

Drosophila Development Requires Spectrin Network Formation

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Abstract. The head-end associations of spectrin give rise to tetramers and make it possible for the molecule to form networks. We analyzed the head-end associations of *Drosophila* spectrin in vitro and in vivo. Immunoprecipitation assays using protein fragments synthesized in vitro from recombinant DNA showed that interchain binding at the head end was mediated by segment 0-1 of α -spectrin and segment 18 of β -spectrin. Point mutations equivalent to erythroid spectrin mutations that are responsible for human hemolytic anemias diminished *Drosophila* spectrin head-end interchain binding in vitro. To test the in vivo consequence of deficient head-end interchain binding, we introduced constructs expressing head-end interchain binding mutant α -spectrin into the *Dro-*

sophila genome and tested for rescue of an α -spectrin null mutation. An α -spectrin minigene lacking the codons for head-end interchain binding failed to rescue the lethality of the null mutant, whereas a minigene with a point mutation in these codons overcame the lethality of the null mutant in a temperature-dependent manner. The rescued flies were viable and fertile at 25°C, but they became sterile because of defects in oogenesis when shifted to 29°C. At 29°C, egg chamber tissue disruption and cell shape changes were evident, even though the mutant spectrin remained stably associated with cell membranes. Our results show that spectrin's capacity to form a network is a crucial aspect of its function in nonerythroid cells.

SPECTRIN is an essential and widely distributed protein in eukaryotes (Hayes and Baines, 1992; Winkelmann and Forget, 1993). Although spectrin has been extensively studied in erythrocytes (Marchesi et al., 1970; Bennett, 1985), there have been few attempts to relate its biochemistry to its function in nonerythroid systems. The timing and localization of spectrin's expression suggest that this molecule may, in some cells, serve static organizational demands (Nelson and Lazarides, 1984; Koenig and Repasky, 1985; Lee et al., 1993), and, in other cells, be involved in dynamic events (Perrin and Aunis, 1985; Schatten et al., 1986; Lee et al., 1988; Sobel et al., 1988; Pasacreta et al., 1989). This functional diversity probably reflects both the heterogeneity in spectrin's primary structure (Moon and McMahon, 1990) and the different balance of components that can regulate and interact with spectrin in different cells (Bennett and Gilligan, 1993). Thus, paradigms derived from studies of the erythrocyte membrane skeleton are likely to suggest only some of spectrin's biochemical features and only some of the ways these features actually function in a nucleated cell that contains transcellular cytoskeletal elements absent in an erythrocyte.

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In erythrocytes, spectrin forms a network that supports the plasma membrane and contributes to cell shape (Elgsaeter et al., 1986; Steck, 1989). The formation and integrity of this network depend on inter- and intramolecular associations at two key points in the spectrin molecule: at spectrin's "tail" end, between spectrin and short actin oligomers and, at its "head" end, between spectrin and itself (Byers and Branton, 1985; Marchesi, 1985; Bennett, 1989). In erythrocytes, the head-end association of two spectrin heterodimers gives rise to tetramers (Shotton et al., 1979; Ungewickell and Gratzner, 1978; Morris and Ralston, 1989), and defects in tetramer formation that prevent normal spectrin network formation are often associated with hemolytic anemias (Liu et al., 1981, 1982; Palek and Lambert, 1990). But in nonerythroid cell types, it is not clear whether spectrin functions include network formation. Here, we characterize the head-end association of *Drosophila* spectrin in vitro, and we show that this association, which is crucial for network formation, is required for normal *Drosophila* somatic cell development and oogenesis.

Materials and Methods

Plasmid Constructs

N8 (Dubreuil et al., 1989) is an in vitro expression plasmid construct derived from vector pNB40 (Brown and Kafatos, 1988). It contains the full-length α -spectrin cDNA driven by the Sp6 polymerase promoter. We used published methods (Dubreuil et al., 1989) to produce a similar expression

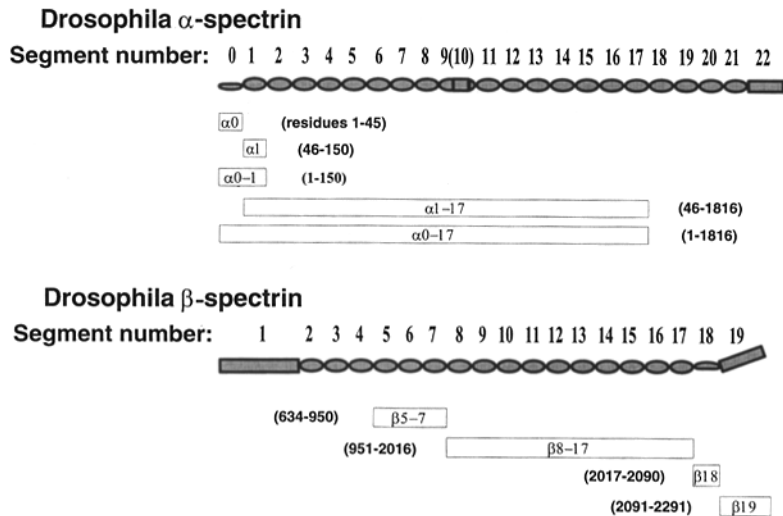


Figure 1. Spectrin fragment nomenclature. Every segment of spectrin, including the nonrepetitive segments, is numbered above the cartoons (gray) of α - and β -spectrin. Below the cartoons, outlines of the corresponding fragments are labeled to show nomenclature. Each fragment's horizontal position under the cartoon indicates the region of the intact subunit from which it is derived. Numbers in parentheses indicate the residue numbers contained in each fragment.

construct, named N β , that contained a full-length β -spectrin cDNA (including the 5' and 3' untranslated sequences) from clone KBA (Byers et al., 1989) in place of the α -spectrin cDNA sequence of N8.

