Spectrin Tetramer Formation Is Not Required for Viable Development in Drosophila

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Background: Underneath the membrane of most animal cells is a network (membrane skeleton) assembled using tetramers of the protein spectrin.

Results: Although spectrins are essential proteins, tetramer formation is surprisingly unimportant for Drosophila development.

Conclusion: The major roles of the membrane skeleton do not require a conventional network.

Significance: The ubiquitous model of the spectrin-based membrane skeleton has limited applicability.

The dominant paradigm for spectrin function is that (αβ)2-spectrin tetramers or higher order oligomers form membrane-associated two-dimensional networks in association with F-actin to reinforce the plasma membrane. Tetramerization is an essential event in such structures. We characterize the tetramerization interaction between α-spectrin and β-spectrins in Drosophila. Wild-type α-spectrin binds to both β- and βH1-chains with high affinity, resembling other non-erythroid spectrins. However, α-specR22S, a tetramerization site mutant homologous to the pathological α-specR22S allele in humans, eliminates detectable binding to β-spectrin and reduces binding to βH1-spectrin ~1000-fold. Even though spectrins are essential proteins, α-spectrinR22S rescues α-spectrin mutants to adulthood with only minor phenotypes indicating that tetramerization, and thus conventional network formation, is not the essential function of non-erythroid spectrin. Our data provide the first rigorous test for the general requirement for tetramer-based non-erythroid spectrin networks throughout an organism and find that they have very limited roles, in direct contrast to the current paradigm.

α- and β-Spectrins form (αβ)2 heterotetramers to cross-link F-actin and form spectrin-based membrane skeletons (SBMS). Analysis of the erythrocyte has provided the dominant paradigm for the SBMS, where short F-actin rods are interconnected by spectrin to form a branching network resembling a fishing net (1, 2). αl/β1-spectrin will also form higher order oligomers (heterotetramers, hexamer, and octamers etc.) (3) and recent cryoelectron microscopy images have indicated that these are common in the red cell (4).

Spectrin oligomerization via the head-to-head (H2H) interaction between the N terminus of αl-spectrin and a C-terminal domain of βl-spectrin is required to form this network. In the erythrocyte, the H2H interaction is of moderate affinity (Kd = 400–800 nm) (5, 6) permitting network formation, but also rearrangement upon shear stress during circulation (7). Mutations that decrease H2H affinity reduce or eliminate oligomerization and cause network disruption and hereditary elliptocytosis or hereditary pyropoikilocytosis (e.g. Refs. 6, 8, and 9), so it is clear that the demands for such a network are acute in red cells.

H2H binding between vertebrate non-erythroid αlI- and βlI-spectrins is much tighter (Kd = 4.5–8.5 nm) (5), but the morphology of the non-erythroid SBMS is less well defined. F-actin cross-linking by spectrin has been observed in vitro (10), in the terminal web (11), on the membrane of inner hair cells (12) and in some axons (13). In these cases the models proposed are all based on oligomeric states that form via the H2H interaction, an interaction that has apparently persisted for some 500 million years (see Ref. 14).

In most cell types the exact form of the SBMS remains undefined (e.g. Refs. 15 and 16), and network organizations may exist. Alternative oligomerization mechanisms based on lateral interactions between α-spectrin and β-spectrin have been proposed (17) but remain equivocal (18). Recently, it was shown that human βH1-spectrin can self-associate (19), raising the possibility that F-actin cross-linking could be mediated by this β isoform in the absence of α-spectrin. Clearly there is potential for diversity although none has yet been demonstrated. For the purposes of this paper we refer to any H2H-dependent oligomerization process that leads to F-actin cross-linking as the “conventional network,” this being the activity upon which all currently visualized networks appear to depend. Genetic investigations have demonstrated that non-erythroid α- and β-spectrins are essential proteins (20–23), but data on the general requirement for oligomerization via the H2H interaction per se, and therefore conventional network formation dependent upon F-actin cross-linking, is lacking.
The analysis of the temperature-sensitive (Ts) α-spec<sup>R22S</sup> mutation appeared to indicate that network formation is essential for viability in *Drosophila* (24). We were interested in utilizing the reported Ts network formation and quantitatively characterized the biochemical consequences of this mutation. We find α-spec<sup>R22S</sup> dramatically affects H2H binding at all temperatures, but that full-length α-spectrin<sup>R22S</sup> rescues adult development in a null background. Reexamination of the phenotypic consequences of this mutation reveals only minor development in a null background. Reexamination of the phenotype is actually due to defects in the ovaries, midgut, and at neuromuscular junctions, not typic consequences of this mutation reveals only minor development in a null background. Reexamination of the phe-

