH</sup> (Fig. 4 K) and  $\beta$ spec <sup>$\alpha$ 13</sup> (not depicted), exhibited the same lateral distribution in both wild-type larvae and mutants lacking endogenous  $\beta$  spectrin. Thus, once again, targeting of spectrin to the plasma membrane did not require the ankyrin-binding domain. However, unlike the copper cell, targeting in the salivary gland did not require the PH domain either, suggesting that there are additional cell type-specific targeting mechanisms.

### The sequence of events in spectrin assembly

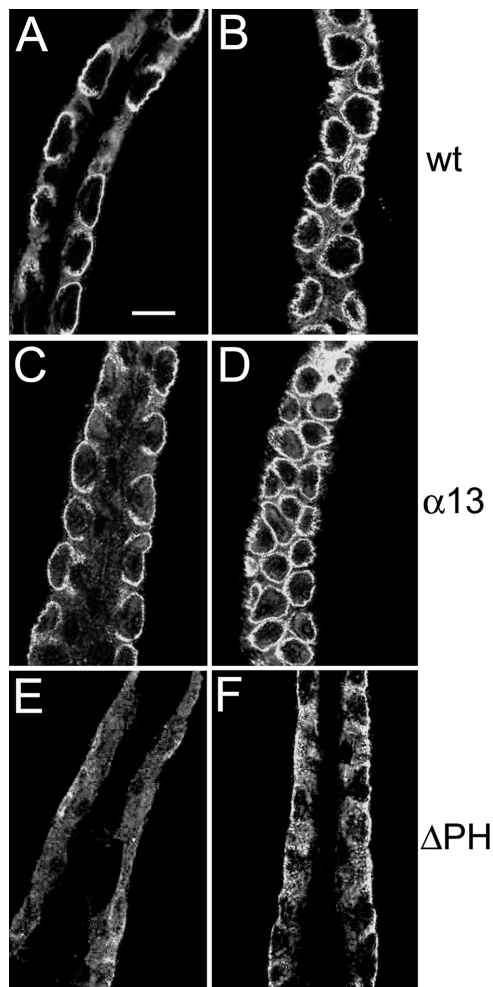
The above results suggested that the targeting of spectrin in copper cells and salivary gland was independent of ankyrin. Further insight into this unexpected observation came from studies of a DAnk1-EGFP fusion protein. The transgene was engineered by splicing the coding sequence of EGFP to the last

codon of the *D. melanogaster* DAnk1 coding region. A Western blot of larvae carrying the UAS transgene and heat shock (HS) Gal4 was probed with a rabbit anti-DAnk1 antibody (Fig. 5 A, right). In addition to the 170-kD DAnk1 band found in control larvae (Fig. 5 A, left), the UAS-DAnk1-GFP flies expressed an  $\sim$ 200-kD product that is the expected size of the DAnk1-EGFP fusion. The HS Gal4 driver was chosen for this experiment because, fortuitously, it was constitutively active in both copper cells and salivary gland.

The distribution of DAnk1-EGFP was examined in the copper cells of wild-type and  $\beta$  spectrin mutant embryos. In wild type, the pattern was identical to the antibody-stained distribution of  $\beta$  spectrin, throughout the basolateral domain, including the septate junction (Fig. 5 B). Thus, there appears to be a 1:1 correspondence between the distribution of ankyrin and  $\beta$  spectrin in these cells. The distribution of DAnk1-EGFP was dramatically altered in copper cells from  $\beta$ spec<sup>*em6*</sup> mutants (Fig. 5 C), becoming diffusely distributed throughout the cytoplasm with no detectable fluorescence at the plasma membrane. A similar effect was observed in salivary gland cells from  $\beta$ spec<sup>*em6*</sup> mutant embryos (Fig. 5 F). DAnk1-EGFP codistributed with  $\beta$  spectrin throughout the lateral membrane domain of wild-type embryos (Fig. 5 E), but its distribution was largely shifted to the cytoplasm of mutant cells. However, unlike copper cells, it was possible to detect some DAnk1-EGFP in the lateral domain of salivary gland cells from mutants, although it was greatly