To provide a general cassette for cloning truncated versions of α - or β -spectrin cDNA, N8 and N β were modified by introducing a KpnI site immediately after the ATG start codon and a SpeI site (ACTAGC), which also serves as the stop codon, at the 3' end of the cDNA sequence (neither the KpnI nor the SpeI site occurs in N8 or N β). The resulting constructs NA and NB were used to subclone truncated α - or β -spectrin cDNA sequences using standard methods (Sambrook et al., 1989). After verifying their sequences, the proteins encoded by these sequences were synthesized in vitro under the control of the Sp6 promoter using the TNT Sp6-coupled reticulocyte lysate system (catalogue No. L4600; Promega Corp., Madison, WI).

Coimmunoprecipitation Assay

In a typical assay, [¹⁴C]leucine labeled fragments of one spectrin subunit synthesized in the TNT lysate were incubated for 2 h at the required temperature with fragments of the other subunit similarly synthesized, but without label. All subsequent additions, incubations, and immunoprecipitations were performed at the same temperature. Polyclonal antibodies against the unlabeled subunit (anti-*Drosophila* α -spectrin antibody No. 354, initially called 905 in Byers et al., 1987, or anti-*Drosophila* β -spectrin antibody No. 337, initially called A089 in Byers et al., 1989) were added to the mixture together with immunoprecipitation buffer (1% Nonidet P-40, 0.25% deoxycholate in PBS, pH 7.3). Incubation continued for 1 h, after which dead *Staphylococcus aureus* cells (catalogue No. 507858; Calbiochem-Novabiochem Corp., La Jolla, CA) were added. The immunoprecipitated proteins were separated from the supernatant by sedimentation at 16,000 g, washed three times with immunoprecipitation buffer, and electrophoresed on Tricine/SDS/10% polyacrylamide gels (Schagger and Jagow, 1987). Radiolabeled proteins were identified by autoradiography. Preliminary experiments, and controls within our experiments (see below), showed that the anti- β antibody precipitated no α -spectrin, and that the anti- α antibody precipitated primarily α -spectrin and only trace amounts of β -spectrin.

Expression of Recombinant Protein in *Escherichia coli*

cDNA sequences of interest were removed from the NA- or NB-derived constructs by digestion with KpnI and SpeI, and subcloned into a modified pGEX-2T expression vector (kindly provided by A. Viel, Branton Laboratory, Harvard University, Cambridge, MA) containing KpnI and SpeI sites downstream of the thrombin cleavage site. Using these pGEX-2T-derived constructs, spectrin protein fragments were produced and purified essentially as described by Winograd et al. (1991). The purified fragments were dialyzed against 100 mM NaCl, 8 mM NaPO₄, pH 7.5, before use.

Chymotrypsin Protection Assays

α -Spectrin and β -spectrin fragments produced in *E. coli* were mixed and incubated together overnight on ice at the stated concentrations and then left at the stated temperature for 1 h. In some cases (explicitly indicated),

fragments were mixed and incubated for 3 h at the stated temperature before the addition of chymotrypsin. α -Chymotrypsin was added at a total substrate/enzyme mass ratio of 100:1. Incubation proceeded at the stated temperature. Starting immediately after addition of chymotrypsin, timed samples were taken, boiled for 5 min in electrophoresis sample buffer, and electrophoresed in Tricine/SDS/10% polyacrylamide gels (Schagger and Jagow, 1987).

Transformation Rescue of the α -Spectrin Null Mutant

Previous work from our laboratories showed that $p[w^+UMS]$, an α -spectrin mini-gene under ubiquitin promoter control, rescued all of the otherwise lethal spectrin mutants in the $l(3)dre3$ complementation group (Lee et al., 1993). For the experiments described here, we again used a germ-line transformation method (Spradling and Rubin, 1982; Lee et al., 1993) and generated transformants bearing a mini-gene that encoded α -spectrin with a portion of the human *c-myc* protein inserted immediately downstream of the ATG initiator codon. The epitope tag was recognized by the 9E10.2 monoclonal antibody (Evan et al., 1985). These ($p[w^+UM-3]$) transformed lines rescued $l(3)dre3^{rs41}$ mutants as efficiently as did lines bearing $p[w^+UMS]$ (not shown). For some experiments, we replaced the wild-type α -spectrin cDNA in $p[w^+UM-3]$ with α -spectrin cDNAs bearing either an arginine to serine mutation at the codon for residue 22 ($\alpha(R22S)$) or a deletion of the first 45 residues ($\alpha(dl-45)$) to create transformant lines bearing $p[w^+UM-3,R22S]$ or $p[w^+UM-3,dl-45]$. The ability of these transformant lines to rescue the α -spectrin null mutant, $l(3)dre3^{rs41}$, was assayed by crossing $w/Y;p[w^+UM-3,R22S]/+;TM6B/Df(3L)R-R2$ to $w/w;+/+;TM6B/l(3)dre3^{rs41}$. The resulting rescued progeny, bearing $p[w^+UM-3,R22S]$ as the only source of α -spectrin, were then crossed inter se at 25°C to generate stocks. In our initial experiments, flies bearing either one copy or two copies of $p[w^+UM-3,R22S]$ (we could not distinguish these two genotypes) were taken from these stocks and shifted to the desired temperature for phenotypic analyses. In later experiments, phenotypes were analyzed in rescued flies generated de novo by crossing $w/Y;p[w^+UM-3,R22S]/+;l(3)dre3^{rs41}/TM6B$ to $w/w;+/+;TM6B/Df(3L)R-R2$. Thus, in all of these later experiments, the rescued flies contained only one copy of $p[w^+UM-3,R22S]$. Phenotypes that were analyzed in early and late experiments were equivalent.

Immunoblot Analysis

Single-fly preparations were solubilized in standard Laemmli sample buffer, electrophoresed on 8% SDS-PAGE gels (Laemmli, 1970), and the proteins were blotted onto nitrocellulose for immunostaining (Burnette, 1981; Dubreuil et al., 1987) in a 1:1,000 dilution of anti- α antibody or in undiluted anti-*c-myc* 9E10.2 culture supernatant (Evan et al., 1985) followed by alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody (Zymed Laboratories, South San Francisco, CA).

Immunocytochemistry

Fly ovaries were prepared for staining according to Grossniklaus et al. (1989). Tissues were stained for α -spectrin as described (Lee et al., 1993).

