

Maintaining epithelial integrity: a function for gigantic spectraplakins isoforms in adherens junctions

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The Short stop (Shot/Kakapo) spectraplakins are giant cytoskeletal proteins, which exist in multiple isoforms with characteristics of both spectrin and plakin superfamilies. Previously characterized Shot isoforms are similar to spectrin and dystrophin, with an actin-binding domain followed by spectrin repeats. We describe a new large exon within the *shot* locus, which encodes a series of plakin repeats similar to the COOH terminus of plakins such as plectin and BPAG1e. We find that the plakin repeats are inserted between the actin-binding domain and spectrin

repeats, generating isoforms as large as 8,846 residues, which could span 400 nm. These novel isoforms localized to adherens junctions of embryonic and follicular epithelia. Loss of Shot within the follicle epithelium leads to double layering and accumulation of actin and ZO-1 in between, and a reduction of Armadillo and Discs lost within, mutant cells, indicative of a disruption of adherens junction integrity. Thus, we identify a new role for spectraplakins in mediating cell–cell adhesion.

Introduction

Members of the spectrin and plakin superfamilies play key roles in the link between the plasma membrane and the cytoskeleton. The protein encoded by the *Drosophila melanogaster* gene *short stop* (Shot, also known as Kakapo) was found to be a hybrid spectrin/plakin molecule, or spectraplakins (for review see Röper et al., 2002). The Shot sequence and its mutant phenotype led us to propose that it is a *Drosophila* version of one of the mammalian plakins, plectin, and instead of linking integrins to intermediate filaments as plectin does, it links integrins to microtubules (Gregory and Brown, 1998; Strumpf and Volk, 1998). This is consistent with observations that microtubules, not intermediate filaments, provide stabilizing function in *Drosophila* epidermal cells, and the confirmation that the *Drosophila* genome sequence does not encode any cytoplasmic intermediate filaments that Shot could interact with (Adams et al., 2000).

The NH₂-terminal third of Shot contains an actin-binding domain (ABD) of the type common to both spectrin and plakin superfamily members, consisting of two calponin homology domains, but is clearly more similar to plakins than spectrin family members (see Fig. 1; Gregory and Brown, 1998). The ABD of plectin binds not only to actin

but also to the unusually long cytoplasmic tail of the $\beta 4$ integrin subunit (Reznicek et al., 1998). All plakins have a related COOH-terminal domain consisting of what are called plakin repeats or plectin repeats (Green et al., 1990; Schultz et al., 1998; Leung et al., 2001a; Bateman et al., 2002). The known function of this domain is to bind to intermediate filaments (Nikolic et al., 1996; Leung et al., 1999; Choi et al., 2002), and because intermediate filaments are not present in *Drosophila* it made sense that this domain was lacking in the Shot isoforms that were initially characterized. Instead, the majority of Shot was found to be composed of spectrin repeats, more related to dystrophin and spectrin (Strumpf and Volk, 1998). In addition Shot has a GAS2 domain at the COOH terminus, which has been found to bind microtubules (Lee et al., 2000; Sun et al., 2001). In embryos lacking Shot, the epidermal cells that attach to the muscles, the tendon cells, are pulled apart by muscle contractions, and the microtubules have lost their connection to the basal cell surface (Prokop et al., 1998). This appears analogous to the cell disruption in the basal layer of the epidermis when BPAG1 or plectin are missing (Guo et al., 1995; McLean et al., 1996). Thus, the region of Shot that is conserved with plectin is the portion that interacts with integrins, whereas the intermediate filament binding

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Abbreviations used in this paper: ABD, actin-binding domain; dlt, Discs lost; FasIII, Fasciclin III; IP, immunoprecipitation; PY, phosphotyrosine; WB, Western blot.