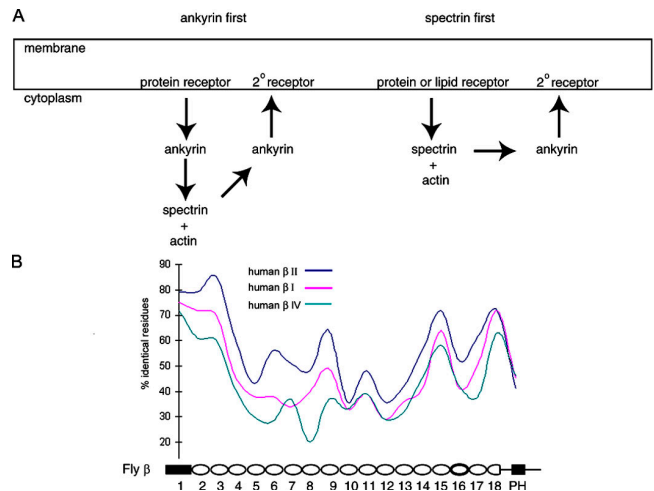
**Figure 5. Targeting of DAnk1-EGFP to the plasma membrane.** Expression of a UAS DAnk1-EGFP transgene in copper cells was driven by HS Gal4. (A) Basal expression without HS was detected in Western blots of total proteins from adult flies probed with a rabbit anti-DAnk1 antibody. The antibody detected the 170-kD endogenous DAnk1 (arrowhead) and the  $\sim$ 200-kD DAnk1-EGFP fusion (arrow). The antibody also cross-reacted with lower molecular mass proteins that increased in abundance after HS (left) relative to no HS (right). Molecular mass markers are shown to the left in kD. (B) DAnk1-EGFP was targeted to the plasma membrane of copper cells in the same pattern as endogenous spectrin. (C) DAnk1-EGFP was not detected at the plasma membrane of copper cells from  $\beta$ spec<sup>*em6*</sup> mutant larvae, indicating that spectrin was required for ankyrin targeting. (D) DAnk1-EGFP also failed to accumulate at the plasma membrane of copper cells from  $\beta$ spec <sup>$\alpha$ 13</sup> larvae, confirming that this transgene product lacked ankyrin-binding activity. DAnk1-EGFP was also detected at the lateral plasma membrane of salivary gland epithelial cells in wild-type embryos (E) and larvae (G). Most, but not all, of the lateral membrane pattern was shifted to the cytoplasm of  $\beta$ spec<sup>*em6*</sup> mutant embryos (F) and larvae either carrying the  $\beta$ spec <sup>$\alpha$ 13</sup> transgene (H) or rescued by the transgene (I). Mean pixel intensities for the lateral contact region (filled bars) and the cytoplasm (open bars) were calculated for each genotype, as indicated (J), using NIH ImageJ ( $n > 100$  cells/class). Bars, 20  $\mu$ m.



**Figure 6. The behavior of the Na,K ATPase in copper cells is linked to spectrin.** (A and B) Staining of the Na,K ATPase in copper cells from wild-type (wt) larvae using monoclonal antibody  $\alpha 5$  (and TRITC-conjugated secondary antibody) detected conspicuous staining of the basolateral membrane below the level of the septate junction. (C and D) A similar distribution was observed in rescue larvae expressing the  $\beta spec^{\alpha 13}$  transgene, where DANK1-EGFP staining was shifted from the plasma membrane to the cytoplasm. (E) The basolateral distribution of the Na,K ATPase was significantly diminished in  $\beta$  spectrin mutants rescued by the  $\beta spec^{\Delta PH}$  transgene. (F) Weak plasma membrane staining of the Na,K ATPase was observed in some cells from 33% of rescued larvae. Bar, 10  $\mu m$ .

reduced compared with wild type. Thus, DANK1 targeting to the plasma membrane was almost entirely dependent on the presence of  $\beta$  spectrin.

The rescue activity of the  $\beta spec^{\alpha 13}$  transgene and its normal basolateral targeting in copper cells suggested that either the mutation did not eliminate ankyrin binding or that ankyrin binding was dispensable for  $\beta$  spectrin function. To distinguish between these possibilities, we examined the distribution of DANK1-EGFP in  $\beta spec^{\alpha 13}$  rescue larvae (in the absence of endogenous  $\beta$  spectrin). The result (Fig. 5 D) showed that DANK1-EGFP was displaced to the cytoplasm of copper cells, as observed in the  $\beta spec^{em6}$ -null mutant. The  $\beta spec^{\alpha 13}$  mutation also had a striking effect on the behavior of DANK1-EGFP in salivary gland. In fact, most of the lateral membrane distribution of DANK1-EGFP observed in control larvae (Fig. 5 G) was