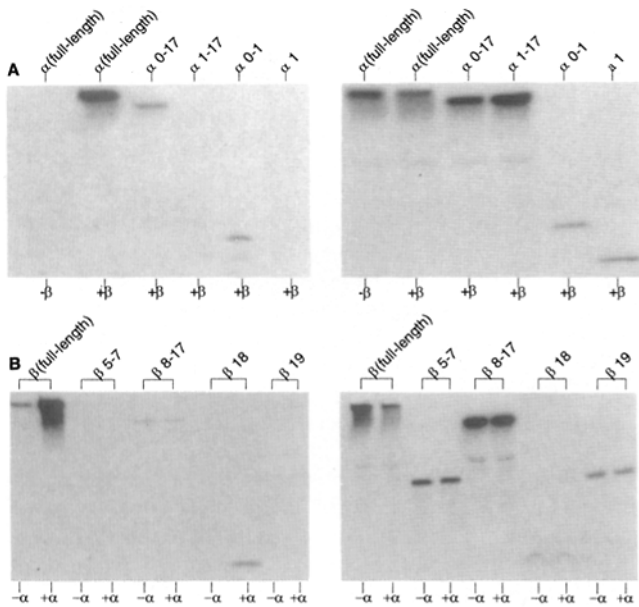


Figure 2. (A) Segment $\alpha 0$ is required to bind the head end of α -spectrin to β -spectrin. ^{14}C -labeled α -spectrin fragments were incubated at 0°C in the presence (+ β) or the absence (- β) of unlabeled full-length β -spectrin, and immunoprecipitated with anti- β -spectrin antibody. Half of the pellet fraction (left panel) and 1/10 of the supernatant fraction (right panel) were loaded onto a 10% tricine gel for autoradiography as described in Materials and Methods. (B) Same as in A, except that ^{14}C -labeled β -spectrin fragments, either in the presence or absence of unlabeled full-length α -spectrin, were immunoprecipitated with anti- α -spectrin antibody.

For propidium iodine staining, tissues were pretreated with 2 mg/ml RNase A (Boehringer Mannheim Biochemicals, Indianapolis, IN) in $2\times$ SSC for 30 min at 37°C , washed in PBS, and subsequently stained with propidium iodine (1 mg/ml) in PBS for 30 min. Tissues were washed again in PBS and mounted in Mowiol (Polyscience, Niles, IL) before viewing. All images were collected on a confocal microscope (MRC-600; Bio Rad Laboratories, Hercules, CA).

Results

Spectrin Fragments

The spectrin fragments we used and their nomenclature (Fig. 1) were based on the phasing of conformational units (Winograd et al., 1991) and the subsequent crystallographic studies (Yan et al., 1993) that show how three α -helices in each conformational unit fold to produce the quasirepetitive segments found in both α - and β -spectrin.

$\alpha 0$ -1 and $\beta 18$ Are Functional, High Affinity, Head-end Interchain Binding Domains

Coimmunoprecipitation experiments with full-length subunits (Fig. 2 A, lanes 1 and 2) showed that the α subunit was sedimented by anti- β antibody only in the presence of unlabeled, full-length β . Since this antibody did not immunoprecipitate full-length α -spectrin in the absence of β -spectrin, we concluded that the presence of α -spectrin in the pellet resulted from the association of α -spectrin with β -spectrin. We then looked for fragments of α -spectrin that bind to full-

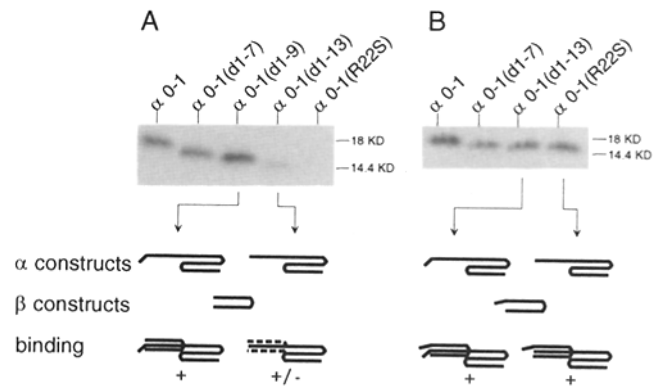


Figure 3. Residues upstream of the potential amino terminal C helix in $\alpha 0$ or upstream of the A helix in $\beta 18$ can promote head-end interchain binding. (A) $\beta 18$ -19 was incubated with labeled peptides corresponding to either wild-type $\alpha 0$ -1, $\alpha 0$ -1 with different NH₂-terminal deletions, or $\alpha 0$ -1 with point mutation R22S, followed by immunoprecipitation with anti- β -spectrin antibody and autoradiography, as in Fig. 2. Note the small amount of $\alpha 0$ -1(d1-13) immunoprecipitated with $\beta 18$ -19. (B) A fragment consisting of the last five residues of $\beta 17$ (ENLQL) followed by $\beta 18$ -19 was incubated with labeled α -spectrin polypeptides, as in A. Note that with the ENLQL residues upstream of $\beta 18$ -19, $\alpha 0$ -1(d1-13) bound as well as the full $\alpha 0$ -1 construct. Autoradiographs of supernatant fractions (not shown) showed equal α construct loading in all lanes. Below the autoradiographs, cartoons in the top row show how the $\alpha 0$ -1 constructs contribute the equivalent of helix C of a three-helix bundle (see Discussion), whereas those in the second row show how the β constructs (simplified in the cartoon to show only $\beta 18$) contribute the equivalent of helices A and B of a three-helix bundle. Based on the known structure of a spectrin segment (Yan et al., 1993), the bottom row shows how the α and β constructs may associate to form a three-helix bundle.

length β and found that, in the absence of α -spectrin's tail (α segments 18-22), $\alpha 0$ was necessary for interchain association (Fig. 2 A, lanes 3-6). Thus, with $\alpha 0$ deleted, a fragment that included a large portion of α -spectrin ($\alpha 1$ -17) had no interchain binding activity. While $\alpha 0$ -1 was sufficient for the head-end binding, and it is clear that $\alpha 0$ is absolutely required, we have been unable to synthesize a stable, radiolabeled fragment of $\alpha 0$ alone. We were, therefore, unable to demonstrate whether $\alpha 0$ alone is sufficient for binding.