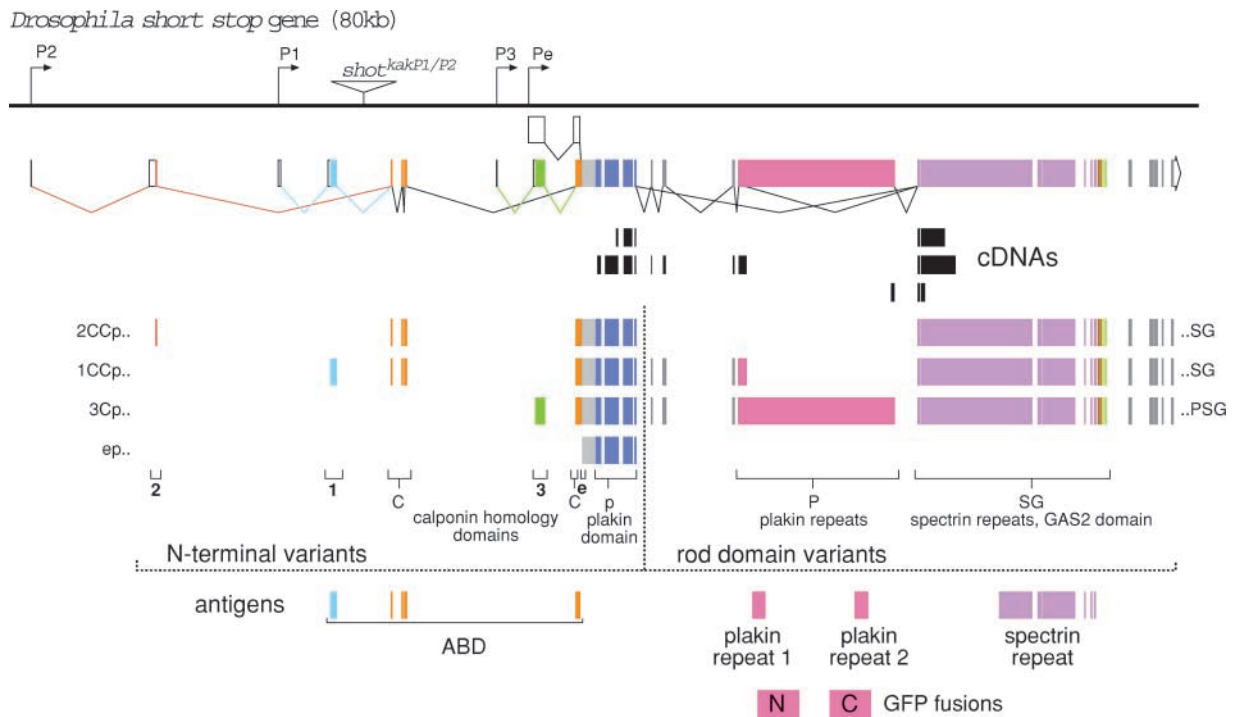


Figure 1. Genomic structure of the *shot* locus and potential protein isoforms. Diagram showing from top to bottom: the four starts of transcription; the insertion of the P-element transposon in the *shot*^{kakP1} and *shot*^{kakP2} alleles; exons of the *shot* gene, solid color indicates coding sequence, with splicing shown in regions of alternate splicing (except for an alternative splice in the Gas2 domain, which is not depicted); cDNAs indicating splicing around the plakin repeat encoding exon; variations in predicted protein products separated into NH₂-terminal variants on the left and variants around the rod domain on the right, which can potentially produce 12 different isoforms; and segments of *shot* used to generate either the GFP fusion constructs or the antibodies used here. The labeling of the protein domains also indicates the nomenclature used to describe the different isoforms. On the right is the description of the NH₂-terminal variants produced by transcription initiation at the four sites. For example, a protein starting at promoter 1 and containing the full ABD (CC), plakin family domain (p), plakin repeats (P), and the spectrin repeats and Gas2 domain (SG) is called 1CCpPSG. Here, we have not been able to distinguish between the isoform completely lacking the plakin repeat domain and the isoform that contains some plakin repeat sequence.

domain of plectin has been replaced with a microtubule binding domain. Although a role in linking integrins to the microtubules remains a likely function of Shot, several observations show that this is not the whole picture.

