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Drosophila β Spectrin Functions Independently of α Spectrin to Polarize the Na,K ATPase in Epithelial Cells

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Abstract. Spectrin has been proposed to function as a sorting machine that concentrates interacting proteins such as the Na,K ATPase within specialized plasma membrane domains of polarized cells. However, little direct evidence to support this model has been obtained. Here we used a genetic approach to directly test the requirement for the β subunit of the αβ spectrin molecule in morphogenesis and function of epithelial cells in *Drosophila*. β Spectrin mutations were lethal during late embryonic/early larval development and they produced subtle defects in midgut morphology and stomach acid secretion. The polarized distributions of $\alpha\beta_H$ spectrin and ankyrin were not significantly altered in β spectrin mutants, indicating that the two isoforms of *Drosophila* spectrin assemble independently of one another, and that ankyrin is upstream of αβ spectrin in the spectrin assembly pathway. In contrast, β spectrin mutations had a striking effect on the basolateral accumulation of the Na,K ATPase. The results establish a role for β spectrin in determining the subcellular distribution of the Na,K ATPase and, unexpectedly, this role is independent of α spectrin.

Key words: cell polarity • cytoskeleton • *Drosophila melanogaster* • plasma membrane • ankyrins

Introduction

Two different mechanisms have been proposed to explain the enrichment of Na,K ATPase within the basolateral membrane domain of polarized epithelial cells. One relies on sorting events in the secretory pathway that separate newly synthesized proteins into transport vesicles destined for either the basolateral or apical membrane domain. This model accounts for the observation that newly synthesized Na,K ATPase molecules are directly delivered to the basolateral domain (Caplan et al., 1986; Gottardi and Caplan, 1993; Zurzolo and Rodriguez-Boulan, 1993). The second relies on selective stabilization of Na,K ATPase molecules once they have arrived at the basolateral domain, whether or not the protein is sorted in the secretory pathway. This model was based on a series of observations: (a) the Na,K ATPase colocalized with sites of polarized spectrin/ankyrin assembly at the cytoplasmic face of the plasma membrane (Nelson and Veshnock, 1986; McNeill et al., 1990); (b) Na,K ATPase was shown to physically interact with ankyrin in vitro (Nelson and Veshnock, 1987); and (c) basolateral accumulation of Na,K ATPase was observed in an MDCK cell line that did not sort this protein in the secretory pathway (Hammerton et al., 1991; Mays et al., 1995). Direct evidence demonstrating that either of these mechanisms is sufficient to explain the steady-state distribution of the Na,K ATPase is lacking.

Genetic methods in *Drosophila* provide an opportunity to directly test these models. Virtually all of the known properties and protein interactions of spectrin are conserved in *Drosophila* relative to the vertebrate systems in which they were first characterized (for reviews see Bennett and Gilligan, 1993; Dubreuil, 1996). The native spectrin molecule consists of high molecular weight α and β subunits that assemble as elongated heterotetramers. Spectrin tetramers associate with ankyrin, which in turn associates with the cell adhesion molecule neuroglian and presumably other integral plasma membrane proteins. The sequence of the ankyrin binding site of the Na,K ATPase is conserved in *Drosophila* (Zhang et al., 1998), and the Na,K ATPase codistributes with ankyrin and spectrin in polarized fly cells (Baumann et al., 1994; Dubreuil et al., 1997). Yet, despite these conserved features, Na,K ATPase polarity was not detectably altered in epithelial cells from null α spectrin mutants (Lee et al., 1993, 1997). These

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results led to the conclusion that basolateral accumulation of the Na,K ATPase in *Drosophila* epithelia did not require a stabilizing interaction with the spectrin membrane skeleton.

Here we describe the production and initial characterization of lethal mutations in the *Drosophila* β spectrin gene. These mutations provide an opportunity to use a genetic approach to directly test the proposed functions of the conventional β spectrin isoform that is broadly expressed in nonerythroid cells. The phenotypes of mutations in the β_H subunit of spectrin in *Drosophila* (Thomas et al., 1998) and *Caenorhabditus elegans* (McKeown et al., 1998) have already been described. β_H is an unusual spectrin isoform found in invertebrates that appears to have specialized functions that are distinct from conventional β spectrins (Dubreuil and Grushko, 1998). We focused attention on midgut copper cells because of their unique form and function, which make them well-suited for studies of the role of the spectrin membrane skeleton in the development and function of polarized cells (Lee et al., 1993; Dubreuil et al., 1998). The results provide the first direct evidence that spectrin contributes to the polarized distribution of the Na,K ATPase in epithelial cells and, unexpectedly, that the β subunit of spectrin carries out this role independently of α spectrin.

Materials and Methods

Fly Stocks and Genetic Screen

A lethal deficiency in the β spectrin region of the X chromosome, *Df(1)SD10*, was produced by γ-ray mutagenesis of flies carrying the ry^+ P-element insertion S6.9-11 (Wakimoto et al., 1986). In brief, adult males were exposed to 3,300 rads from a Cs^{137} source, crossed to ry^{506}/ry^{506} females, then scored for ry^- female progeny. A total of 32 ry^- revertant females were recovered from 145,000 females screened, and three hemizygous lethal lines were obtained. *Df(1)SD10* was the only cytologically visible deficiency (16A-16DE) that lacked the β spectrin gene by polytene hybridization. Duplications of the β spectrin region on chromosome 3 were produced by γ-ray mutagenesis of the stock $Tp(1;3)B^{S3i}$, which is marked with the *Bar-super* dominant visible eye mutation. $Tp(1;3)B^{S3i}$ males were irradiated with 4,000 rad, then crossed to Oregon R females. *Bar*-eye male progeny retaining the duplication were mated with *ywf/ywf* females, and crosses were scored for reversion of the male-sterile phenotype. Screening of 36,000 progeny yielded three male-fertile *Bar*-eye lines: $Dp(1;3)B^{S3i}D1$, $Dp(1;3)B^{S3i}D2$, and $Dp(1;3)B^{S3i}D3$. The extent of the duplications was determined by in situ hybridization and conventional cytology and balanced stocks of the recessive-lethal duplications were maintained over the *TM6b* chromosome.

A germ-line tranformant expressing an *myc* epitope-tagged wild-type β spectrin transgene on chromosome 2 was produced using a previously described strategy (Dubreuil et al., 1996). A BamHI-NotI fragment of the full-length β spectrin cDNA (Byers et al., 1992) was subcloned into the vector pWUMB. The vector was assembled from the w^+ transformation vector pW8 (Klemenz et al., 1987), a 2-kb fragment of the *Drosophila* ubiquitin promoter (Lee et al., 1988), and a linker sequence encoding the *myc* epitope tag at the translation start site. The resulting construct encodes full-length wild-type β spectrin, except that the first 10 amino acids of β spectrin are replaced with the 10–amino acid myc epitope tag, which reacts with the mouse mAb 9E10 (Evan et al., 1985). The pWUMB-βspec construct was introduced into germ-line DNA by standard embyro microinjection. A single autosomal transformant P[$\beta spec^{T3I}$] was recovered on chromosome 2.