To determine the regions of β -spectrin responsible for head-end binding, we carried out similar experiments with anti- α spectrin antibodies using labeled β -spectrin fragments and unlabeled, full-length α -spectrin (Fig. 2 B). Only $\beta 18$ had significant interchain binding activity; no interchain binding was evident in either $\beta 5$ -7 or $\beta 8$ -17. The trace of $\beta 8$ -17 immunoprecipitated in the presence of α -spectrin never exceeded the amount immunoprecipitated in the absence of α -spectrin. Segment $\beta 19$ is a nonrepetitive domain and exhibited no interchain binding activity (Fig. 2 B, lanes 9 and 10).

Drosophila α -Spectrin Amino Acids 10-150 Have Full Interchain Binding Activity

To further define the amino acid sequence of $\alpha 0$ that is involved in interchain binding, we synthesized $\alpha 0$ -1 with different NH₂-terminal-end truncations: $\alpha 0$ -1(d1-7), $\alpha 0$ -1(d1-

Table I. The Association of α - and β -Spectrin Fragments

Fragments	Incubation temperature		
	0°C	25°C	29°C
α 0-1 and β 18-19	+++	++	+
α 0-1(R22A) and β 18-19	-	-	-
α 0-1(R22S) and β 18-19	-	-	-
α 0-1(L43H) and β 18-19	-	-	-
α 0-1(L43F) and β 18-19	++	-	-
α 0-1 and β 18-19(A2062P)	-	-	-

Effects of point mutation on *Drosophila* head-end interchain binding are shown. In vitro-synthesized wild-type or mutant α 0-1 and β 18-19 fragments were incubated together at the specified temperatures, and their association was assayed by immunoprecipitation, as described in Materials and Methods. The plus or minus signs approximate the relative amounts of labeled α 0-1 immunoprecipitated by anti β -spectrin antibody.

9), and α 0-1(d1-13), where (dn₁-n₂) indicates deletion of amino acids n₁ through n₂. Both α 0-1(d1-7) and α 0-1(d1-9) exhibited binding activity similar to that of α 0-1, but amino acid deletions beyond residue 9, as in α 0-1(d1-13), greatly diminished binding activity (Fig. 3 A). Loss of binding in α 0-1(d1-13) could not be attributed to gross misfolding because, in the absence of β 18, α 0-1(d1-13) had the same pattern of chymotrypsin resistance as did α 0-1 (not shown). Furthermore, whereas α 0-1(d1-13) bound very poorly to β 18-19, it exhibited full binding activity with a fragment that consisted of the last five residues of β 17 followed by β 18-19 (Fig. 3 B) or with full-length β -spectrin (not shown). We concluded that α -spectrin residues 1-9 do not contribute to binding activity, whereas residues 10-14 (KILET) may do so when α 0-1 is assayed against β 18-19 alone. Thus, the presence of either residues 10-14 of α 0-1 (KILET), or the five residues upstream of β 18-19 (ENLQL) appears to be important for head-end binding. These residues may stabilize the conformation or interactions of the presumed α -helical structures that give rise to head-end binding.

Point Mutations Equivalent to Erythroid Spectrin Mutations Responsible for Human Hemolytic Anemias Modify Head-end Interchain Binding in *Drosophila* Spectrin

Previous studies have associated hereditary elliptocytosis and pyropoikilocytosis with impaired erythroid spectrin tetramer formation (Delaunay and Dhermy, 1993). Codon 28 of human α -spectrin cDNA (homologous to Arg22 of *Drosophila* spectrin) was identified as a "hot spot" at which mutations that encoded amino acid substitutions (Arg to Leu, Ser, Cys, or His) were frequently associated with impaired erythroid spectrin tetramer formation (Coetzer et al., 1991). Other changes associated with impaired tetramer formation included α -spectrin Leu49 to Phe (Morle et al., 1990) and β -spectrin Ala2053 to Pro (Tse et al., 1990). To demonstrate that a single amino acid substitution is sufficient to affect the interaction between α 0-1 and β 18, we mutated the sequence of *Drosophila* spectrin cDNA fragments to introduce amino acid substitutions, some of which correspond to those associated with impaired erythroid spectrin tetramer formation (i.e., α -spectrin R22S, L43F, and β -spectrin A2062P). Because the head-end interchain associations of erythroid spectrin are known to be temperature dependent

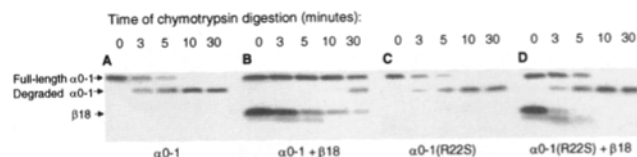


Figure 4. Bacterially expressed α 0-1 or α 0-1(R22S) and β 18 associate with each other. 2.4 μ M of α 0-1 (A and B) or 2.4 μ M of α 0-1(R22S) (C and D) was incubated alone (A and C) or with 15 μ M of β 18 (B and D) for 3 h at room temperature. Chymotrypsin was then added to the mixture, and timed samples were taken directly into SDS sample buffer for electrophoresis. Comparison of equally digested sample, e.g., 5 min lanes, shows that β 18 protected both α 0-1 and α 0-1(R22S).

(Ungewickell and Gratzer, 1978; DeSilva et al., 1992), we performed these assays at 0, 22, and 29°C. All of the amino acid substitutions were introduced in either α 0-1 or β 18 produced *Drosophila* spectrin fragments with significantly reduced binding capacity (Table I).