The identification of vertebrate orthologues of Shot rapidly demonstrated that this protein is not a specialized version of plectin unique to invertebrates (Leung et al., 2002; for review see Röper et al., 2002). Two spectraplakin genes have been found in mammals: *MACF1* and *BPAG1*. Several of the diverse mutant phenotypes of the *shot* locus, or the mouse *BPAG1* gene, *dystonia musculorum*, appear not directly related to integrin function. The discovery that prompted the work described here was the identification of a novel exon within the *shot* gene that encodes an extended set of plakin repeats. Integration of this domain into Shot protein isoforms could further multiply the isoform variability and potentially generate isoforms with new functions that do not involve integrins. The discovery of the plakin repeat encoding region in the *shot* locus is curious, as the only known function of these repeats so far is to interact with intermediate filaments. We were especially interested to see whether they had adopted a different function in the fly that could potentially shed light on additional functions of plakin repeat regions in vertebrate proteins.

Results

A new large exon within the *shot* locus encodes plakin repeats

In the process of characterizing the gene structure of *shot*, we noted a large intron right at the point in the *shot* mRNA sequence where the encoded protein changes from being most similar in sequence to plectin to more related to dystrophin. Sequencing through this intron revealed a large exon of 10,497 nucleotides (Fig. 1; Gregory, S.L., personal communication and unpublished data), which was confirmed in the completed *Drosophila* genome sequence (Adams et al., 2000). A single EST (Rubin et al., 2000) contains sequences from this exon, which splices the 5' end of it to the downstream spectrin repeat-containing exons (Fig. 1). A previously characterized cDNA contains a short exon consisting of the start of this large exon (Gregory and Brown, 1998). Therefore, the new exon can be incorporated into a 27-kb transcript containing all exons. Four starts of transcription have been identified to date (Gregory and Brown, 1998; Lee et al., 2000), all upstream of this new exon. This suggests that the inclusion of this exon into mRNAs will be regulated by alternative splicing rather than an alternative transcriptional start. The plakin repeat-containing exon is also conserved in the single *Caenorhabditis elegans spectraplakin* locus (unpublished data; Boshier et al., 2003). However, in con-