Mutations in the *Drosophila* β spectrin gene were produced by chemical mutagenesis. The screen was based on recovery of X-linked lethal mutations by complementation with $Dp(1;3)B^{S3i}D3$, a duplication of the 16A-F region of the X chromosome on chromosome 3. Males from an isogenized Oregon R stock were fed 24.5 mM ethyl methane sulfonate using standard methods (Grigliatti, 1986), then mated to *C(1)DX/y;* $Dp(1;3)B^{S3i}/+$ females. F_1 male progenies were selected for presence of the duplication by their *Bar*-eye phenotype and crossed in single pair matings with *C(1)DX/y* females. Recessive X-linked lethal mutations in the 16A-F region were identified as crosses that yielded exclusively *Bar*-eye male progeny. 20 new mutations were recovered from a total of 10,744 chromosomes screened. The mutants were ordered into three intervals (I–III) by complementation tests with two additional duplications: $Dp(1;3)B^{S3i}D1$ and $Dp(1;3)B^{S3i}D2$. The mutants were assigned to six complementation groups by standard complementation tests. The complementation group representing the β spectrin gene was identified by rescue with the P[$\beta spec^{T3I}$] transgene.

Balanced stocks of each mutant over a *FM7*[*Kruppel*-GFP] chromosome (Casso and Kornberg, 1999) were used to recover *β-spec*$^-$ embryos in all experiments. Embryos carrying the balancer chromosome express green fluorescent protein (GFP)[1] with the characteristic *Kruppel* pattern (Gaul et al., 1987). Embryos were collected from each *β-spec*$^-$*/FM7*[*Kr*-GFP] × *FM7*[*Kr*-GFP]/y line for 2 h at 25°C. Embryos were aged overnight at 22°C, dechorionated in 50% bleach, and transferred to microscope slides to score GFP expression by fluorescence microscopy. *β-spec*$^-$ male embryos were identified by their lack of GFP expression. The mutants and their wild-type siblings were separately transferred to apple juice agar plates for further development at 22°C.

Phenotype Analysis

Hatching rates of mutants and wild-type siblings were scored 48 h after egg-laying. Embryo samples for gel electrophoresis (20/lane) were collected as late embryos, just before hatching of wild-type larvae. Electrophoresis, transfer to nitrocellulose, and staining with rabbit anti–β spectrin antibody were carried out as described previously (Byers et al., 1989).

Sequence data were obtained from cloned mutant genomic DNA amplified by PCR. An ~2-kb fragment of genomic DNA spanning positions 3105–5192 of the published cDNA sequence (Byers et al., 1992), was amplified from each of the four mutants and the isogenic parent strain. Duplicate clones from independent PCR reactions were sequenced on both strands by automated sequencing in the University of Chicago Cancer Center core facility. DNA sequences were analyzed using the UWGCG computer programs (Devereux et al., 1984).

Immunofluorescent staining of ankyrin, α and β_H spectrin, and the Na,K ATPase were carried out as described previously (Dubreuil et al., 1997, 1998). The distributions of ankyrin and β spectrin in copper cells were compared in double-labeling experiments with tissue from larvae expressing the myc epitope–tagged P[$\beta spec^{T3I}$] β spectrin transgene, because the available antibodies against endogenous β spectrin generate an intractable background in the dissected midgut (our unpublished observation). Larvae were raised at 29°C for staining of the Na,K ATPase to maximize its expression (Feng et al., 1997). Specimens shown in Figs. 3 and 4 were viewed and photographed with an ausJena Jenalumar photomicroscope. Images were captured on photographic film, digitized using a Polaroid SprintScan scanner, and edited using Adobe Photoshop. Images shown in Figs. 5 and 6 were captured digitally using an Olympus IX70 confocal microscope with Fluoview software. Colocalization ratios were calculated using MetaMorph software (Universal Imaging Corp.).

Midgut acidification was scored by feeding larvae with bromphenol blue–impregnated yeast paste as described previously (Dubreuil et al., 1998). Since wild-type larvae were found to develop more rapidly than β spectrin mutants, independent collections were used to isolate pools of larvae at comparable stages of development. Many of the mutant larvae were manually dissected from the vitelline envelope because of their low frequency of hatching. Midgut acidification was scored by viewing the dissected digestive tract under a dissecting microscope several hours after transfer of larvae to bromphenol blue–dyed yeast.

Results

Identification and Characterization of β Spectrin Mutants

Chemically-induced X-linked lethal mutations in the region of the β spectrin gene were identified in a genetic screen for rescue by $Dp(1;3)B^{S3i}D3$ (a duplication that

[1] *Abbreviation used in this paper*: GFP, green fluorescent protein.

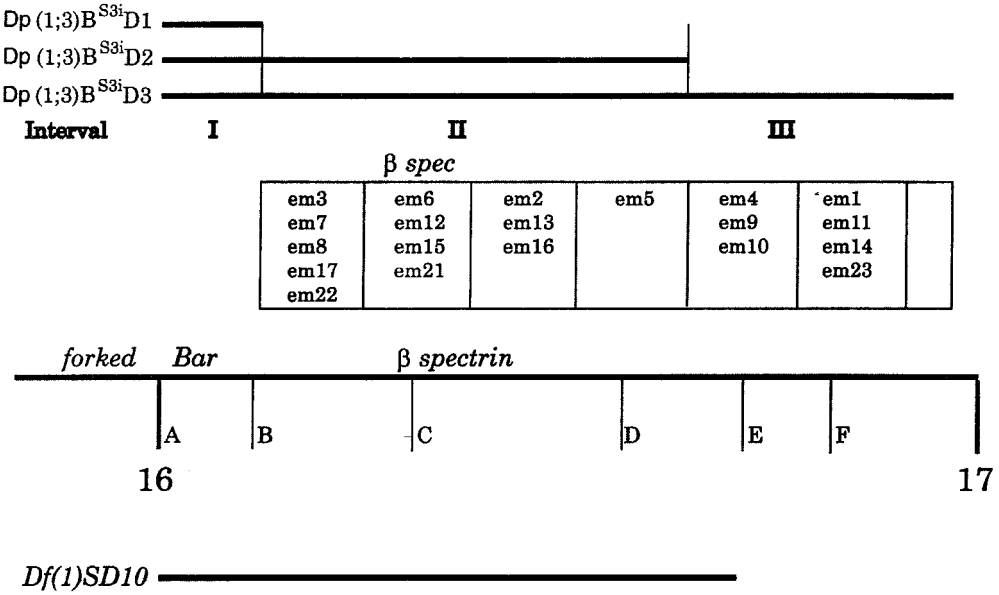

Figure 1. Cytogenetic map of the β spectrin region of the X chromosome. Three overlapping duplications define intervals of the X chromosome. 20 ethyl methane sulfonate–induced mutations in the β spectrin region were ordered into six complementation groups within these intervals (vertical rows). None of the interval II complementation groups complemented the X chromosome deficiency *Df(1)SD10*. Note that the order of complementation groups within intervals II and III is not known.