Chymotrypsin Digestion Assays Refine the Analysis of Associations between β 18 and α 0-1

In the absence of β 18, the α 0 region of α 0-1 was very sensitive to chymotrypsin digestion: within 10 min at room temperature, α 0-1 alone was nearly completely degraded into a fragment whose size corresponded to α 1 (Fig. 4 A). The presence of β 18 provided significant protection: >90% of α 0-1 remained intact after 10 min of chymotrypsin digestion (Fig. 4 B). The chymotrypsin digestion assay also suggested a significant association between α 0-1(R22S) and β 18 (Fig. 4, c and d, compare 5 min lanes), even though binding of α 0-1(R22S) to β 18 was not detected in the stringent immunoprecipitation assays (Table I; Fig. 3, lane 5). This suggestion was substantiated by quantitative evaluation of the chymotrypsin protection assays: in the absence of β 18, the α 0 regions of α 0-1 and α 0-1(R22S) were equally sensitive to digestion, whereas in the presence of β 18, both were protected from digestion in a temperature-sensitive manner (Table II). Similar experiments with similar results have been repeated four times. Since β 18 alone does not inhibit the ac-

Table II. Chymotrypsin Protection of α 0-1 by β 18

	Percent intact			
	19°C		29°C	
β 18 present	α 0-1	α 0-1(R22S)	α 0-1	α 0-1(R22S)
No	12.7	11.3	12.2	12.4
Yes	91.4	61.2	86.1	22.4

Temperature-dependent associations protect against chymotrypsin degradation. Bacterially expressed α 0-1 or α 0-1(R22S) (2.4 μ M) was incubated at 19°C or 29°C with or without 15 μ M β 18, and then subjected to chymotrypsin digestion followed by electrophoresis (see Materials and Methods). The extent to which α 0-1 or α 0-1(R22S) was protected against digestion by the presence of β 18 is stated as "percentage intact," and was determined by densitometry of Coomassie blue-stained gel bands. Percentage intact was calculated as the molar amount of undegraded α 0-1 (or α 0-1[R22S]) divided by the sum of the molar amount of undegraded α 0-1 (or α 0-1[R22S]) plus the molar amount of degraded α 0-1 (or α 0-1[R22S]). The molar stain density is defined as the integrated stain density of a particular polypeptide band divided by the molecular weight of that polypeptide. Digestion at 19°C was for 220 s; digestion at 29°C was for 120 s.

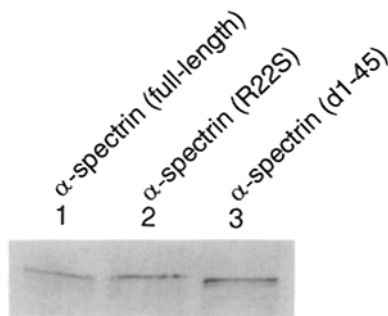


Figure 5. Transformant lines bearing modified α -spectrin express stable levels of protein. Single female flies carrying $p[w^+UM-3]$ (lane 1), $p[w^+UM-3, R22S]$ (lane 2), or $p[w^+UM-3, dl-45]$ (lane 3) in a wild-type background for α -spectrin were electrophoresed, blotted, and stained with anti-*c-myc* antibody (9E10.2 supernatant). As expected, α -spectrin with a 45-amino acid deletion (lane 3) migrated slightly faster than full-length α -spectrin (lanes 1 and 2).

tivity of chymotrypsin and other full segments of spectrin do not substitute for $\beta 18$ (not shown), these chymotrypsin protection results confirm an association between either $\alpha 0-1$ or $\alpha 0-1(R22S)$ and $\beta 18$.

Because the digestion assays contained only chymotrypsin and purified spectrin fragments, they further showed that no accessory components, such as might be found in the coupled reticulocyte lysate system used in the immunoprecipitation assays, were needed for the association between $\beta 18$ and $\alpha 0-1$.

The chymotrypsin protection assays also provided an estimate of the minimum affinity between $\alpha 0-1$ and $\beta 18$. Fig. 4 B shows that, at room temperature, most of the $\alpha 0-1$ (present at $2.4 \mu M$) was associated with the approximately sixfold excess of $\beta 18$ ($15 \mu M$); any $\alpha 0-1$ not associated with $\beta 18$ would have been digested as in Fig. 4 A. Thus, $\alpha 0-1$ associated with $\beta 18$ with an affinity that was equal to or greater than the $2.4\text{-}\mu M$ association constant measured at $25^\circ C$ for the head-end association of human erythroid α - and β -spectrin (DeSilva et al., 1992).

Spectrin Tetramer Formation Is Essential for *Drosophila* Development and Oogenesis

To examine the role of head-end interchain interactions in *Drosophila* development, germ-line transformants expressing the $\alpha(R22S)$ mutation and the $\alpha(dl-45)$ deletion were established. Lysates from representatives of each of the transformant lines bearing $p[w^+UM-3, R22S]$ or $p[w^+UM-3, dl-45]$ showed clear reactivity with anti-*myc* antibody 9E10.2 (Fig. 5) in Western blots, indicating that the flies were expressing a stable *myc*-tagged product from the integrated mini-gene. Flies containing either construct in a wild-type background were normal. Hence, neither mutation had detectable dominant effects. Three independent $\alpha(R22S)$ lines were tested for their ability to rescue the first instar lethality of the α -spectrin null allele, $I(3)dre^{3841}$. At $19^\circ C$ and $25^\circ C$, all $\alpha(R22S)$ lines rescued the embryos to the adult stage, albeit with slightly less efficiency than lines bearing wild-type spectrin (Fig. 6). Although the $\alpha(R22S)$ rescued progeny exhibited normal morphology at $19^\circ C$ and $25^\circ C$, they were