**EXPERIMENTAL PROCEDURES**

**Constructs and Spectrin Fragment Purification—**Spectrin gene fragments encoding the tetramerization domain were amplified (Table 1) from preexisting cDNA clones (α-spectrin, β-spectrin, and β<sub>H</sub>-spectrin) or mutated constructs (α-spec<sup>R22S</sup>), cloned into pGEX4T-1, sequence verified, and expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL (Agilent Technologies, Santa Clara, CA) using standard methods. Bacteria were grown to log phase in LB and induced using 1 mM isopropyl 1-thio-β-d-galactopyranoside. Fusion proteins were extracted in Lysis Buffer (50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1 mM β-mercaptoethanol, 5 mM EDTA, 150 μM PMSF, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 μg/ml of diisopropyl fluorophosphate) by sonication on ice. After centrifugation the supernatant was mixed with glutathione-agarose beads (Sigma) that had been previously equilibrated in PBEP (10 mM NaPO<sub>4</sub>, 130 mM NaCl, 1 mM β-mercaptoethanol, 150 mM EDTA, and 150 μM PMSF). After 1 h of tumbling, the beads were packed into a column and washed with ≥10 bed volumes of PBPE, followed by ≥10 bed volumes of PBPE without PMSF, and eluted using G-buffer (50 mM Tris-Cl, pH 8.0, 10 mM reduced glutathione, 1 mM β-mercaptoethanol, 5 mM EDTA, 1 μg/ml of pepstatin). Thrombin (Sigma) was dissolved in dH<sub>2</sub>O, added at an empirically determined ratio to release GST from each spectrin fragment and stopped by the addition of 300 μM PMSF. Following extensive dialysis in PBPE the GST/spectrin mixture was again applied to glutathione beads as described above to remove the GST. Flow-through from this column was concentrated and residual contaminants were removed by passing it through a preparative HiLoad 16/60 Superdex 75 column. MALDI analysis of each protein verified that the mass of all fragments was as expected (Table 1). Circular dichroism and analytical ultracentrifugation were performed as previously described (6) to verify that each protein had a prototypical helical content for such spectrin fragments and that they behaved as monomers. Proteins were stored on ice until used.

**HPLC Analysis of Complex Formation—**Complex formation between α-spectrin and β-spectrin fragments was verified by gel filtration. ~1 nmol of each fragment alone or in combination was combined, allowed to assemble on ice (assembly was complete in <1 min), then loaded directly onto two analytical (7.8 × 300 mm) TSK gel columns (G3000SWXL and G2000SWXL) in series (Tosoh, King of Prussia, PA) equilibrated in 10 mM NaPO<sub>4</sub>, 130 mM NaCl, 1 mM EDTA, 0.15 mM PMSF, 1 mM β-mercaptoethanol, pH 7.3.

**Differential Scanning Calorimetry—**Thermal denaturation analyses of spectrin recombinant peptides were performed using an MCS differential scanning calorimeter (MicroCal, Northampton, MA). Purified proteins were exhaustively dialyzed against 10 mM NaPO<sub>4</sub>, 130 mM NaCl, 1 mM β-mercaptoethanol, pH 7.4. Samples were degassed for 5 min, and protein samples at 0.6 to 1.0 mg/ml in the sample cell were compared with dialysate buffer in the reference cell by scanning from 25 to 100 °C at a scan rate of 90 °C/h. Data analysis was performed using the ORIGIN (version 3.2) software provided with the instrument.