spans the β spectrin locus; Fig. 1). Mutant alleles from six independent complementation groups met the criterion of survival only in the presence of the duplication. Four complementation groups were rescued by the smaller duplication *Dp(1;3)B^{S3i}D2*, which defines interval II. None of the mutations were rescued by the smallest duplication, *Dp(1;3)B^{S3i}D1*. The complementation groups in interval II failed to complement the deficiency *Df(1)SD10*, which spans the β spectrin locus. The mutations in interval II were further tested for their ability to be rescued by a β spectrin transgene, P[β-spec^{T3I}], composed of the cDNA coding sequence for β spectrin under control of the *Drosophila* ubiquitin promoter. Using this strategy, complementation group 2 from interval II was identified as the β spectrin locus. Hereafter, these four mutant alleles are referred to as *β-spec^{em6}*, *β-spec^{em12}*, *β-spec^{em15}*, and *β-spec^{em21}*. None of the other mutations in interval II or III were rescued by the β spectrin transgene.

Initial examination of the β spectrin mutants indicated that they were embryonic lethal. To further characterize them, balanced stocks of each allele over a GFP-marked *FM7* balancer chromosome were generated. Heterozygous mutant females in these stocks produced mutant male progeny that were identified by their lack of GFP expression. Using this strategy, it was found that β spectrin mutant males developed to the point of hatching (Table I). However, most of the mutants died before hatching, even though they appeared to be fully developed and motile. The *β-spec^{em6}* and *β-spec^{em15}* alleles had the lowest hatching rates (~10%), and the *β-spec^{em12}* and *β-spec^{em21}* alleles had a somewhat higher hatching rate (~25%) relative to their wild-type siblings. Whether or not they hatched, the mutant larvae survived for 1–3 d. A null mutation in *Drosophila* α spectrin (*α-spec^{rg41}*) was characterized previously as a first instar larval lethal (Lee et al., 1993). Here we quantified the hatching rate of *α-spec^{rg41}* (Table I) and found that it was on average approximately fivefold greater than the strongest β spectrin allele (*β-spec^{em6}*). The β spectrin mutants that hatched were generally similar in appearance and behavior to null α spectrin mutants. Newly hatched larvae initially crawled, ate, and responded to touch, but they grew increasingly sluggish over time, failed to grow, and eventually died.

The same *FM7*[*Kr*-GFP]-based strategy was used to isolate mutant embryos for Western blot analysis using a rabbit anti–β spectrin antibody. Staining of wild-type embryos revealed the expected 273-kD full-length β spectrin polypeptide (Fig. 2, lanes 1, 3, 5, and 7) as well as an ~150-kD proteolytic fragment. The *β-spec^{em6}* (Fig. 2, lane 2) and *β-spec^{em15}* (Fig. 2, lane 6) mutants exhibited no significant reactivity with the antibody, indicating that all of the proteins detected in wild-type siblings are encoded by the β spectrin gene. Truncated immunoreactive β spectrin products were observed in *β-spec^{em12}* and *β-spec^{em21}* (Fig. 2, lanes 4 and 6, asterisks), with estimated mobilities of 190 kD and 170 kD, respectively. The ~150-kD proteolytic fragment of β spectrin was observed in the latter two mutants as well. The truncated fragments were faintly visible in wild-type siblings (Fig. 2, lanes 3 and 7), but were underrepresented here because the embryo collection included homozygotes for the *β-spec^{+}* balancer chromosome as well as *β-spec^{-}/FM7*[*Kr*-GFP] heterozygotes.

Based on the size of the truncated products in *β-spec^{em12}* and *β-spec^{em21}*, a PCR-based strategy was developed to an-

Table I. Hatching Rates of Spectrin Mutant Embryos

Genotype	Hatching frequency	No.
	%	
βspec^{em6}/+	89 ± 1	211
βspec^{em6}/y	12 ± 3	215
βspec^{em12}/+	88 ± 1	122
βspec^{em12}/y	28 ± 2	154
βspec^{em15}/+	92 ± 0	100
βspec^{em15}/y	13 ± 0	106
βspec^{em21}/+	89 ± 2	152
βspec^{em21}/y	22 ± 6	167
α-spec^{rg41}/α-spec^{rg41}	53 ± 29	99