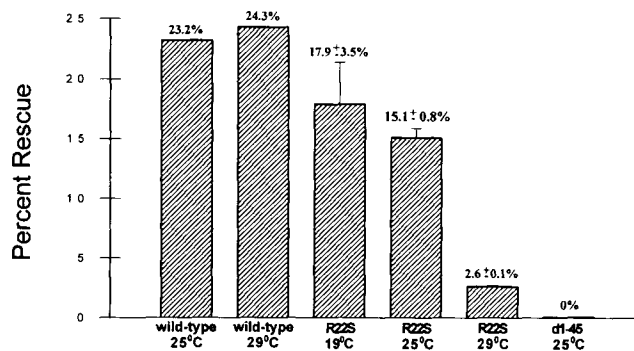


Figure 6. Constructs with alterations in the $\alpha 0$ segment either failed to rescue, or rescued the α -spectrin null mutant in a temperature-dependent manner. Germ-line transformants carrying either the wild-type α -spectrin mini-gene, $p[w^+UM-3]$, or the mutant mini-genes, $p[w^+UM-3, R22S]$ or $p[w^+UM-3, dl-45]$, (as shown at the bottom of the bar graph), were tested for their ability to rescue the first instar lethality of $I(3)dre^{3841}/Df(3L)R-R2$ at 19 , 25 , or $29^\circ C$. Values are expressed as the percentage of rescued progeny relative to the total number of progeny observed. The expected percentage of rescued progeny based on Mendelian proportions is 20%. 200–400 progeny were scored for each experiment.

generally lethargic, exhibited lower fecundity than wild-type flies, and walked about but did not fly. When shifted to $29^\circ C$, they essentially ceased to lay viable eggs. To verify that α -spectrin(R22S) was the only source of α -spectrin in the rescued progeny, blot overlays of lysates from flies rescued by the wild-type- ($p[w^+UM-3]$) or mutant- ($p[w^+UM-3, R22S]$) bearing lines were probed with the $\beta 18$ spectrin fragment. The $p[w^+UM-3]$ -transformed lines, containing full-length, wild-type α -spectrin, bound the $\beta 18$ probe, whereas lysates from the rescued progeny bearing only $p[w^+UM-3, R22S]$ as a source of α -spectrin message expressed a stable form of α -spectrin, but did not bind the probe (not shown). Thus, α -spectrin(R22S) was the only α -spectrin in the rescued progeny.

Because the three independent $\alpha(R22S)$ lines showed similar temperature sensitivity in their ability to rescue development of the α -spectrin null alleles, only one line was used as a representative for subsequent studies. Rescue efficiency by the $\alpha(R22S)$ line was reduced from 18% at $19^\circ C$ to $<3\%$ at $29^\circ C$ (Fig. 6). In contrast, neither of two transformed lines that expressed α -spectrin with a deleted $\alpha 0$ segment was able to rescue the null mutation at 19° , 25° , or $29^\circ C$ (Fig. 6, only the $25^\circ C$ rescue is shown). Together, our results suggest that (a) the head-end interchain binding site was critical for survival and (b) changing codon 22 from arginine to serine imparted temperature sensitivity.

Although the rescued progeny expressing α -spectrin (R22S) displayed normal gross morphology and were fertile at 19° , 21° , and $25^\circ C$, the fertility of the rescued females was temperature sensitive. 5 d after shifting the flies from $21^\circ C$ to $29^\circ C$, the number of eggs laid, and the number of hatching larvae from these point mutant females were greatly diminished relative to similar flies held at $21^\circ C$ or to wild-type flies held at $29^\circ C$ (data not shown). To determine the causes of decreased fecundity, we examined the cellular distribution of α -spectrin in egg chambers from flies that had been reared