**Isothermal Titration Calorimetry—**Thermodynamic parameters for the binding of the α-spectrin 0–1 recombinant fragments to wild-type 16–17 were determined using a MicroCal VP-ITC or MicroCal Auto-iTC200 isothermal titration calorimeters (GE Healthcare). Samples were dialyzed overnight in 10 mM NaPO<sub>4</sub>, 130 mM NaCl, 1 mM β-mercaptoethanol, pH 7.4, before analysis, and wild-type β-spectrin(16–17) or wild-type β<sub>H</sub>-spectrin(31–32) at 10–15 mM or dialysate buffer (controls) were loaded into the reaction cell. Wild-type or mutant α-spectrin 0–1 at a concentration of 95–185 mM were used as the titrant for experimental measurements and controls at 18, 23, or 29 °C stirring at 300 rpm in the reaction cell. Typically, 26 injections of titrant were used (10 μl each with VP-ITC; 1.5–2 μl each with Auto iTC200). Protein concentrations were determined from absorbance at 280 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to verify the absence of degradation products before and after each experiment. Data were analyzed using OriginLab (version 5.0) software as previously described (25, 26) to calculate dissociation constants. All interactions were characterized at least three times.

**Fly Strains—**The transformation host strain y w was used as a wild-type control. The wild-type UAS-myc-α-spectrin<sup>37</sup> strain (27) and lines containing α-spec<sup>Jm32</sup> α-spec<sup>Jm57</sup>, and α-spec<sup>Jm111</sup> alleles were a gift from Dr. Ronald Dubreuil (University of Illinois, Chicago, IL). Lines containing α-spec<sup>gs12</sup> and Df(3L)R<sub>E2</sub> were a gift from Dr. Daniel Branton (Harvard University, Cambridge, MA).

All α-spectrin alleles were originally generated on a *roughest scarlet ebony* chromosome (28); as a result lm/lm or lm/rg allelic combinations exhibit these phenotypes. α-spec<sup>gs12</sup> is a null allele that is known to be a deletion that results in a frame-shift after just 91 residues (20, 29, 30). α-spec<sup>gs12</sup> and α-spec<sup>Jm111</sup> have not been sequenced but do not produce detectable protein and are either null or strong hypomorphs (20, 29, 30). α-spec<sup>gs12</sup> is lethal over Df(3L)R<sub>E2</sub> and other α-spectrin alleles, but is otherwise uncharacterized (20, 29, 30).

The germline MTD-Gal4 driver was obtained from the Bloomington stock center (BL #31777; Indiana University, Bloomington, IN). The α-spectrin GFP line contains GFP inserted between amino acids 1330 and 1331 (repeat 12) at the endogenous locus after a weeP GFP-exon trap insertion (31). This strain is homozygous viable, exhibits wild-type α-spectrin distributions, and was a gift from Dr. Melissa Rolls (Penn State, University Park, PA).
Drosophila Development without Spectrin Tetramerization

All available α-specR22S fly lines were no longer expressed in α-spectrin (see text). Because, we sought to achieve the same Ts rescue of α-spectrin null mutations shown by Deng (32) we replicated exactly their construct. Plasmid P[w+ UAS] containing a full-length wild-type α-spectrin cDNA (20) (a gift from Dr. Ronald Dubreuil, University of Illinois, Chicago, IL) was mutated using the QuikChange All XL mutagenesis kit (Agilent Technologies) to create the R22S substitution. In addition, we similarly transformed an Ncol site at the start codon to KpnI to permit cloning into P[w+ UM-2], a P-element vector containing the constitutive ubiquitin promoter and Myc tag as previously described (24, 32). The entire gene was sequenced after each round of mutagenesis and in the final construct to ensure there were no non-synonymous substitutions. Rainbow Transgenic Flies, Inc. (Camarillo, CA) created fly strains according to standard methods using a y w host. As soon as possible all inserts were combined with a copy of the α-specH9251 null allele to make stocks that should be less susceptible to the effects of chronic α-spectrin overexpression.

To create α-spectrinR22S rescued flies the crossing scheme outlined in Fig. 3 was followed. With these two crosses rescued flies containing 1, 2, or 3 copies of the α-specR22S transgene were generated and designated 1, 2, or 3 copy rescue (1cr, 2cr, and 3cr), respectively. In addition, siblings that contained 1, 2 or 3 copies of the α-specR22S transgene in the presence of one wild-type allele of α-spectrin were generated and designated 1, 2, and 3 copy coexpression (1cx, 2cx, and 3cx), respectively. Rescue to adulthood was quantified as a percentage of the one offspring (range: 277–721) and included only enclosed and active flies.