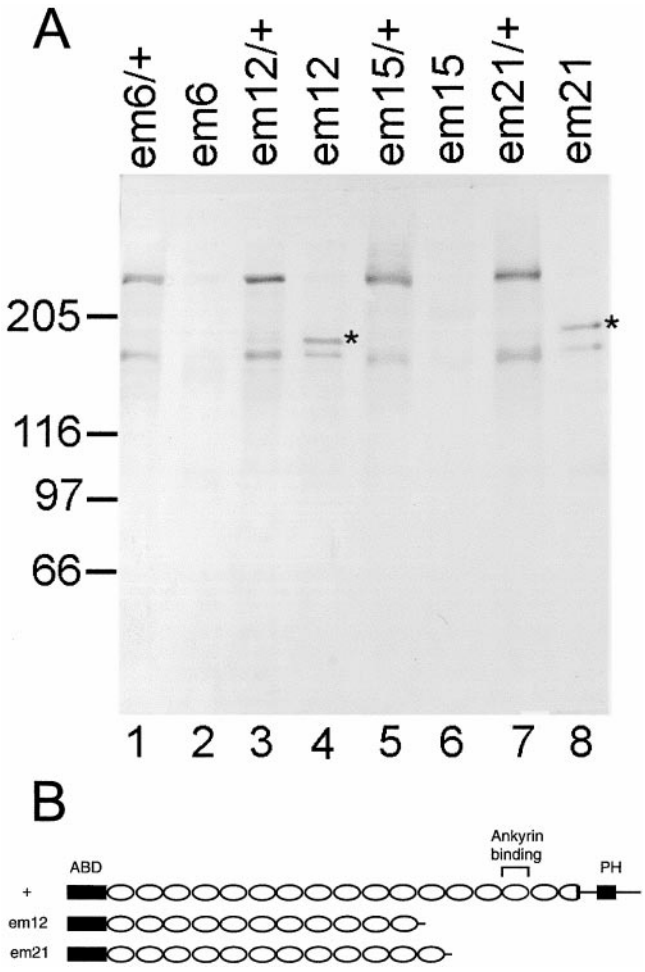

Figure 2. (A) Western blot analysis of β spectrin mutant embryos. Mutant male embryos (lanes 2, 4, 6 and 8) and wild-type female siblings (lanes 1, 3, 5, and 7) were homogenized in SDS sample buffer and stained with rabbit anti–*Drosophila* β spectrin antibody after electrophoresis and transfer to nitrocellulose. Alkaline phosphatase–conjugated goat anti–rabbit antibody was used as secondary label. Mobilities of molecular weight standards are shown to the left in kD. An asterisk marks truncated products observed in two of the mutants. (B) Results of genomic sequence analysis from the β spectrin mutants is shown schematically. (+) The domain structure of wild-type β spectrin includes the actin binding domain (ABD), a series of ~106 amino acid spectrin repeats, an ankyrin binding site, and a plekstrin homology region (PH). The *β-spec^{em12}* mutation was caused by a nonsense mutation within the codon for a highly conserved tryptophan residue in repeat 12. The *β-spec^{em21}* mutation was caused by a nonsense mutation at the same conserved tryptophan codon in repeat 13.

alyze the genomic sequence of each mutant allele. An ~2-kb fragment of genomic DNA from each mutant was amplified, cloned in duplicate from independent PCR reactions, and sequenced on both DNA strands. The nonmutagenized parent chromosome was used as a control in these reactions. The sequence of the parent chromosome was identical in this region to the previously published β spectrin cDNA sequence (Byers et al., 1992), except for several silent third base substitutions. The *β-spec^{em12}* and *β-spec^{em21}* sequences revealed nonsense stop codons (TGA) at the position of the highly conserved tyrosine TGG codon found within the spectrin repeat domain (Fig. 2 B). The nonsense mutations occurred near the start of repeat 12 in *β-spec^{em12}* and near the start of repeat 13 in *β-spec^{em21}*. The predicted molecular masses of these truncated products (175 kD and 188 kD, respectively) agreed well with the estimated sizes of the truncated products observed in Western blots. Both of these truncations deleted the ankyrin binding site of β spectrin (Kennedy et al., 1991).

Role of β Spectrin in Epithelial Differentiation

The cellular consequences of the β spectrin mutations were analyzed in epithelial cells of the larval middle midgut. The copper cells in particular were shown previously to require α spectrin for their normal differentiation and function in stomach acid secretion (Lee et al., 1993; Dubreuil et al., 1998). These cells have a peculiar invaginated morphology in which the apical cell surface is tucked within the cell body (Fig. 3 A). The invagination is connected to the gut lumen through a pore formed by neighboring interstitial cells. Smooth septate junctions (Dubreuil, R.R., T. Grushko, O. Baumann, manuscript submitted for publication) occupy the apicolateral contact region between copper cells and interstitial cells, forming a collar that surrounds the pore. Despite their unusual morphology, copper cells exhibit many of the properties of conventional epithelia. The apical surface, extending inward from the collar, displays densely packed microvilli toward the gut lumen. The basolateral domain, including the apicolateral collar, is the site of contact with neighboring cells in the epithelial sheet. All plasma membrane markers that have been examined so far are segregated within either the apical or the basolateral domain.

Double-label immunofluorescent staining was used to compare the relative distributions of ankyrin and β spectrin within the basolateral membrane domain of copper cells. β spectrin, encoded here by an epitope-tagged transgene, was detected throughout the basolateral region in first instar larvae (Fig. 3 B, arrow), and was especially concentrated in the apicolateral collar (Fig. 3 B, arrowhead). Ankyrin was also concentrated at the collar, with only faint staining visible in the rest of the basolateral domain. Ankyrin staining appeared as comma shapes on either side of the entrance to the apical invagination in favorable optical sections (Fig. 3 C, arrowhead). As larvae grew and copper cells increased in size, ankyrin staining became visible throughout the basolateral domain (Fig. 3 D, arrow), although ankyrin remained relatively concentrated at the apicolateral contacts (Fig. 3 D, arrowhead). These results are consistent with a role for ankyrin in attaching αβ spectrin to the plasma membrane, both at the apicolateral contact region and throughout the rest of the basolateral domain of copper cells. However, ankyrin staining outside of the apicolateral collar was relatively weak and near the threshold of detection in first instar larvae.

Ankyrin staining was used to monitor the effect of β spectrin mutations on cell pattern in the middle midgut epithelium. The en face pattern of ankyrin staining in the first instar middle midgut provides a convenient map of cell outlines in the epithelial sheet (Fig. 4 A) (Dubreuil et