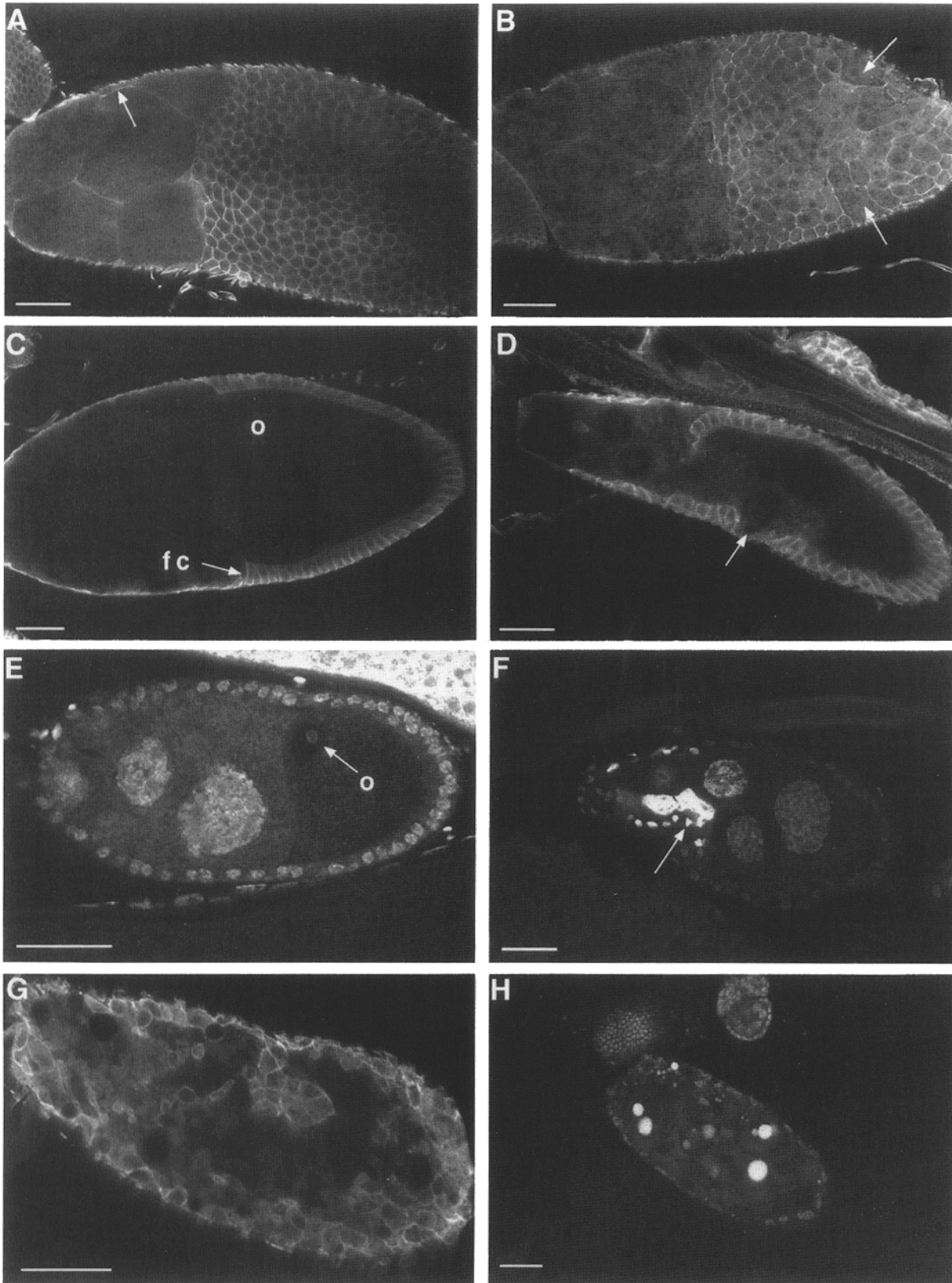


Figure 7. α -Spectrin(R22S) disrupted egg chamber development in a temperature-sensitive manner. Optical sections of egg chambers from point mutant flies bearing only α -spectrin(R22S) grown at permissive (21°C) (A, C, and E) or restrictive (29°C) (B, D, F, G, and H) temperatures. Cells were stained with antispectrin antibody (A–D and G) or with propidium iodide (E, F, and H). At 21°C, spectrin was normally concentrated along the apical membranes of nurse cells (A, arrow), and lateral membranes of follicle cell (C, arrow; fc, follicle cells; o, oocyte). The continuity of the follicle cell monolayer evident in grazing (A) or sagittal (C) sections at 21°C was disrupted in egg chambers from adults held at 29°C for 24 h (B and D, arrows); such chambers also exhibited pyknotic nuclei (F, arrow). Egg chambers from adults held at 29°C for 5 d contained a grossly disrupted follicle cell layer (G) and many necrotic cells (H). Bars, 25 μ m.

at 21°C until eclosion and then held at 29°C, the restrictive temperature, for ≤ 5 d (Fig. 7).

In egg chambers from wild-type females reared at 29°C or point mutant females (bearing α -spectrin(R22S) as the only α -spectrin) reared at 21° or 25°C, the ovarioles showed typical plasma membrane localization of α -spectrin (Pesacreta et al., 1989; Lin et al., 1994) (Fig. 7, A and C) and normal cellular arrangements (Fig. 7 E). Spectrin was also seen in cytoplasmic aggregate structures in the germaria (not shown). The latter staining pattern has been attributed to the adducin-containing fusome (Lin et al., 1994).

In comparison to the normal spectrin distribution and cellular organization seen in wild-type flies at 29°C, or $p[w+UM-3]$ -rescued flies at 29°C, or point mutant flies at 21° or 25°C, egg chambers from point mutant flies that were shifted to 29°C showed progressively altered cellular organization. After 24 h at 29°C, regional losses of spectrin staining and cellular disorganization were evident in the follicle cell layer and immediately adjacent cystocyte cells (Fig. 7, B and D, arrows). Affected regions showed a loss of cell polarity resulting in areas of overlapped follicle cells next to regions devoid of follicle cells (Fig. 7 D, arrow) and follicle cells and underlying nurse cells with pyknotic nuclei (Fig. 7 F, arrow). These condensed nuclei, characteristic of dying or dead cells, were most apparent in egg chambers at stage 8 and older. These rearrangements were accompanied by shape changes of the egg chambers at the sites of monolayer loss.

Point mutant flies held at 29°C for 5 d contained ovarioles that generally displayed a succession of developing egg chambers up to stage 7 of development. Older egg chambers contained the remnants of the follicle cell layer (Fig. 7 G) and were filled with necrotic cells with pyknotic nuclei (Fig. 7 G). Despite these gross structural changes, spectrin staining was still observable at the plasma membrane of the discernible follicle cells (Fig. 7 G), and there appeared to be few structural defects associated with the previtellogenic stages or the cystocyte-fusome arrangements in the germaria (not shown).

α -Spectrin(R22S) Is Stable in an α -Spectrin Null Background

The persistence of α -spectrin(R22S) at the plasma membrane of aberrant egg chambers from α (R22S) flies that had been held at 29°C (Fig. 7 G) suggested that this point mutant protein was stable, even if partially dysfunctional. To verify that α -spectrin(R22S) protein was stable throughout adult flies at 29°C, antibody was used to stain immunoblots of mutant or wild-type flies that had been held at 29°C for 7 d. No consistent differences in spectrin content were observed (not shown).

Discussion

Structural Basis for Head-end Interaction. Recent studies (Speicher et al., 1993; Kotula et al., 1993; Kennedy et al., 1994) have shown that the head-end interchain binding regions of human spectrins are located within an NH₂-terminal 158-amino acid region of the α chain and a COOH-terminal region of the β chain. Our results, using well-defined recombinant polypeptides, show that the *Drosophila* α - and β -spectrin head-end binding regions are perfectly homologous to those of the erythroid α - and β -spectrins, and extend the analysis of interacting sites by investigating truncations and point mutations that further define the essential sequences.

Crystallographic results have shown that the ~ 106 -residue conformational units, or repeating segments, of spectrin fold as three-helix bundles (Yan et al., 1993). Comparing the sequences of $\beta 18$ and $\alpha 0$ to one of these repeating segments shows that each contains residues that are homologous to part of a repeating segment (Fig. 8). Thus, $\beta 18$ has the potential to fold into the equivalent of helices A and B of a three-helix bundle, and $\alpha 0$ has the potential to fold into the equivalent of helix C of a three-helix bundle (Fig. 8). In view of these sequence comparisons and the structural similarities they imply, our data showing that $\beta 18$ and $\alpha 0$ are necessary for head-end interchain binding suggest that hydrophobic and electrostatic interactions that correspond to those that stabilize the three-helix bundle of a repeating segment also underlie the head-end binding between the α and β chains of spectrin (as cartooned in Fig. 3). Others have advanced similar proposals to account for the head-end associations that create tetramers or closed heterodimers in mammalian spectrin (Tse et al., 1990; Speicher et al., 1993; Kotula et al., 1993), and have shown that deleting those residues that form the carboxy-terminal C helix of a three-helix bundle can convert a passive β -spectrin segment into a site that associates with the head end of a α -spectrin (Kennedy et al., 1994). Although we have not been able to duplicate such results by deleting *Drosophila* spectrin helices, our evidence that segment $\beta 18$ is both necessary and sufficient for interchain binding, together with insight into the atomic structure of spectrin's repeating segments (Yan et al., 1993), provides a firm basis for the hypothesis that a three-helix bundle underlies the head-end binding between α - and β -spectrin.