**Figure 7. Pathways of spectrin assembly and conserved sequences within the  $\beta$  subunit of spectrin.** (A) Two models for targeted assembly of the spectrin cytoskeleton in polarized cells. (left) Ankyrin-first model. Ankyrin may serve as a multifunctional adaptor that couples spectrin to the cue that triggers polarized assembly and to the downstream (secondary) receptors that are normally stabilized by their interaction with the spectrin cytoskeleton through ankyrin. (right) Spectrin-first model. The results of the present study suggest a model in which targeting of assembly of the spectrin cytoskeleton occurs through a direct interaction between the PH domain of  $\beta$  spectrin and a protein or lipid cue. In this simpler scheme, ankyrin serves as an adaptor that stabilizes interacting membrane proteins by attaching them to a preassembled spectrin cytoskeleton. (B) Segment-by-segment comparison of the amino acid sequences of *D. melanogaster*  $\beta$  spectrin and human  $\beta I$ ,  $\beta II$ , and  $\beta IV$  spectrins. The percentage of identity was scored for each segment by GAP alignments using GCG software.

displaced to the cytoplasm in heterozygotes expressing both the transgene and wild-type  $\beta$  spectrin (Fig. 5 H). The membrane-associated population of DANK1-EGFP was reduced further in rescue larvae expressing only  $\beta spec^{\alpha 13}$ , comparable to the level of membrane binding observed in the absence of  $\beta$  spectrin (Fig. 5 F). Quantitative analysis of >100 cells from each genotype established that mean pixel intensity at cell contacts was substantially reduced in both heterozygotes and in rescued mutants. As the mean cytoplasmic level rose (Fig. 5 J), the amount of ankyrin at cell contacts was reduced so that it was only slightly greater than the cytoplasmic pool. These results establish that removal of the ankyrin-binding domain in the  $\beta spec^{\alpha 13}$  mutant transgene had a dramatic effect on the behavior of ankyrin, presumably by disrupting ankyrin binding to spectrin.

#### An ankyrin-independent effect of spectrin on the Na,K ATPase

We previously demonstrated a striking effect of  $\beta$  spectrin mutations on the behavior of the Na,K ATPase in copper cells (Dubreuil et al., 2000). In wild-type larvae, the sodium pump was concentrated within the basal and lateral domains of copper cells (Fig. 6, A and B; two different planes of focus). The same distribution was observed in rescued larvae expressing the  $\beta spec^{\alpha 13}$  transgene (Fig. 6, C and D). However, the basolateral pattern of plasma membrane staining was greatly diminished in rescued larvae expressing the  $\beta spec^{\Delta PH}$  transgene (Fig. 6 E). Many of these larvae survive beyond hatching. Weak plasma membrane staining could be detected in a few cells from 33% of



the mutant guts, indicating that the phenotype was not completely penetrant (Fig. 6 F). But the association of the Na,K ATPase with the plasma membrane was dramatically altered in the majority of cells. Thus, behavior of the Na,K ATPase was more closely associated with the assembled state of  $\beta$  spectrin than with ankyrin.

## Discussion

### The spectrin cytoskeleton assembly pathway

The results presented here provide several novel insights into the assembly and function of spectrin. There are two general models to explain the assembly of the spectrin cytoskeleton in polarized cells. Both models incorporate ankyrin as an adaptor that couples integral membrane proteins to the spectrin cytoskeleton. In the first case (Fig. 7 A, left), assembly begins with a protein receptor that recruits ankyrin to a specific region of the plasma membrane. In this model, ankyrin serves two distinct roles: (1) as an adaptor that couples spectrin to a cue for assembly and (2) as an adaptor that links interacting proteins such as the Na,K ATPase and voltage-dependent sodium channels to the preassembled spectrin cytoskeleton. In the “spectrin first” model (Fig. 7 A, right), ankyrin functions as an adaptor that couples interacting membrane proteins to a preassembled spectrin cytoskeleton (Lombardo et al., 1994). In this model, the site of assembly is determined directly by spectrin and the role of ankyrin is to couple the diverse membrane proteins that interact with ankyrin to that site.