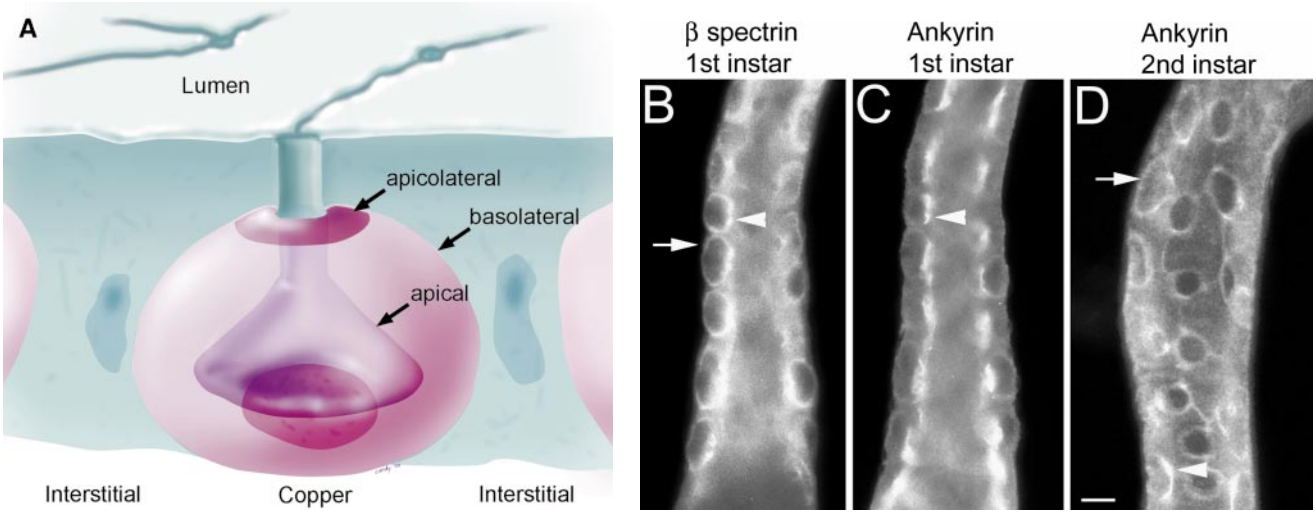

Figure 3. Relative distributions of β spectrin and ankyrin in copper cells. (A) Copper cell morphology. Copper cells alternate with interstitial cells in the larval middle midgut epithelium. The apical surface of the copper cell is invaginated and opens into the gut lumen through a pore formed by surrounding interstitial cells. The basolateral surface is spheroid in shape and completely surrounds the invaginated apical domain, except at the pore. The basolateral domain immediately surrounding the pore (apicolateral, arrow) forms a septate junction with the adjacent region of the interstitial cells. (B) Dissected middle midgut from a first instar larva expressing the *myc* epitope–tagged β spectrin transgene stained with mouse mAb against the epitope tag and Texas red–labeled goat anti–mouse secondary antibody. The most intense staining was found at the apicolateral domain of copper cells (arrowhead), with weaker staining of the rest of the basolateral plasma membrane (arrow). (C) Double labeling of the same sample as in A, stained with affinity-purified rabbit anti-ankyrin antibody and FITC-conjugated secondary antibody. Arrowhead marks comma-shaped staining of the apicolateral contacts between copper cells and neighboring interstitial cells in this optical section. (D) Staining of a second instar *β-spec*$^{+}$ larva with ankyrin antibody as in C. Arrow marks staining of the basolateral domain of copper cells and arrowhead marks the relatively intense staining of the apicolateral contact region. Bar, 10 μM.

al., 1998). The apicolateral contacts between wild-type copper cells and interstitial cells appeared as small rings (Fig. 4 A, arrow) interconnected by lines that represent contacts between adjacent interstitial cells (Fig. 4 A, arrowhead). Ankyrin staining revealed the same overall pattern of cell contacts in *β-spec*^{em6} (Fig. 4, B and C) and *β-spec*^{em21} (not shown) male first instar larvae, indicating that development of the cell pattern was normal in the mutants and that the association of ankyrin with the plasma membrane was independent of β spectrin. However, whereas the ring-shaped profiles were consistently small in the posterior region of the wild-type middle midgut (Fig. 4 A), the rings from β spectrin mutants were large and irregular. In some cases, the diameter of the pore remained relatively small, whereas the zone of ankyrin staining was broadened into a wide collar (Fig. 4 B). In other cases, the thickness of the ring of ankyrin staining remained narrow, but the pore size was expanded (Fig. 4 C) as in most anterior copper cells of the wild-type middle midgut (Dubreuil et al., 1998). Thus, the size and shape of the apicolateral contact between copper cells and interstitial cells was dependent on β spectrin function.

The effects of β spectrin mutations on α and β_H spectrin assembly were also examined by immunofluorescence. The α subunit of spectrin was associated with both the apical and basolateral domains of the wild-type copper cell plasma membrane (Fig. 5 A) (Lee et al., 1993). The β_H subunit, in contrast, was exclusively associated with the apical invagination (Fig. 5 B) where it colocalized with 30% of pixels in the α spectrin channel. The spacing and organization of cells in the wild-type midgut was remarkably consistent. In contrast, α and β_H spectrin staining patterns in the mutants were highly irregular and it was difficult to find fields of cells that were all in the same plane of focus. There was a conspicuous shift in the α spectrin staining pattern of *β-spec*^{em6} copper cells (Fig. 5 D). Much of the α spectrin signal in the mutants colocalized with β_H spectrin in the apical invagination with 78% pixel overlap of β_H spectrin (Fig. 5 E) with α spectrin. There was also conspicuous cytoplasmic staining of α spectrin in the mutants that was not typically observed in wild-type. Some residual α spectrin staining of the basolateral membrane observed in *β-spec*^{em6} may be due to perduring maternal β spectrin. In some cells (Fig. 5 E, arrowhead), there appeared to be mislocalization of β_H spectrin to the cytoplasm and to the basolateral plasma membrane. However, the most consistent feature observed with both antibodies was staining of the apical invagination. Identical results were obtained with *β-spec*^{em21} mutants (data not shown). Thus, β spectrin appeared to be required for efficient basolateral targeting of the α subunit, but not for the apical assembly of $\alpha\beta_H$ spectrin.

The effect of β spectrin mutations on plasma membrane polarity was monitored by staining for the Na,K ATPase, which is normally concentrated in the basolateral membrane domain of copper cells (Lee et al., 1993). In wild-type larvae, the en face Na,K ATPase pattern appeared as rings representing the copper cell basolateral domain (Fig. 6 A). Optical sections through the central region of the gut revealed that basolateral staining of copper cells extended

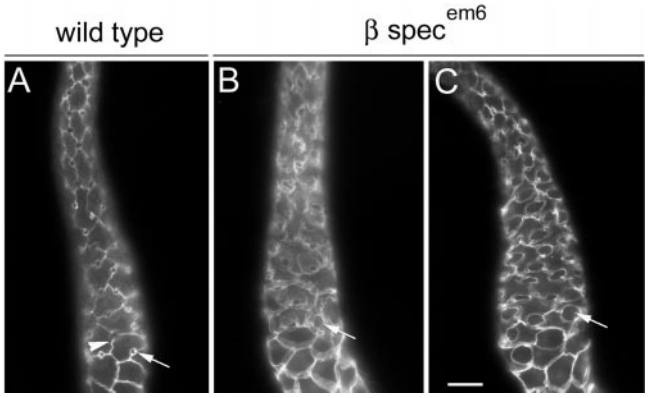

Figure 4. Localization of ankyrin in β spectrin mutants. Ankyrin staining (as in Fig. 3) was used to generate en face views of cell pattern in the posterior region of dissected wild-type (A) and *β-spec*^{em6} mutant (B and C) middle midguts. Circular profiles (arrows) represent apicolateral contacts between copper cells and interstitial cells, which are consistently small in wild-type but large and irregular in mutants. Lines connecting circles (arrowhead) represent staining of apicolateral contact sites between neighboring interstitial cells. Bar, 10 μM.

up to the point of apicolateral contact with interstitial cells (Fig. 6 B). A fine reticular pattern of cytoplasmic staining was also observed, but most of the signal was associated with the plasma membrane. A striking change in the distribution of Na,K ATPase staining was observed in *β-spec*^{em6} mutants (Fig. 6, C and D). Identical results were observed in *β-spec*^{em21} mutants (data not shown). The nature of the change was dependent on the region of the gut examined. The most anterior copper cells (arrowheads) exhibited occasional plasma membrane staining, although in most cells the Na,K ATPase was associated with intracellular compartments. In the most posterior cells, Na,K ATPase staining was typically punctate and irregular (arrows). The large puncta of staining were often closely apposed to the nucleus, indicating that the Na,K ATPase was intracellular rather than clumped at the plasma membrane. Copper cells in between these two regions exhibited very weak staining that was not obviously associated with the plasma membrane. Thus, it appears that there are different fates of the Na,K ATPase within copper cell subpopulations in the β spectrin mutants. However, in all cases, the normal accumulation of Na,K ATPase at the plasma membrane was severely perturbed by the loss of β spectrin function.