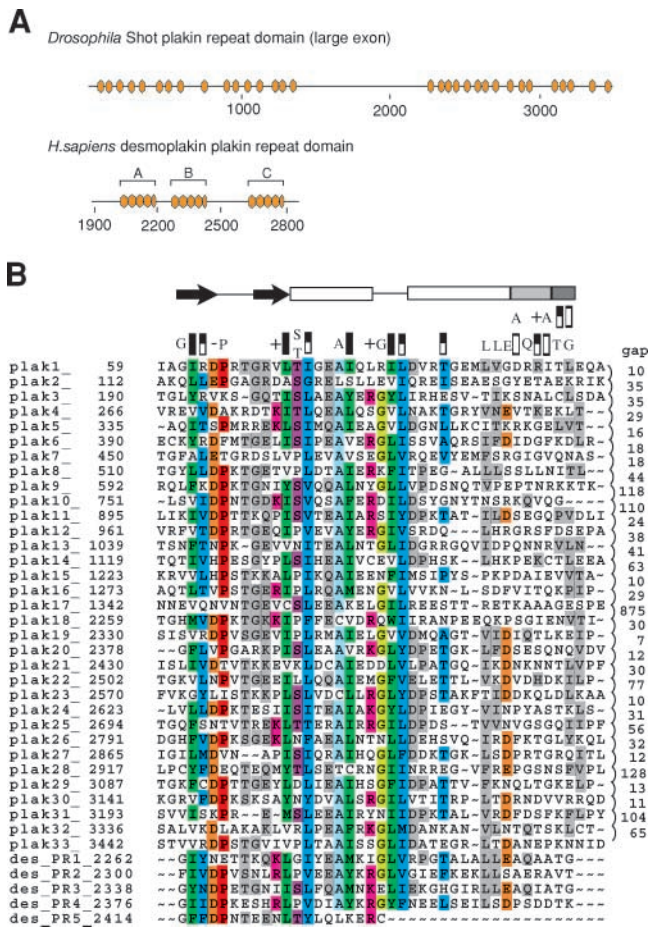


Figure 2. Analysis of the plakin repeat domain of Shot. (A) Arrangement of the plakin repeats encoded by the large exon of *shot*, which are not clustered into domains containing 4.5 repeats as they are in other plakins like desmoplakin (domains labeled A, B, and C). (B) Comparison of the 33 Shot plakin repeats with the 4.5 plakin repeats of desmoplakin domain B, the structure of which has been solved (Choi et al., 2002). The scheme on the top depicts the secondary structural elements found in desmoplakin: a β -fold (arrows) followed by two α -helices of varying length depending on the repeat (white and gray boxes). The structural consensus sequence is indicated above the alignment, as defined by Choi et al. (2002): single letter code indicates highly conserved residues. Shaded boxes indicate large hydrophobic residues (F, I, L, M, Y, and W), open boxes indicate small hydrophobic residues (A, C, P, T, and V), and half-filled boxes indicate general hydrophobic residues. A plus sign indicates basic residues (H, K, and R), and a minus sign indicates acidic residues (D and E). Two consensus sequences are shown for the end of the second α -helix; the top is for repeat 2 and the bottom for repeat 1 and 3. Conserved residues are color-coded in the alignment. Numbers on the left indicate the first residue of each repeat (for Shot plakin repeats counted from the beginning of the large exon). Numbers on the right indicate the length of the gaps found in between individual plakin repeats in Shot, whereas the desmoplakin repeats run into each other without gaps.

trast to the fly, this exon encodes an alternative COOH terminus in the worm protein.

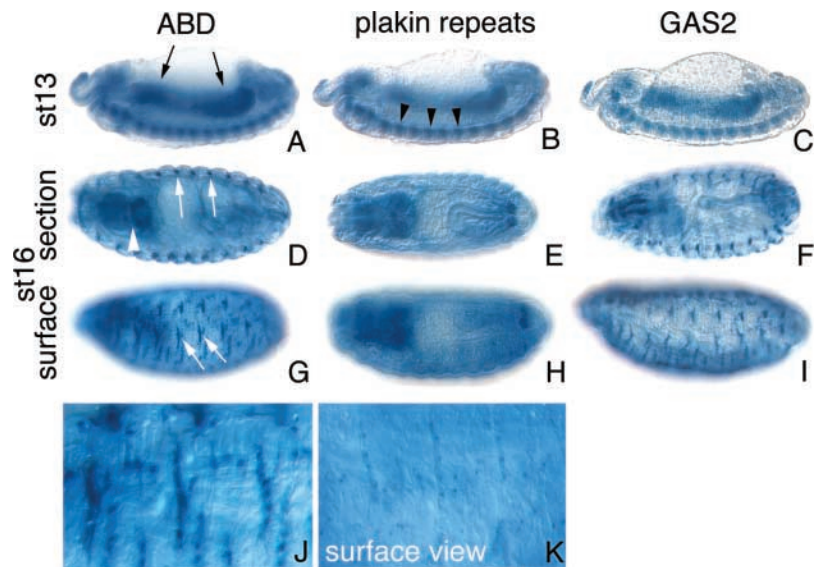
This new exon of the *shot* gene encodes for 33 plakin/plectin repeats according to SMART and Pfam predictions (Fig. 2 A; Schultz et al., 1998; Bateman et al., 2002). As the structure of domains B and C of desmoplakin, each of which are formed by 4.5 plakin repeats, has been solved (Choi et al.,