The results of the present study provide the first direct evidence supporting the spectrin-first model. Ankyrin assembly at the basolateral membrane domain of copper cells was dependent on spectrin. Spectrin in turn was dependent on the PH domain of the  $\beta$  subunit in copper cells and on an as-yet-unidentified signal in salivary gland cells. There are examples of ankyrin-independent assembly of spectrin in other systems: (1) During erythrocyte differentiation, ankyrin assembly occurs after the stable assembly of spectrin (Lazarides, 1987). (2) A related observation is that spectrin assembly appears remarkably normal in erythrocytes that lack band 3, the major membrane receptor for ankyrin in the erythrocyte (Peters et al., 1996; Southgate et al., 1996). Thus, even in the best-characterized membrane model, it has been difficult to ascertain the sequence of events that leads to spectrin assembly. (3) Targeting of the  $\alpha\beta_H$  isoforms of spectrin is thought to occur by an ankyrin-independent mechanism (Dubreuil and Grushko, 1998). These spectrins have unusually large and divergent  $\beta$  subunits and are targeted to the apical membrane domain of polarized epithelia in *D. melanogaster*. Together with the current results, it appears that targeting to the plasma membrane is a shared property of spectrins, whether or not they interact with ankyrin.

PH domains have been detected in hundreds of different proteins and in many cases they have physiological roles in binding to phosphoinositides (for review see Lemmon and Ferguson, 2000). Structures have been determined for spectrin PH domains from mammals (Macias et al., 1994) and *D. melanogaster* (Zhang et al., 1995), and binding to phosphoinositides has been

demonstrated (Hyvonen et al., 1995; Wang and Shaw, 1995). The PH domain of spectrins does not have the expected lipid specificity of a protein that mediates phosphatidylinositol (PtdIns)-3-kinase signaling (Lemmon and Ferguson, 2000). Although the structure of the spectrin PH domain appears to be compatible with binding to PtdIns(3,4,5)P<sub>3</sub>, a more likely binding partner in vivo is PtdIns(4,5)P<sub>2</sub> (Hyvonen et al., 1995; Rameh et al., 1997). The six residues that contact PtdIns(4,5)P<sub>2</sub> are conserved in the *D. melanogaster* PH domain and out of 37 amino acid identities among PH domains from mammalian  $\beta$ I,  $\beta$ II,  $\beta$ III, and  $\beta$ IV spectrins, 31 are conserved in *D. melanogaster*. Overall, there was 44% identity between the fly PH domain and mammalian PH domains. For comparison, there was a mean of 62% identity between the PH domains of the four mammalian  $\beta$  spectrin isoforms. Interestingly, in comparisons of full-length sequences, there was greater identity between *D. melanogaster*  $\beta$  spectrin and human  $\beta$ II spectrin (57%) than between human  $\beta$ II and  $\beta$ IV (53%) or between  $\beta$ I and  $\beta$ IV (49%). Therefore, it appears likely that the functions of  $\beta$  spectrin, including the lipid-binding function of the PH domain, are conserved between *D. melanogaster* and mammals.

The PH domain of spectrin may also mediate membrane targeting through interactions with protein receptors. For example, the membrane-binding activity originally described for brain  $\beta$  spectrin was protease sensitive (Steiner and Bennett, 1988). Interactions between PH domains and protein ligands such as protein kinase C and G protein  $\beta\gamma$  subunits have been reported (Lemmon and Ferguson, 2000). However, the interaction between the PH domain of a mammalian  $\beta$  spectrin and  $\beta\gamma$  subunits was tested and found to be comparatively weak (Touhara et al., 1994). We do not yet know the reason for differential targeting of the  $\beta$ spec<sup>APH</sup> product in a mutant versus a wild-type background. Further experiments will be necessary to determine whether mixed tetramers form and whether other sites in the molecule affect their targeting.

Although the PH domain was required for the targeting of spectrin in copper cells, neither the PH domain nor ankyrin binding were required for targeting in the salivary gland. One obvious candidate to explain the recruitment observed in salivary gland is the ankyrin-independent membrane-binding site identified near the N terminus of mammalian  $\beta$  spectrin (Davis and Bennett, 1994; Lombardo et al., 1994). It is also possible that multiple membrane-binding sites contribute to targeting in some cells, even though the PH domain alone appears to be critical in copper cells. To help resolve these questions, it will be important in future studies to identify sites that are sufficient for membrane targeting in different cell types and to produce mutant transgenes in which multiple candidate targeting activities have been knocked out simultaneously.