On the other hand, our data show that at least five $\alpha 0$ residues (K I (or V) L E T) that can contribute to interchain binding lie beyond the boundaries of the residues that are homologous to those that form the C helix in other repeating segments (Fig. 8). This raises the possibility that there are subtle but significant differences between the packing of the C helix in a repetitive segment and the packing of the potential C helix of $\alpha 0$ with the presumed A and B helices in $\beta 18$. Indeed, comparison of the NH₂-terminal polypeptide se-

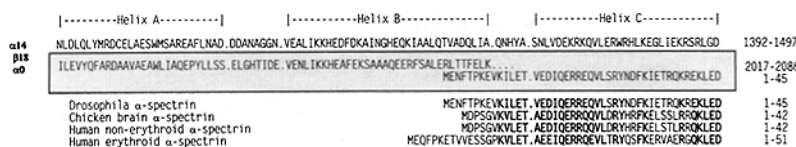


Figure 8. Amino acid sequence alignment of *Drosophila* $\beta 18$ and $\alpha 0$ (shaded box) with *Drosophila* $\alpha 14$ and with $\alpha 0$ from other spectrins (below box). $\alpha 14$ is known to form a three-helix bundle whose helix boundaries are bracketed. Residue numbers are at the right.

quences of four α -spectrins (Fig. 8) shows that, among them, a region of near identity extends upstream of the potential amino terminal C helix to include the five-residue sequence, (K I (or V) L E T), which is not readily accommodated in any alignments with the helices found within the repetitive segments. The homology among these NH₂-terminal polypeptides suggests that the five residues may contribute to interchain association, and it is consistent with our finding, and with that of Kotula et al. (1993), that their removal severely affects head-end binding when assayed against β 18 (Fig. 3, lanes 3 vs 4). It will, therefore, be important to examine the position and interactions of these five residues in a crystal equivalent to β 18 + α 0-1.

Because network formation is a critical aspect of spectrin's function in erythrocytes, and because spectrin's ability to form a network depends on the association between head-end interchain binding sites on α - and β -spectrin, it was not surprising that transformants bearing α -spectrin mini-gene constructs with no head-end binding region ($p[w^+UM-3, dl-45]$) were unable to rescue α -spectrin null mutants (Fig. 6). On the other hand, the identification of α -spectrin(R22S) as an α chain whose association with β -spectrin is strongly temperature dependent (Table II) has allowed us to examine the requirements for a spectrin-based membrane skeleton at stages of development previously inaccessible with the null mutation. Thus, the temperature-sensitive failure of oogenesis caused by α -spectrin(R22S) has related a subset of morphological events to a molecular alteration in spectrin that affects head-end interchain binding.

A critical point during oogenesis appears to occur when yolk formation in the oocyte requires an increase of total egg chamber volume (Mahowald and Kambyzellis, 1980). At this stage, diminished interchain binding at the head-end of the dimer apparently results in the disruption of the follicle cell monolayer, changes in cell shape, and subsequently, cell death. That these disruptions begin in a localized manner where the follicle cells and cystocytes abut suggests that an interaction between the two cell types is disrupted. One explanation for our observations is that an intact spectrin network is required to sustain the convoluted plasma membrane projections that normally interlock follicle cells and cystocytes (Mahowald, 1972). These structures, which are filled with actin filaments, are thought to facilitate the exchange of nutrients for eventual yolk formation in the oocyte. The perturbation of head-end interchain binding of spectrin and consequent membrane disruption may lead to the simultaneous death of interacting somatic and germline cells.

We argue that the specific loss of interchain binding activity at the restrictive temperature is the cause of the phenotypes we have observed. Our data show no generalized α -spectrin instability or dysfunction in flies rescued with the point mutant spectrin construct. Despite evident morphological anomalies in egg chambers, the α -spectrin(R22S) is associated with the plasma membrane, even at 29°C, indicating that the protein is able to bind to the cell membrane. Furthermore, adult females carrying α (R22S) in a null background and held at the restrictive temperature for 7 d (twice the time required for a previtellogenic egg chamber to fully develop) contained comparable levels of total spectrin as did wild-type flies. This observation suggests that, in vivo, α -spectrin(R22S) remains stable at the restrictive tem-

perature and that this point mutant protein is not simply equivalent to a spectrin null mutation.

Further evidence that the R22S mutation is not simply causing complete loss of spectrin function comes from our examination of intestinal cuprophilic cells. In previous work we showed that complete removal of zygotically expressed α -spectrin led to death during the first larval instar (Lee et al., 1993). This death was accompanied by disruption of the intestinal cuprophilic cells' shape and adhesive properties. In contrast, shifting developing embryos containing only α -spectrin(R22S) from permissive to restrictive temperature produced no defects in intestinal cuprophilic cell morphology or adhesion, even after several days at the restrictive temperature. Hence, we believe that α (R22S) is not equivalent to a null mutation.

Our data showing that a deletion of the α -spectrin head-end binding segment, or even a single point mutation in this segment, can disrupt normal development strongly implies that at least one of spectrin's roles in *Drosophila* involves the formation of a membrane-skeleton network. The observation that point mutant flies exhibited oogenesis defects but no noticeable defect in larval cuprophilic cell shape implies that the functional demand on the spectrin-based membrane skeleton varies from one tissue type to another. Furthermore, the observation that α (R22S) exhibits a temperature-dependent change in rescue efficiency makes it evident that other post-oogenic developmental stages may also be susceptible to the loss of spectrin network formations. Whether there are a number of defined periods during development in which spectrin tetramerization and network formation are critical for progression to the next phase is currently being addressed.

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