2002), we compared the sequences found in Shot with the structural consensus (Fig. 2 B), which is shifted relative to the SMART and Pfam consensus by 12 aa. In the desmoplakin domains, repeats 1–4 consist of a β -hairpin followed by two α -helices, with the last half repeat of each domain lacking the second α -helix. These 4.5 repeats fold into a larger globular structure, mediated by contacts between the second α -helix of one repeat with conserved residues of the following repeat (Choi et al., 2002). Fig. 2 B shows the alignment of the 33 Shot repeats and 4.5 desmoplakin repeats from domain B. In general, Shot sequences retain the structural consensus for the β -fold and the first α -helix, but the second α -helix is poorly conserved. In contrast to desmoplakin, where each plakin repeat within a domain is adjacent to the next, the plakin repeats in Shot are separated by between 7 and 128 residues. The site of insertion of these intervening sequences is on the outside of the desmoplakin structure, and, therefore, they could be tolerated in a similar structure (Weis, W.I., personal communication). However, the divergence within the second α -helix makes it unlikely that the repeats found in Shot form a similar 4.5 repeat-containing globular structure as seen in desmoplakin. To summarize, the Shot locus contains a previously unidentified large exon encoding for plakin repeats. Evidence from incomplete cDNAs suggests that the exon is incorporated into Shot mRNAs. Shot's plakin repeats are clearly related to the repeats found in typical plakins such as desmoplakin, but their divergence from the structural consensus sequence in a critical position makes it uncertain whether it could adopt the same tertiary structure.

mRNAs containing the plakin repeat exon are expressed in the *Drosophila* embryo

To determine whether the plakin repeat exon is incorporated into *shot* mRNAs, in situ hybridization on whole mount *Drosophila* embryos was performed. Two different RNA probes from this exon were used and gave identical patterns (Fig. 3, plakin repeats, and not depicted). Their pattern of staining was compared with that seen with probes directed against the exons encoding the NH₂-terminal ABD (Fig. 3, ABD) and the COOH-terminal GAS2 domain (Fig. 3, GAS2). At mid-embryogenesis (i.e., developmental stages 10–13), the mRNAs containing these exons were all expressed in the same pattern, with staining in the epidermis, the midgut primordia (Fig. 3 A, arrows), and the central nervous system (Fig. 3 B, arrowheads). At the end of embryogenesis, some tissues still contained mRNAs detectable with probes for all three regions: the brain (Fig. 3 D, arrowhead), pharynx, and proventriculus. However, in the epidermis the mRNAs were expressed differently. The mRNAs encoding the ABD and the GAS2 domains were most strongly expressed in the epidermal tendon cells (Fig. 3, D and G, arrows; Fig. 3, F, I, and J). In contrast, the mRNAs encoding the plakin repeat domain remained evenly expressed in all epidermal cells (Fig. 3, E and H). As the staining patterns of the different probes clearly differed, this rules out the possibility that the signal from the plakin repeat probe was simply derived from its expression as an intron in the pre-mRNAs of the other isoforms. Expression as an intron was visible by the nuclear labeling with the plakin repeat probe in the tendon cells (Fig. 3 K). Thus, the in situ analysis has demonstrated that the plakin repeat exon is ex-

Figure 3. Analysis of Shot protein domain expression at the mRNA level. Whole mount in situ hybridization with probes directed against the regions encoding the ABD (A, D, G, and J), plakin repeats (B, E, H, and K) and GAS2 domain (C, F, and I). (A–C) Widespread staining was detected with all three probes in stage 13 embryos, with elevated levels in the midgut primordia (A, arrows) and segmental central nervous system precursors (B, arrowheads). (D–K) In late stage embryos (st16) the ABD and GAS2 domain probes strongly label epidermal tendon cells (D and G, arrows), whereas plakin repeat probe labeling remains uniform in the epidermis (E and H). All probes label the embryonic brain (D, arrowhead). At high power, the elevated expression of the form lacking the plakin repeats in the tendon cells is demonstrated by the cytoplasmic staining with the ABD probe (J) and elevated labeling of the plakin repeat region as an intron, revealed by the nuclear labeling with the plakin repeat probe (K).



pressed during embryogenesis, and that the ratio of transcripts produced with and without this exon is regulated differently in tendon cells versus other epidermal cells.