Antibodies and Imaging—The following primary antibodies were used at the dilutions indicated in parentheses (immunoblot/immunofluorescence): one of two preparations of mouse anti-α-Spectrin were used: Ascites #N3 (1:100,000/1:10,000; from Dr. Daniel Branton, Harvard University, Cambridge, MA) or culture supernatant 3A9 (1:1,000–10,000/1:20; from The Developmental Studies Hybridoma Bank); mouse anti-c-myc 9E10 (1:100/1:10; Enzo Life Sciences, Farmingdale, NY); Alexa Fluor 488-conjugated goat anti-horseradish peroxidase (1:500; Jackson ImmunoResearch); rabbit anti-Synaptotagmin DsytCL1 (1:5000; Dr. Noreen Reist, Colorado State University, Fort Collins, CO), mouse anti-Hts (Adducin, 1B1; 1:250) developed by Dr. Howard Lipshitz, and mouse anti-Discs large (4F3; 1:50) developed by Dr. Corey Goodman were obtained from The Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biology, University of Iowa. HRP-conjugated anti-mouse secondary antibodies for immunoblots (Jackson ImmunoResearch) were used at 1:2500. Alexa Fluor 405-, 488-, and 594-labeled goat secondary antibodies (Invitrogen) were preadsorbed against fixed embryos and used at 1:250.

Immunoblots used enhanced chemiluminescent substrate (Pierce/Thermo Fisher) and were quantified either using a blot scanner (Liorc, Lincoln, NE) or with appropriate film exposures (that utilized serial dilutions to verify linear response of the emulsion), followed by imaging and densitometry in ImageJ.

Immunostaining of ovaries was performed as previously described (33). To visualize F-actin staining in the brush border, guts from wandering third instar larvae midguts were dissected and accumulated on ice in PBS (130 mM NaCl, 10 mM NaPO4, pH 7.4), then fixed for 20 min in 8% formaldehyde in PBS at room temperature. Phalloidin staining was performed as previously described (34).

For immunocytochemistry of neuromuscular junctions (NMJ), wandering third instar larvae were dissected leaving the CNS in place and pinned out to expose the body wall muscles, immediately fixed in 4% formaldehyde in PBS for 20 min, rinsed with PBS, and the CNS was removed and discarded. The remaining body wall muscle “pelt” preparation was unpinned and further processed for immunocytochemistry in glass depression dishes. PBS containing 0.1% Triton X-100 and 5% normal goat serum was used for subsequent antibody incubations and washes.

Samples were imaged on a CARV II spinning disc confocal (BD Biosystems) with a Retiga EXi camera (Q Imaging systems, Surrey, BC) mounted on an Olympus BX50 microscope (Olympus America, Center Valley, PA) and used iVision 4.5 software for control and capture (Biovision, Exton PA). Figures were assembled using Adobe CS4 Design Standard (Adobe Systems Inc., San Jose, CA).

RESULTS

Characterizing the Fly Spectrin Head-to-Head Interactions—The α/β-spectrin H2H interaction can be recapitulated using small two-repeat fragments (Fig. 1A) of each subunit (35, 36). We cloned and expressed these fragments of the fly α-, β-, and βH-spectrin using homologous end points (Table 1) (35). The α-specR22S construct that was reported to have a Ts phenotype contained a Myc tag at its N terminus, but it was not shown whether the presence of the tag was important for this. This addition probably has a minimal effect on the H2H interaction because it resides at the end of an unstructured region N-terminal to the tetramerization site in α-spectrin (35) and is fully functional in rescue experiments (20, 29, 30). Nonetheless, we felt it was important to reproduce the previous construct exactly and therefore included the tag. Solubility was good, purity was high (Fig. 1B), and differential scanning calorimetry experiments (Fig. 1C) yielded melting profiles very comparable with published spectrin repeat data (6, 37) indicating that they are in the native conformation.