Role of β Spectrin in Epithelial Function

The physiological role of copper cells is to secrete stomach acid (Dubreuil et al., 1998). Acid secretion is easily monitored by feeding larvae with yeast paste containing bromphenol blue. The dye changes from a brilliant blue color (pH $>$ 4) to a bright yellow color (pH $<$ 2.35) in the copper cell region of wild-type larvae. In between these pH ranges, the dye exhibits a variable green color. α Spectrin mutants were previously found to lack detectable midgut acidification, presumably because of defects within the apical or basolateral domain, or both, of copper cells (Dubreuil et al., 1998). Results of bromphenol blue feeding experiments with β spectrin mutants and their wild-type siblings are summarized in Fig. 7. As expected, most *yw* control larvae and larvae carrying the *β-spec*$^{+}$*-FM7*[*Kr*-GFP] balancer chromosome exhibited strong (pH $<$ 2.3) midgut acidification. A significant fraction of the *β-spec*^{em6} and *β-spec*^{em15} mutant larvae also exhibited acidification below pH 2.3. Interestingly, the *β-spec*^{em12} and *β-spec*^{em21} mutants, which express large truncated fragments of β spectrin, were less efficient in acid secretion than the mutants that altogether lack detectable β spectrin. Nevertheless, the effect on midgut acidification in β spectrin mutants was small compared with the previously described null α spectrin mutants (Dubreuil et al., 1998). Based on these results we conclude that the β spectrin mutations had little effect on plasma membrane integrity or the ac-

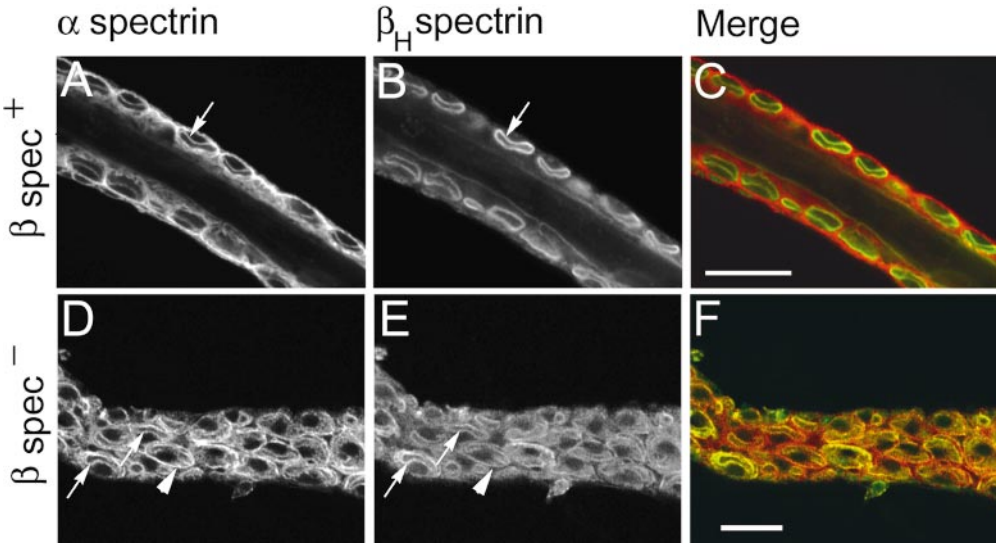

Figure 5. Localization of α and β$_H$ spectrin in β spectrin mutants by confocal microscopy. Dissected preparations of middle midgut were double-labeled with mouse monoclonal anti–α spectrin (A and D) and rabbit anti–β$_H$ spectrin (B and E) and fluorescent secondary antibodies as in Fig. 3. The two subunits colocalized in the apical membrane domain of copper cells in wild-type (A–C, arrows) and in *β-spec*^{em6} copper cells (D and E, arrows). Although α spectrin was most prominently visible at the basolateral surface of wild-type copper cells (A), staining of the apical surface predominated in the β spectrin mutants (D). In some cases, α and β$_H$ spectrin colocalized at the basolateral surface of mutant copper cells (D and E, arrowheads). Bar, 20 μM.

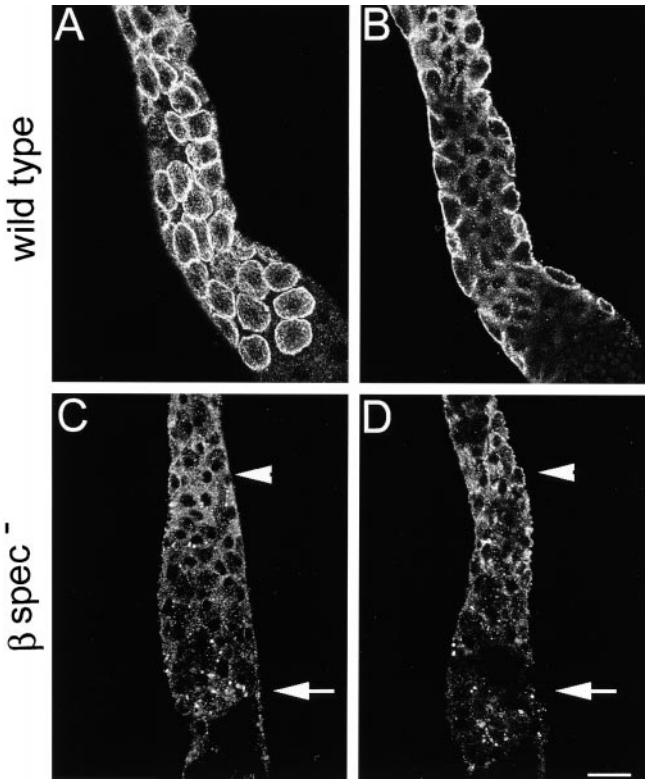

Figure 6. Localization of the Na,K ATPase in β spectrin mutants by confocal microscopy. Dissected preparations of the middle midgut from wild-type control larvae (A and B) and *β-spec*^{em6} mutants (C and D) were stained with mouse monoclonal anti–Na,K ATPase α subunit and fluorescent secondary antibody. The basolateral staining pattern of the Na,K ATPase in wild-type appeared as rings in en face views (A), or as horseshoe shapes in optical sections near the center of the midgut (B). Neither pattern was detectable in the *β-spec*^{em6} mutants (C and D). Toward the anterior (C and D, arrowhead), Na,K ATPase staining was distributed throughout the cytoplasm. Toward the posterior (C and D, arrow), staining appeared as large puncta and did not appear to be associated with the plasma membrane. In between these two regions, staining was relatively weak or in some cases absent. Bar, 10 μM.