Shot isoforms with plakin repeats also contain spectrin repeats

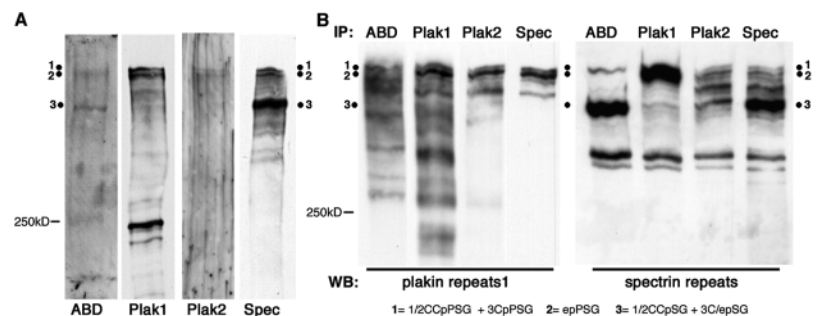
To analyze Shot isoforms containing the plakin repeats, antibodies were generated against two segments of the Shot plakin repeat domain (Fig. 1). These were used in combination with previously described antibodies against the ABD and the spectrin repeats (Gregory and Brown, 1998; Strumpf and Volk, 1998). In Western blots (WBs) of whole embryo lysates (Fig. 4 A) the ABD domain antibody recognized two isoforms, one substantially larger than the other. Both anti-plakin repeat antibodies recognized a high molecular mass doublet, the top band of which co-migrated with the larger of the two bands containing the ABD. The plakin repeat 1 antibody also recognized a prominent band at just under 250 kD, due to a spurious cross reactivity with the *Drosophila* band 4.1 orthologue Coracle (unpublished data). The spectrin repeat antibody recognized the same high molecular mass doublet as the two plakin repeat antibodies and the same lower band as the ABD antibody. Thus, the WB revealed three isoforms: the top band that contains all domains examined (iso-

forms 1CCpPSG, 2CCpPSG, and possibly 3CpPSG, with predicted molecular masses of 995, 982, and 989 kD, respectively; see Fig. 1 for description of nomenclature); the next band lacks the ABD (epPSG, 951 kD); whereas the third band represents the previously characterized forms lacking plakin repeats (1CCpSG, 2CCpSG, and 3CpSG, with predicted molecular weights of 595, 582, and 588 kD, respectively). We confirmed the identity of these bands by immunoprecipitating with each of the four antibodies, and probing with either the plakin repeat antibody 1 or the spectrin repeat antibody (Fig. 4). Additional shorter proteins were detected, but these may be degradation products rather than genuine short isoforms. The different antibodies gave different estimates of the relative abundance of the different isoforms: probing WBs of total lysates or immunoprecipitates with the spectrin repeat antibody suggested that the form lacking plakin repeats is more abundant, but the WB with anti-ABD suggested they are of equivalent abundance (Fig. 4 B).

Shot isoforms containing the plakin repeats localize to cell–cell junctions

To gain insight into the potential function of these new forms of Shot we examined their subcellular distribution. In late stage 16 embryos with fully developed muscle attach-

Figure 4. Western analysis of Shot protein isoforms. (A) WB of embryo lysates using four different antibodies raised against different segments of Shot (Fig. 1): an antibody against the ABD, two different antibodies against plakin repeats (Plak1 and Plak2), and an antibody against the spectrin repeats (Spec). (B) Combinatorial IP/Western analysis of Shot protein isoforms. Shot was immunoprecipitated (IP) from embryo lysates with the four anti-Shot antibodies, run on a gel and transferred to a filter, which was then probed with each antibody. Two examples are shown (plakin repeats1 and spectrin repeats). In both experiments, three different molecular mass forms are reproducibly seen, labeled 1–3. Corresponding isoforms, based on predicted molecular masses, are indicated.