### Ankyrin-independent function of spectrin

We expected that loss of ankyrin binding would severely compromise the function of  $\beta$  spectrin. Although there was a relatively low viability of flies rescued by the  $\beta$ spec<sup>al3</sup> transgene, and they often had wing phenotypes and appeared less healthy than their wild-type siblings (unpublished data), a surprising number of these flies survived as fertile adults. In contrast, the

four loss-of-function mutations that we previously characterized all exhibited embryonic lethality (Dubreuil et al., 2000). The rescue result reinforces the conclusion that spectrin targeting is independent of its interaction with ankyrin and further suggests that some important aspects of spectrin function are independent of its association with ankyrin.

There are several possible interpretations of  $\beta\text{spec}^{\alpha 13}$  rescue that will require further experiments to resolve. (1) There may be redundant cellular mechanisms that can partially compensate for loss of the adaptor function of ankyrin. (2) The modification of the ankyrin-binding domain of  $\beta\text{spec}^{\alpha 13}$  may have selectively blocked its association with DAnk1 but left Dank2 binding intact. Once appropriate tools become available it will be important to test whether DAnk2 has an essential function and whether that function depends on the ankyrin-binding site defined here. (3) There may be residual ankyrin-binding activity in  $\beta\text{spec}^{\alpha 13}$  that was below the threshold of detection in our experiments. One reason to consider this possibility emerged from sequence comparisons between fly and human  $\beta$  spectrins (Fig. 7 B). Current structural models indicate that part of the putative ankyrin-binding site may include part of segment 15 (repeat 14), where there is striking sequence conservation. Future experiments will address whether this conservation represents part of the ankyrin-binding site or whether it is an as-yet-unidentified functional site in the  $\beta$  spectrin molecule. In any case, it's apparent that most of the ankyrin 1-binding activity of spectrin was removed in  $\beta\text{spec}^{\alpha 13}$ . (4) Finally, it is also formally possible, although unlikely given the degree of sequence conservation, that spectrins and ankyrins in vertebrates and invertebrates have fundamentally different roles in plasma membrane organization and function.

Another surprising result in the present study was the finding that the behavior of the Na,K ATPase was more closely linked to the behavior of spectrin than to ankyrin. Based on biochemical evidence showing that purified mammalian ankyrin and Na,K ATPase directly interact with one another in vitro (Nelson and Veshnock, 1987), we previously assumed that any effects of spectrin mutations on the behavior of the Na,K ATPase in vivo were likely to be mediated through ankyrin (Dubreuil et al., 2000). Current evidence raises the possibility that the Na,K ATPase may be linked to spectrin either directly or perhaps by some other indirect mechanism. It is possible that vertebrates and invertebrates evolved independent mechanisms to link the Na,K ATPase to the spectrin cytoskeleton. It was recently suggested that mammalian KCNQ potassium channels and voltage-dependent sodium channels acquired their functional interaction with ankyrin through a process of convergent molecular evolution, after the split between vertebrates and invertebrates (Pan et al., 2006). The conserved ankyrin-binding sequence found in these mammalian proteins is not present in their *D. melanogaster* homologues. That does not appear to be the case with the Na,K ATPase, as the amino acid sequence that mediates interaction with ankyrins in vertebrates (Zhang et al., 1998) is remarkably conserved in the *D. melanogaster* Na,K ATPase (Lebovitz et al., 1989). Future studies should address the possibility that, even though there is a direct interaction between ankyrin and the Na,K ATPase in vitro, there may be an

important functional interaction with mammalian spectrin in vivo that is ankyrin independent.

### Functional properties of the spectrin repeats

The greatest sequence identity between *D. melanogaster* and mammalian  $\beta$  spectrins occurs in the first three segments of the protein (Fig. 7 B), which includes the actin-binding site (Banuelos et al., 1998) and tail-end subunit interaction sites in segments 2 and 3 (Harper et al., 2001). Another site of striking sequence conservation among  $\beta$  spectrins is the partial repeat 18, where  $\alpha$  and  $\beta$  spectrin interact to form tetramers (Fig. 7 B). Spectrin is thought to have evolved from a large single-subunit ancestor by fracturing of the coding sequence at a site within an ancestral structural repeat (Tse et al., 1990). A point mutation in the N-terminal partial repeat of  $\alpha$  spectrin (R22S) produced a temperature-sensitive defect in spectrin tetramer formation (Deng et al., 1995). Here, we tested the effect of a comparable mutation in the  $\beta$  subunit that was also identified by its role in human anemia (Nicolas et al., 1998). The W2033R mutation corresponds to the W2024 position of human erythroid  $\beta$  spectrin, and that residue is also conserved in human  $\beta$ II,  $\beta$ III, and  $\beta$ IV, but not in  $\beta$ V spectrin. This tryptophan residue was dispensable for  $\beta$  spectrin function in *D. melanogaster*, presumably because it does not affect tetramer formation. Polarized targeting of the transgene product was also unaffected by the mutation (unpublished data). Another tryptophan at position 2061 of human erythroid  $\beta$  spectrin that has been implicated in hereditary elliptocytosis (Nicolas et al., 1998) is conserved in human  $\beta$ II,  $\beta$ III, and  $\beta$ IV spectrin, but not in *D. melanogaster*  $\beta$  or in human  $\beta$ V spectrin. The importance of these tryptophan residues in nonerythroid spectrin tetramer formation and function has not been tested.