HPLC analysis indicates that α0–1 forms a complex with both β16–17 and β31–32 (Fig. 2A). The Ka for these interactions was determined by calorimetry (Fig. 2) (6) and indicates that the wild-type interactions have a Ka (23 °C) of 14 nM for α0–1/β16–17 and 5 nM for α0–1/β31–32 (Fig. 2C). These values are most similar to other non-erythroid α/β interactions (5, 6) rather than that of the red cell where the Ka is 400–840 nM (5, 6). These results reinforce the notion that the high Ka for red cell α/β-spectrin binding is a special case.

In contrast, α0–1R22S binding to β16–17 is not detectable (Fig. 2B) and binding to β31–32 is reduced ~1000-fold to 3.7–15 μM (Fig. 2, B and C), across the reported permissive to non-permissive temperature range for the α-specR22S phenotypes (Fig. 2D) (24). Although no H2H interaction between


**TABLE 1**

Spectrin fragments expressed and purified for this study. Indicated are the relevant codons included by use of the indicated primers. Myc indicates that a Myc tag was present on this construct.

| Spectrin fragment | Codons From-to | Primers (5′-3′)* | M<sub>p</sub> predicted/MALDI† |
|-------------------|----------------|------------------|-----------------------------|
| α 0–1            | 2..152         | gpeENFT          | 18441.58/18441.41           |
| α myc0–1R22S     | Myc..2..R22S..152 | gpeEQKL         | 19702.0/19700.14            |
| β 16–17          | 1911..2093    | ggEQKL          | 21651.61/21637.03          |
| βH 31–32         | 3412..3594    | gAEQKL          | 21666.50/21666.80          |

* Amino acids in lower case were added by the construct and remain present following thrombin cleavage.
† MALDI estimates of the molecular weights of the purified proteins.

α/β-spectrins seems likely in vivo, the residual interaction between α0–1R22S and βH31–32 might permit some tetramer formation depending upon the local spectrin concentration. However, in the red cell, which probably represents an extreme formation depending upon the local spectrin concentration. The R28S substitution similarly reduces the K<sub>d</sub> (6) and has pathological consequences (38). So it seems unlikely that the residual binding between α0–1R22S and βH31–32 would sustain a wild-type network in vivo. We conclude that the reported Ts phenotype of the α-spectrin<sup>R22S</sup> allele is not due to a reversible H2H interaction.

**α-Spectrin<sup>R22S</sup> Rescues α-Spectrin Null Flies to Adulthood—** Existing stocks expressing α-spectrin<sup>R22S</sup> were made in the mid 1990s (24), no longer express Myc-tagged α-spectrin, and contain promoter-deleted transgenes, suggesting that long term constitutive expression of this protein has been selected against in the stocks (not shown). We therefore regenerated exactly the same construct starting with the same full-length α-spectrin cDNA (24) including the Myc tag, which might participate in the reported temperature sensitivity. To protect the construct from eventual deletion, we immediately crossed each new line into a background heterozygous for the α-spec<sup>g41</sup> null allele.

The crossing schemes shown in Fig. 3 were used to generate rescued flies. In this scheme flies were produced that express up to 3 copies of the transgene in an α-spectrin mutant background (referred to as 1–3 copy rescue flies). Sibling flies heterozygous for each mutant allele containing the rescue construct are referred to as 1–3 copy coexpression flies. Rescued flies were readily obtained at 18, 25, and 29 °C and with 1, 2, and 3, although the frequency was
FIGURE 2. Complex formation between α- and β-spectrin tetramerization domains. A, HPLC gel filtration profiles. Solid lines show an equimolar mixture of α0–1 and β16–17 (top) or β31–32 (bottom); dashed lines, α0–1 alone; dotted lines, β16–17 alone (top) and β31–32 alone (bottom). Complex formation is robust in both cases. B, representative ITC analyses for binding between α0–1 (wt) or α0–1R22S (αR22S) and β16–17 (β) or β31–32 (βH) as indicated. C, table showing the $K_D$ (with 95% confidence intervals) determined for each interaction at three different temperatures. n, number of replicates; UND, interaction undetectable. The α-specR22S mutation eliminates interaction with β16–17, whereas a reduction in binding of ~1000-fold is seen with β31–32.