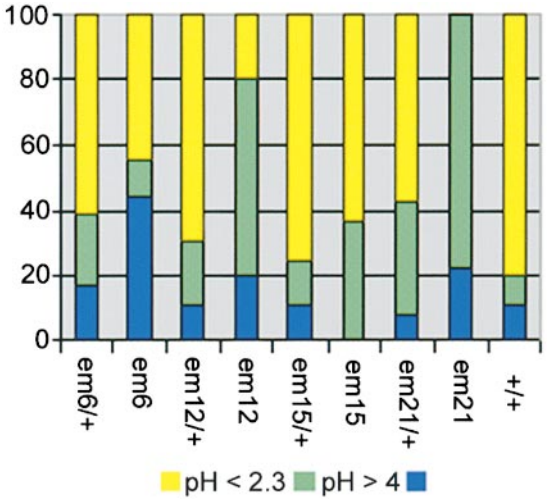

Figure 7. Stomach acidification was analyzed by feeding β spectrin mutant larvae and their wild-type siblings with bromphenol blue–dyed yeast paste. Acidification was scored by examining the dye color in dissected midguts. Results are presented as the percentage of larvae with strong (yellow), moderate (green), or no detectable midgut acidification (blue).

tivity of copper cells, despite their effects on cell morphology and Na,K ATPase localization.

Discussion

The results of this study provide a number of novel insights into αβ spectrin assembly and function. (a) The most striking new observation was the discovery that the basolateral distribution of the Na,K ATPase in larval copper cells was dependent upon β spectrin function, but not on α spectrin. (b) β Spectrin is essential: mutations in the β spectrin gene were lethal early in development. (c) β Spectrin is downstream of ankyrin in the membrane skeleton assembly pathway, since ankyrin remained associated with the plasma membrane in β spectrin mutants. (d) Spectrin isoforms assemble independently of one another. The $\alpha\beta_H$ isoform of spectrin remained associated with the apical membrane domain of epithelial cells in β spectrin mutants. (e) β Spectrin mutations produced a relatively modest effect on the acid secretion activity of midgut copper cells, in contrast to the severe defect observed in α spectrin mutants. Thus, the latter phenotype appears largely attributable to loss of $\alpha\beta_H$ spectrin function from the apical domain of copper cells.

Essential Function of β Spectrin

β Spectrin mutants complete much of embryonic development before they ultimately die as fully formed larvae. The broad expression pattern of αβ spectrin in the developing embryo (Pesacreta et al., 1989) makes it likely that many different tissues are affected by the mutations, any of which could be responsible for lethality. The β spectrin lethal phenotype is remarkably similar to mutations in genes that affect nervous system function, such as synaptotagmin (DiAntonio et al., 1993). Indeed, spectrin is a major structural protein in neurons, comprising ~2.4% of total protein in mammalian brain homogenates (Davis and Bennett, 1983). Future studies of the *Drosophila* nervous system in α and β spectrin mutants are therefore likely to provide valuable insights into the function of spectrin in the brain.

The lethality of the β spectrin mutations reflects an essential requirement for αβ spectrin function. Mutations in the α subunit affect both spectrin isoforms and they result in death early in larval development (Lee et al., 1993). In contrast, mutations in β_H spectrin produce a more subtle phenotype (Thomas et al., 1998). Occasionally mutants altogether lacking β_H spectrin survive to adulthood and reproduce, although many die as larvae. The early larval-lethal phenotypes of α and β spectrin mutations suggest a stringent requirement for αβ spectrin function early in development, with a less stringent requirement for $\alpha\beta_H$ spectrin at later stages.

The β spectrin mutants die shortly before larval hatching, whereas α spectrin mutants generally die after hatch-

ing. Survival through embryonic development in both cases is thought to depend on maternally derived protein. There are two possible explanations for the observed differences in lethal phase. There may be functions of β spectrin that are independent of α spectrin, including a role in larval hatching. The differential effects of α and β spectrin mutations on Na,K ATPase polarity (discussed below) support this interpretation. Or there may simply be a larger pool of maternally contributed α spectrin that outlasts the β spectrin pool, allowing α spectrin mutants to survive longer. Further experiments will be necessary to distinguish between these possibilities.

Spectrin in Copper Cell Differentiation and Function

Although mutations in the α and β subunits of spectrin were both lethal, they produced remarkably different phenotypes in the larval midgut epithelium. It was previously shown that α spectrin mutants had severe defects in the apical morphology and acid secretion activity of copper cells, but there was no apparent effect on basolateral accumulation of the Na,K ATPase (Lee et al., 1993, 1997; Dubreuil et al., 1998). Thus, it appears that the α subunit is critical to the function of $\alpha\beta_H$ spectrin in the apical domain of copper cells. In contrast, the finding that Na,K ATPase accumulation was perturbed in β spectrin mutants, but not in α spectrin mutants, suggests that β spectrin functions independently of α spectrin in the basolateral region of copper cells. β Spectrin mutations also affected morphogenesis of the apicolateral domain of copper cells. The severity of the apical defect in α spectrin mutants precludes analysis of whether or not this structural role of β spectrin is also independent of α spectrin.

Copper cells differentiate in response to extracellular gradients of *wingless* and *decapentaplegic* during embryonic development, which in turn activate expression of the homeotic gene *labial* within copper cell progenitors (Hoppler and Bienz, 1994, 1995). The opening of the apical invagination of copper cells to the gut lumen is constricted into a narrow pore in the most posterior cells, which are closest to the source of the *wingless* signal. The opening gradually broadens toward the anterior, as cells receive a weaker *wingless* signal and express less *labial* protein (Hoppler and Bienz, 1994, 1995; Dubreuil et al., 1998). Ankyrin and β spectrin are both highly enriched in the apicolateral collar that surrounds the pore where they probably associate with components of the smooth septate junction. We speculate that spectrin and ankyrin couple these components to a cytoplasmic contractile mechanism that determines the diameter of the apical pore as well as the size and shape of the surrounding septate junction. Interestingly, the phenotype of a weak *labial* mutant allele in adult *Drosophila* copper cells includes a defect in apicolateral domain pattern that is remarkably similar to the *β-spec*$^-$ phenotype described here (Dubreuil, R.R., T. Grushko, O. Baumann, manuscript submitted for publication).