Relatively little is known about the function of repetitive segments 4–14 in  $\beta$  spectrin. This region has more limited sequence conservation than segments near the ends of the molecule (Fig. 7 B). Further studies using smaller scale modifications will be required to identify the functional sites that explain the mutant phenotype of  $\beta\text{spec}^{\alpha 2-9}$ . For now, this transgene helps to establish that not all functional defects in  $\beta$  spectrin result in mislocalization. Thus, gene modifications that do affect protein targeting, such as  $\beta\text{spec}^{\Delta PH}$ , are probably identifying functional sites that are responsible for targeting.

### Future prospects

The domain swap strategy described here takes advantage of the powerful genetic tools available in *D. melanogaster* to study protein function. This approach is especially well suited to studies of a modular protein such as spectrin and has provided valuable insights into both the function and the targeting of the protein in vivo. Combined with the fact that *D. melanogaster* is a simpler system, having only three spectrin genes and two ankyrin genes, this approach should continue to provide valuable information that will be more difficult to obtain in mammalian systems. One intriguing observation in this study was the effect of the  $\Delta PH$  mutation on the size of the rare flies that survived to adulthood. This phenotype was reminiscent of *D. melanogaster*



mutations that affect PH domain-containing components of the insulin/insulin-like growth factor signaling pathway (Oldham and Hafen, 2003). Given the importance of a phosphoinositide-binding PH domain to  $\beta$  spectrin function, it will be interesting to genetically test the possibility that spectrin has a functional interaction with growth factor signaling pathways.

## Materials and methods

### Antibodies

A rabbit anti- $\beta$  spectrin serum (Kcar) used for immunolocalization experiments and affinity-pure rabbit anti-ankyrin1 (DAnk1) antibody were previously described (Dubreuil and Yu, 1994). Another rabbit anti- $\beta$  spectrin serum (337; Byers et al., 1989) was used on Western blots. Myc epitope-tagged  $\beta$  spectrin transgenes were detected using the 9E10 mouse monoclonal antibody (Evan et al., 1985). Mouse anti-Dlg (discs large) antibody 4F3 was obtained from the Developmental Studies Hybridoma Bank. Rabbit anti-Scribble was a gift from C. Doe (University of Oregon, Eugene, OR; Albertson and Doe, 2003). Monoclonal antibody  $\alpha$ 5 against the  $\alpha$  subunit of the Na,K ATPase was a gift from D. Fambrough (Johns Hopkins University, Baltimore, MD).

### Fly stocks and transgenes

The WUMB- $\beta$  spectrin transgene was described previously (Dubreuil et al., 2000). All of the following were produced by PCR-based modifications and/or mutagenesis of the WUMB- $\beta$  spectrin plasmid (as indicated), and the modifications were confirmed by DNA sequencing. Primers were obtained from Operon, and DNA sequencing was performed by the DNA Services Facility at the University of Illinois at Chicago Research Resource Center. All of the transgenic lines described here were produced by standard embryo microinjection into  $w^{1118}$  by Genetic Services, Inc.

**$\beta$ spec<sup>3A</sup>.** The myc-tagged  $\beta$  spectrin transgene line that we described previously (Dubreuil et al., 2000) had a dominant male sterile phenotype, making it difficult to maintain a working stock. We produced five new transgenic lines in this study using the same construct. One of the five new lines,  $\beta$ spec<sup>KW3A</sup>, was a recessive lethal insertion that was balanced over the TM3 chromosome as a stable line. The four other insertions had fertility problems comparable to the original line. In fact, we have observed a high frequency of male sterile lines with many of our transgenes that encode  $\beta$  spectrins, but not with any other transgenes we have produced.