Parental genotypes

| α-spectrin | Relevant progeny genotypes | Name used in paper (selection criteria) |
|------------|---------------------------|----------------------------------------|
| +          | α-spectrin$^{m11}$        | One copy rescue (1xP[w$^+$], Cy$^+$ Hu$^+$) |
| +          | TM6B, Tb Hu e             |                                        |
| P[Ubi-α-spectrinR22S] | α-spectrin$^{m11}$        | Two copy rescue (2xP[w$^+$], Cy$^+$ Hu$^+$) |
| CyO, Cy    | P[Ubi-α-spectrinR22S]     |                                        |
|            | α-spectrin$^{m11}$        | One copy coexpression (1xP[w$^+$], Cy Hu) |
|            | TM6B, Tb Hu e             |                                        |
| P[Ubi-α-spectrinR22S] | α-spectrin$^{m11}$        | One copy coexpression (1xP[w$^+$], Cy Hu) |
| CyO, Cy    | P[Ubi-α-spectrinR22S]     |                                        |
|            | α-spectrin$^{m11}$        | Two copy coexpression (2xP[w$^+$], Cy$^+$ Hu) |
|            | TM6B, Tb Hu e             |                                        |

FIGURE 3. Genetics of crosses resulting in α-spectrinR22S rescued and coexpressing flies.
highly variable in both cases. Flies were normal in morphology, and we do not see evidence of the sluggish behavior in rescued flies reported by Deng et al. (24) (supplemental Movie S1).

We speculate that the difference between our results and the previous study by Deng et al. (24) may have arisen because of over- or under-expression from their original transgenic insertion(s), which would almost certainly have had different levels of expression from our lines due to position effects. We cannot do a direct comparison because the original transgenic lines were lost; however, we sought to verify that our transgenes were producing α-spectrin R22S at physiological levels by comparison to wild-type lines using quantitative immunoblots of whole fly extracts (Fig. 4). The results appear to indicate that our single and dual copy rescue flies express α-spectrin R22S at ~80% wild-type levels and that coexpression with one copy of wild-type α-spectrin results in mild overexpression. However, these results are quite variable and do not achieve statistical significance (one sample t test versus normalized control 0.07 < p < 0.21, n = 7). Because two copies of the transgene produce a better rescue frequency, whereas the size of the stable α-spectrin R22S pool does not change, we suggest that the rate of α-spectrin production may influence viability. We conclude that our transgenes are producing near wild-type amounts of α-spectrin R22S protein.

Probing with the anti-Myc antibody for the tagged α-spectrin R22S protein reveals that the presence of wild-type α-spectrin often appears to cause the selective destabilization of α-spectrin R22S (Fig. 4C) or that the ability to form tetramers results in the selective stabilization of wild-type α-spectrin. However, this result is also highly variable and although striking on some blots, again does not reach statistical significance (one sample t test versus normalized control 0.09 < p < 0.15, n = 6).

α-Spectrin R22S Rescued α-Spectrin Null Flies Exhibit Subtle Tissue Abnormalities—Rescued males and females raised at any temperature mated and viable eggs were produced, indicating that early development (prior to zygotic α-spectrin transcription) also does not require tetramerization. We examined the ovaries of rescued flies closely because of the previously reported degeneration phenotype in post-stage 7 egg chambers (24). Such chambers were rare in our hands and are identical to chambers degenerating in response to the nutritional checkpoint (Fig. 5) (39). The existence of this checkpoint and the importance of using well-fed females to examine ovaries were noted until after Deng et al. published their paper (39).

We conclude that there is no such phenotype in the α-spectrin R22S rescued females. However, there are more subtle abnormalities in oogenesis not previously described. First, we noticed that the fusome structure is altered. The fusome is a cytoplasmic membrane "network" that contains α/β-spectrin and Adducin (40), forms at sites of incomplete cytokinesis, associates with spindle poles during cystoblast division, and reaches its greatest complexity in germline regions 2a/b before starting to disappear. Staining for Adducin (1B1) reveals fusome morphology (Fig. 6A) (see Ref. 41). In rescued α-spectrin ε1111/α-spectrin 711 flies 1B1 staining becomes fragmentary in region 2 (Fig. 6, B and B′) suggesting that spectrin tetramerization is required for late fusome integrity. We also see a similar fragmentation when one copy of a wild-type α-spectrin gene is present (Fig. 6, C and C′) showing that the α-spectrin R22S can dominantly disrupt the fusome. This is not a fixation artifact because fragmentation was also seen with coexpression of an α-spectrin::GFP gene trap line when we imaged freshly dissected germaria (Fig. 6, D and D′). It is also possible that expression of α-spectrin above wild-type levels is responsible for this