Spectrin as a Determinant of Plasma Membrane Polarity

The quantity and distribution of the Na,K ATPase at the basolateral membrane of copper cells was significantly altered in β spectrin mutants. Based on the known protein interactions of the spectrin membrane skeleton in vertebrates and its conserved properties in *Drosophila*, this effect is likely to involve disruption of a ternary complex between the Na,K ATPase, ankyrin, and spectrin. This population of spectrin and ankyrin appears to be distinct from the apicolateral population described above, since the Na,K ATPase was excluded from the apicolateral region of copper cells (Fig. 6 B).

The proposed role of spectrin in the development of plasma membrane polarity is based on its capacity to transduce subcellular positional information. The term positional information is traditionally used to describe the cues that govern tissue patterning in developing organisms. Virtually the same problem of establishing and interpreting spatial coordinates is faced during the differentiation of polarized cells (Drubin and Nelson, 1996). The αβ isoform of spectrin assembles in response to the positional cue of cell adhesion during the differentiation of many polarized epithelial cells. Once targeted to a discrete plasma membrane domain, the spectrin membrane skeleton is thought to capture and stabilize additional interacting membrane proteins, thereby altering the composition and function of that domain (Drubin and Nelson, 1996; Dubreuil, 1996).

The model requires that spectrin simultaneously receive and transmit subcellular positional information. That requirement is potentially met by the spectrin tetramer, which includes two ankyrin binding sites (Fig. 8 a). One ankyrin molecule may interact with a source of positional information such as a cell adhesion molecule, whereas a second ankyrin molecule transmits positional information to the Na,K ATPase, resulting in its stable accumulation within the basolateral domain. The positional cue responsible for ankyrin and αβ spectrin assembly at the basolateral domain of copper cells is not known. By analogy to other systems, it is likely to be a cell adhesion molecule.

Biochemical studies have shown that ankyrin has the capacity to simultaneously interact with multiple integral membrane proteins such as the cell adhesion molecule neurofascin and the anion exchanger (Michaely and Bennett, 1995). From this result, one might predict that ankyrin functions as a transmitter of positional information independently of spectrin (Fig. 8 b). However, the current results demonstrate that the Na,K ATPase also relies on β spectrin to acquire its polarized distribution. One possible explanation of this requirement is that the membrane binding activity of ankyrin is dependent on its physical association with β spectrin, perhaps through an allosteric mechanism. It has also been suggested that ankyrin-independent membrane binding sites convey positional information to β spectrin, which then transmits the information to ankyrin and the Na,K ATPase (Devarajan and Morrow, 1996). This latter alternative seems unlikely, since the current results demonstrate that ankyrin can acquire positional information in the absence of β spectrin. Another possible explanation is that β spectrin may be required to cross-link ankyrin molecules that independently associate with the source of positional information and the Na,K ATPase (Fig. 8 c). For example, spectrin–ankyrin complexes could be linked together through the actin binding activity of β spectrin. Similar models have been

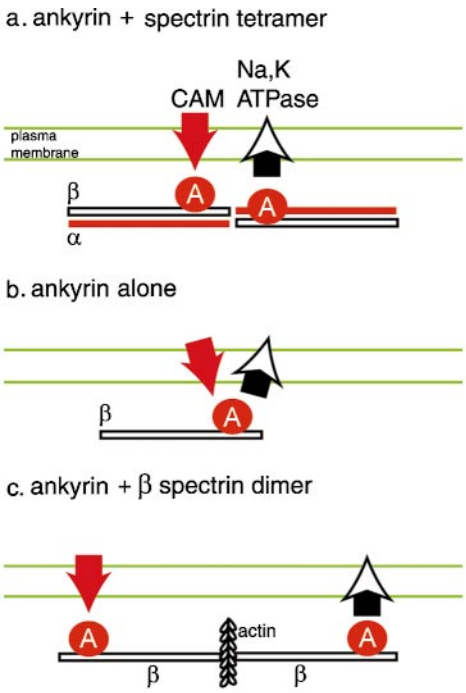

Figure 8. Schematic models for the flow of positional information through the spectrin membrane skeleton. (a) The spectrin tetramer includes two ankyrin binding sites that provide potential interaction sites for a source of positional information (e.g., a cell adhesion molecule [CAM]) and for other membrane markers such as the Na,K ATPase. (b) Biochemical studies indicate that mammalian ankyrin can simultaneously interact with two different integral membrane proteins. The results shown here indicate that ankyrin alone, in the absence of β spectrin, is not sufficient to direct the normal basolateral accumulation of Na,K ATPase. β spectrin may indirectly affect the activity of ankyrin, perhaps through an allosteric mechanism. (c) Alternatively, β spectrin dimers may couple two ankyrin molecules that independently associate with a source and a target of positional information. One possible mechanism of β spectrin dimer formation is through its NH_2-terminal actin binding activity.

proposed to explain the autonomous biochemical properties of β spectrin in vertebrate muscle (Bloch and Morrow, 1989; Porter et al., 1997). Further genetic studies aimed at identifying the minimal functional unit of β spectrin that can support polarized accumulation of the Na,K ATPase will provide a powerful experimental approach with which to distinguish between these possibilities.

The exact fate of the Na,K ATPase in the β spectrin mutants is not yet known. Indeed, there appear to be multiple fates that correlate with the morphology, and perhaps the physiology, of the affected cell. The anterior-most copper cells often exhibited some detectable plasma membrane staining of the Na,K ATPase, whereas the more posterior copper cells exhibited either complete loss of staining or dense aggregates. There may be differences between copper cell populations in the timing of their differentiation during development, or perhaps different rates of degeneration in the absence of β spectrin. Interestingly, overexpression of the ankyrin binding domain of β spectrin in mammalian Caco-2 cells also caused the disappearance of Na,K ATPase staining at the plasma membrane along with the appearance of diffuse cytoplasmic staining and cytoplasmic aggregates (Hu et al., 1995). Recent studies of an ankyrin knockout mouse demonstrated that two other ankyrin-associated membrane proteins, the voltage-dependent sodium channel and neurofascin, rely on the spectrin membrane skeleton for their normal accumulation at the axon initial segment of Purkinje neurons (Zhou et al., 1998). These observations are consistent with a role for the spectrin membrane skeleton in stabilizing membrane activities and/or preventing their endocytosis after delivery to the plasma membrane. However, based on the demonstration that β spectrin interacts with the Na,K ATPase within the secretory pathway (Devarajan et al., 1997), it will be important in future studies to address the possibility that the β spectrin mutants described here affect the accumulation of the Na,K ATPase before its arrival at the cell surface.

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