**$\beta$ spec<sup>13</sup>.** The 15th repeat of  $\beta$  spectrin (segment 16) was excised using Apol and FspA1, which cut the  $\beta$  spectrin cDNA at codons 1810 and 1921, respectively. The excised fragment was eight codons out of phase with the 106-residue structural repeat of spectrin (Yan et al., 1993). A PCR product corresponding to segment 13 was amplified from *D. melanogaster*  $\alpha$  spectrin cDNA N8 (Dubreuil et al., 1989) using a sense primer that added an Apol site to the 5' end of the product (AAATTCCTCAGCGACTACCGC) and an antisense primer that added an FspA1 site to the 3' end of the product (GATGCGCACGTACGCATGTAGAGCTG). The resulting Apol-FspA1 fragment of the cloned PCR product was inserted into WUMB- $\beta$  spectrin, replacing 109 residues beginning at the 8th codon of segment 16 through the 10th codon of segment 17. Three independent insertions of this transgene all exhibited dominant or recessive male sterility. An X-linked insertion was recombined with the  $\beta$ spec<sup>em21</sup> chromosome for the studies described here.

**$\beta$ spec<sup>12-9</sup>.** An 844-codon BglII-ApaI fragment of  $\beta$  spectrin extending from the start of segment 4 to the last third of segment 11 was replaced with an in-phase fragment of  $\alpha$  spectrin amplified by PCR. The  $\alpha$  spectrin sense primer introduced a BglII site at the start of  $\alpha$  repeat 2 (GAGATCTCGCTCGTCTGGTGCAGTTCCTG), and the antisense primer introduced an ApaI site at the corresponding position of repeat 9 (beginning 16 amino acids from the SH3 insertion; GTGCACGGATTCTCCGTGTAGTCGTA). Four autosomal insertions of this transgene exhibited dominant male sterility. Two X-linked insertions were fertile, and one of them was recombined with the mutant  $\beta$ spec<sup>em21</sup> chromosome for the studies described here.

**$\beta$ spec<sup>1PH</sup>.** Codon 2144 at the start of the PH domain was replaced by QuikChange mutagenesis (Stratagene) with a TGA stop codon, generating a truncated coding sequence that lacked the C-terminal 148 residues. The primer sequence used was GAACGAGGCGGAACCTGAGGCGCCGGCGAGGG. Two of three independent lines produced were homozygous viable and fertile.

**$\beta$ spec<sup>W2033R</sup>.** The conserved trp residue at codon 2024 in human erythrocyte  $\beta$  spectrin is mutated to arg in some human probands with hereditary elliptocytosis. This missense mutation, which completely eliminates the in vitro tetramerization reaction of  $\beta$  and  $\alpha$  spectrin (Nicolas et al., 1998), was introduced at the corresponding position of fly  $\beta$  spectrin by QuikChange mutagenesis using the primer sequence CCTGGGCGATCAGCCTGGCCTCGGCCACGGC. Two of four insertions were unstable, one was homozygous viable on X, and the other was recessive sterile on chromosome 3.

### Rescue crosses

The four  $\beta$  spectrin mutant alleles that were produced in our genetic screen (Dubreuil et al., 2000) were initially maintained as balanced stocks with the *C(1)Dx* compound X chromosome. Subsequently, the mutant X chromosomes were rebalanced using the *FM7[Kr-GFP]* chromosome to allow identification of mutant embryos. Females from this rebalanced line were outcrossed to males carrying a test transgene on one of the autosomes to ask whether the transgene would allow recovery of F1 males carrying the  $\beta$ spec<sup>-</sup> chromosome. Without rescue, the only surviving F1 males are those that inherited the marked *FM7* balancer (carrying the wild-type  $\beta$  spectrin gene from their mother). Unexpectedly, all of the mutant transgenes that we tested produced at least a low level of rescue. Ultimately, when we analyzed rescue progeny from crosses with the  $\beta$ spec<sup>1PH</sup> transgene on Western blots stained with the anti- $\beta$  spectrin antibody 337, we found flies that expressed only the rescue transgene (and not the full-length wild-type protein). However, we also found male progeny with the rescue phenotype (i.e., they lacked the *FM7* marker *Bar*), but on Western blots these flies had both the rescue transgene product and the full-length wild-type product (unpublished data). Thus, these were not true rescue flies and ultimately we were able to trace the source of these flies to transmission of wild-type X chromosomes from fathers to sons in the rescue cross. This unusual transmission pattern could arise through the presence of XXY females in the *FM7* balanced population or through nondisjunction of *FM7*.