![FIGURE 4. Analysis of α-spectrin levels by quantitative immunoblot. A, an example blot where fly extracts from wild-type, α-spectrin R22S rescued (one or two copies of transgene) and α-spectrin R22S coexpressing (one or two copies of transgene) flies were probed for α-spectrin R22S (via its myc tag), total α-spectrin and α-tubulin (for normalization). B, comparison of total α-spectrin levels with wild-type. Rescued flies have ~80% of the α-spectrin seen in wild-type, whereas coexpressed α-spectrin R22S is not additive with wild-type, probably reflecting the observation that it is destabilized in the presence of wild-type protein (panel C); C, comparison of α-spectrin R22S levels in coexpressing and rescued flies. There is less α-spectrin R22S when wild-type α-spectrin is present. Genotype rescued was α-spectrin ε1111/TM6B. Background for coexpression was α-spectrin α-B). Error bars represent 95% confidence intervals.](image-url)
phenotype. We therefore overexpressed wild-type \(\alpha\)-spectrin in the germline in a wild-type \(\alpha\)-spectrin background using the Gal4/UAS system (42) with the MTD-Gal4 driver. Staining MTD-Gal4\(\rightarrow\)UAS\-\(\alpha\)-spectrin\({}^{37}\) ovarioles for 1B1 reveals fusions of normal complexity (Fig. 6E).

The second phenotype seen in the ovaries of rescued females is an occasional gap in the follicle cell monolayer (Fig. 6, F and F\'). These are mostly just one or two cell diameters in size, although a handful of much larger ones have been observed. Given the fertility of the rescued females and the low frequency of degenerating chambers we assume that most of these resolve themselves.

A number of other tissues are affected by loss of function \(\alpha\)-spectrin alleles in the fly. In particular the spectrins are particularly necessary in the nervous system (e.g. Refs. 27, 43, and 44) and in the midgut (34). We therefore performed initial phenotypic analyses on the morphology of the NMJ and brush border of rescued third instar larvae. In NMJ of rescued third instar larvae all our marker proteins (including \(\alpha\)-spectrin) localized normally (Fig. 6, H–J). From the location and complexity of the NMJ they reveal, we further infer, that the motor neurons find and invade the musculature normally with little difficulty. However, we do see that the NMJ itself has a slightly altered morphology, with some of the boutons in rescued larvae often being less individuated (Fig. 6, I and J). Similarly, phalloidin staining of the brush border of the middle midgut reveals no conspicuous abnormalities (Fig. 6, K and L). The role of tetramerization at both of these locations is therefore not of sufficient importance to compromise the tissue under culture conditions. In future studies it will be interesting to see if any specific functionalities at either of these locations is perturbed by the absence of tetramerization.

**DISCUSSION**

The wild-type dissociation constants for the tetramerization reaction between fly \(\alpha/\beta\)-spectrin and \(\alpha/\beta_4\)-spectrin are both in the low nanomolar range and about 100-fold lower than that of red cell spectrin. This is in line with all other results for non-erythroid spectrins to date (5), and is consistent with the notion that the lower tetramerization affinity associated with red cell spectrin results from the specialized nature of this cell type.

Our biochemical data strongly suggests that tetramerization will not occur between \(\alpha\)-spectrin\({}^{R22S}\) and \(\beta\)-spectrin *in vivo*, and that the \(\alpha\)-spectrin\({}^{R22S}/\beta_4\)-spectrin interaction is severely compromised. The analysis of the recombinant peptides used to determine the binding affinities between the \(\alpha\)-spectrin and \(\beta/\beta_4\)-spectrin tetramerization domains represents an accepted model since its inception over 20 years ago (36), and analyses of larger proteolytic fragments or full-length proteins has yielded the same properties and binding affinities as the short fragments used here (46). Furthermore, our results are fully in line with other estimates of non-erythroid spectrin tetramerization interactions (5). This strongly suggests that our measurements reflect the tetramerization potential of the full-length proteins *in vivo*. Of course, it is formally possible that *in vivo* conditions (e.g. local concentration or interaction with other proteins) could lead to tetramerization despite the presence of the R22S substitution. However, the magnitude of the effects of this mutation on tetramerization are similar to that seen in the homologous mutation in red cell spectrin, where the effective spectrin concentration has been estimated to be as high as 230 mg/ml (~380 \(\mu\)M) (47), and yet an erythrocyte pathology still results. Therefore, the simplest explanation for our results is

![Image](Image.png)
that the functionality of a conventional red cell-type network is not that important in fly development in any vital tissue.

Previous results suggesting that a conventional network involving spectrin cross-linking of F-actin might not be so important in non-erythroid tissues that came from examination of dominant H2H-disrupting mutations in βI-spectrin that lead to hereditary elliptocytosis and hereditary pyropoikilocytosis (see Ref. 48 and references therein). Despite the expression of the non-erythroid βII/II spliceform of this protein in other tissues, these dominant effects are confined to the red cell. Homozygosity for the βI-spectrinProvidence allele in the tetramerization domain is lethal (49), but other tissues, notably muscles, nonetheless, develop normally in affected individuals (50). However, the issue of isoform redundancy could not be addressed in this study because βIII- and βIV-spectrins were unknown at the time.

In the fly, several articles (21, 51, 52) suggest that β-spectrin function in neurons and epithelial cells is independent of α-spectrin and need not therefore depend upon a conventional network. Also in fly, the W2033R mutation in the βIII-spectrin tetramerization domain was reported to rescue β-spect mutant flies (53) although this was not backed up with a biochemical demonstra-
Drosophila Development without Spectrin Tetramerization

...tion that the mutation had the homologous effect. Vertebrate \( \beta \)-spectrin unaccompanied by \( \alpha \)-spectrin can be found in high molecular weight complexes (54), but it is not clear whether these are oligomers or complexes with other proteins. In all these cases there is currently no published data that indicates whether \( \beta \)-spectrin that is not bound to \( \alpha \)-spectrin is in any kind of network or is operating in a monomeric or oligomeric form.

It has been suggested that the \( \alpha/\beta \)-spectrin might form oligomeric structures without the H2H interaction (3) and it was recently shown that human \( \beta_{1C} \)-spectrin can self-associate (19). Such results raise the possibility that non-conventional networks may exist, and clearly their role would not be tested by the current work.

The relative contribution of conventionally networked spectrin to all the tissues in an organism is directly assayed by this study for the first time because all of the \( \alpha \)-spectrin in the fly is affected by the R22S substitution, there are no other isoforms present. Because a viable and functional adult is produced, H2H oligomerization is by definition not a vital function, but because we do detect minor morphological defects in several tissues, it would appear that there is a role for the conventional network, one of refinement. In the normal environment of the fly, the role of H2H oligomerization must be sufficiently significant to provide a selective pressure for the maintenance of this interaction. Because the structure of the spectrins, including the tetramerization domains, has been maintained for some 500 million years (14), there is clearly selective pressure for its maintenance. However, the selective advantage need only be small over this period of time to result in the present day high affinity interaction. It will be of considerable interest for future studies to identify what changes in functionality are present (e.g. at the NMI) in the rescued fly background that underlie this selective pressure.

Our results change our perspective on these essential proteins. The red cell model and its variants in the terminal web and axons all depend upon F-actin cross-linking by spectrin oligomers that are mediated by the H2H interaction and are widely assumed to apply in all non-erythroid tissues. Although there is a role for a network of this type, our results clearly indicate that this is not perhaps the core function of spectrin in non-erythroid tissue, and further reinforces the notion that the dependence of the red cell on a fully networked SBMS is a special case. Several studies indicate a significant role for spectrin in endomembrane trafficking (45, 55–58). In this context our results make sense, given that the size of most transport organelles is far too small to accommodate a traditional spectrin network.

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