To bypass the false rescue problem, we crossed autosomal transgenes into a *C(1)Dx* background. In this compound X line, X chromosomes are transmitted from mothers to daughters and from fathers to sons, and females transmit Y chromosomes to their sons. *C(1)Dx* females carrying autosomal transgenes to be tested were crossed to  $\beta$ spec<sup>em21</sup> mutant males that also carried a translocation of the X chromosome (including a wild-type  $\beta$  spectrin gene) on chromosome 3: *Dp(1;3)Bar<sup>S31</sup>D3* (Dubreuil et al., 2000). These males transmitted the mutant  $\beta$  spectrin gene on X to their sons, and we could score the ability of a test transgene to replace the translocation by the presence of male progeny that lacked the dominant *Bar* eye phenotype. Progeny that inherited both the test transgene and the translocation were not scored as rescue because they had a *Bar* eye phenotype. Thus, a transgene with 100% rescue efficiency would yield a 2:1 ratio of *Bar* eye/non-*Bar* eye rescue progeny, as observed with the control wild-type transgene.

### UAS DAnk1 transgene

An EcoRI-NotI fragment of a previously described DAnk1 ankyrin-EGFP construct (Jefford and Dubreuil, 2000) was subcloned in the vector pUAST (Brand and Perrimon, 1993). An upstream EcoRI fragment was subcloned from DAnk1 cDNA 11 (Dubreuil and Yu, 1994) to complete the coding sequence. Expression of this transgene was driven by the HS Gal4 line 89-2-1 (Bloomington stock 1799), which caused constitutive expression of DAnk1-EGFP in copper cells and salivary gland.

### Microscopy

Whole flies were photographed with a camera (Coolpix 5700; Nikon) on a dissecting microscope (MZFL111; Leica). Larval midguts and salivary glands were dissected and stained as previously described (Dubreuil et al., 2000) and mounted using Vectashield medium (Vector Laboratories) for viewing at room temperature. Images were captured on a confocal microscope (IX70 or FV500; Olympus) using a 60 $\times$  Plan-Apo oil-immersion objective (NA 1.4) and Fluoview 2.1 software. Brightness settings were adjusted for control specimens (either wild-type siblings or expression level-matched control transgenes) using the photomultiplier, and settings were kept constant for capturing data for mutants. Images were saved as experiments in Fluoview and were converted to jpeg format by NIH ImageJ. Montages were assembled using Photoshop 6.0 (Adobe), and gamma adjustments after conversion to grayscale were performed for all panels simultaneously. The freehand line tool of NIH ImageJ was used for fluorescence intensity measurements of 10–14 salivary glands of each genotype. Intensity values were plotted as a histogram using Excel (Microsoft) with the control value set to 100.

## Western blots

Adult flies were homogenized in SDS gel sample buffer in a dounce homogenizer and immediately boiled. A clarified supernatant corresponding to 0.8 flies per sample was subjected to electrophoresis, blotted to nitrocellulose, reacted with primary antibody and alkaline phosphatase-coupled secondary antibody, and stained with bromochloroindolyl phosphate as previously described (Dubreuil and Yu, 1994). Expression of UAS DANK1-EGFP driven by HS Gal4 was found to be independent of HS (unpublished data). However, the DANK1 antibody did cross-react with polypeptides in the 70–80-kD range in a HS-dependent manner (Fig. 5).

## Sequence comparisons

DNA sequence analysis was performed using the Wisconsin package (Devereux et al., 1984), and sequence alignments were performed using Gap. The sequence of *D. melanogaster*  $\beta$  spectrin (Byers et al., 1992) was compared with human  $\beta$ specII (P11277; Winkelmann et al., 1990),  $\beta$ specI (G01082; Hu et al., 1992),  $\beta$ III spectrin (NP008877; Stankewich et al., 1998),  $\beta$ IV spectrin (NP079489; Tse et al., 2001), and  $\beta$ V spectrin (NP057726; Stabach and Morrow, 2000). Plotted points in Fig. 7 B were aligned with the center of each segment.

## Online supplemental material

Fig. S1 shows that polyclonal rabbit anti- $\beta$  spectrin antibody stained the entire basolateral domain of wild-type midgut copper cells and that plasma membrane staining was abolished in  $\beta$ spec<sup>emb</sup> mutants. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200602095/DC1>.

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