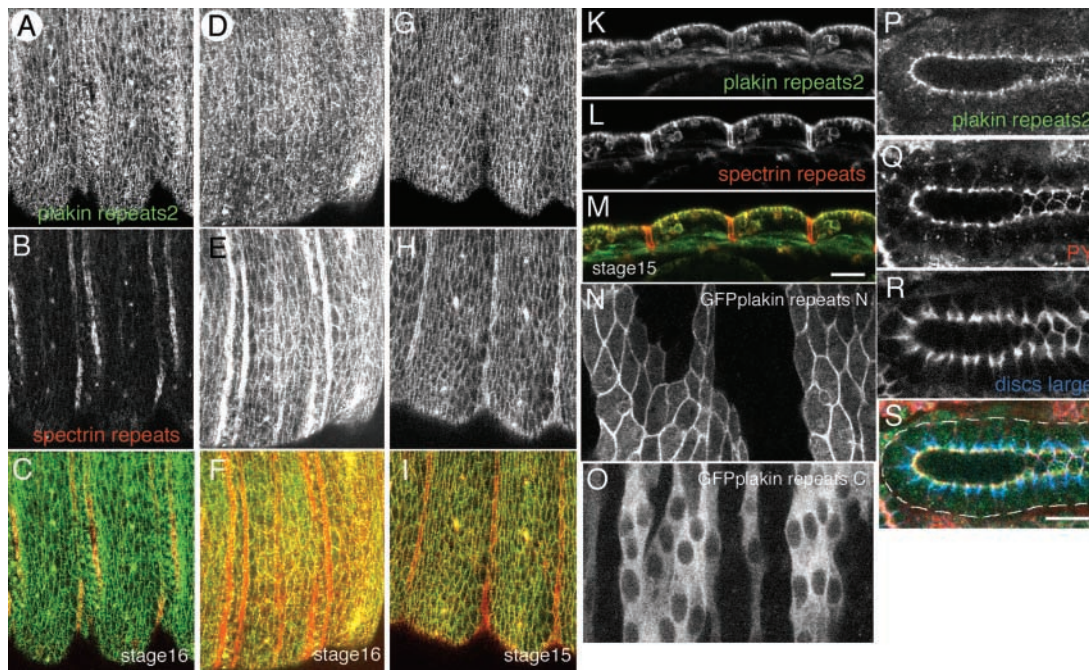


Figure 5. Distribution of Shot isoforms in the *Drosophila* embryo. (A–F) In late stage 16 embryos the spectrin repeat antibody strongly labels the circumference of the tendon cells, and more weakly the rest of the epidermal cells (B and E; and C and F, red). In contrast, the plakin repeat 2 antibody labels the circumference of epidermal cells and is not enriched in tendon cells (A and D; and C and F, green). E is scanned at higher laser power relative to B to reveal the cortical spectrin repeat antibody staining. (G–M) In embryos at stage 15, the elevated levels of tendon cell labeling with the spectrin repeat antibody are just detectable, allowing better visualization of the cortical staining in all epidermal cells (H and L; and I and M, red). Cortical staining was also seen with the plakin repeat antibody (G and K; and I and M, green). Note in the optical sections shown in K–M that the labeling with the spectrin repeat antibody extended from the apical (top) to basal surface of the tendon cells, whereas the labeling with both antibodies was only apical in the other epidermal cells. (N and O) Fusion proteins containing different segments of the plakin repeats fused to GFP (Fig. 1) were expressed in stripes in the epidermis and visualized in live embryos. Note the targeting of GFPplakin repeats N to junctional areas. (P–S) Shot isoforms containing the plakin repeats colocalize with PY in adherens junctions. An embryonic salivary gland is shown labeled with the plakin repeat 2 antibody (P; and S, green) anti-PY (PY20; Q; and S, red), and the septate junction marker Discs large (R; and S, blue). The dotted line in S marks the basal surface. Bars, 20 μ m.

ment sites, antibodies against the ABD and spectrin repeats have been shown previously to strongly label the epidermal tendon cells, in agreement with the *in situ* hybridization data (Gregory and Brown, 1998; Strumpf and Volk, 1998; Lee et al., 2000). In contrast, the plakin repeat antibodies labeled the circumference of all epidermal cells and the labeling was not concentrated in tendon cells (Fig. 5, A, D, and G). The anti-spectrin repeat antibody also stained the circumference of all epidermal cells but at much lower levels than tendon cell labeling (Fig. 5, B and E). Earlier in embryogenesis (stage 15 and early stage 16), the staining with the two antibodies was much more similar with cortical staining of all epidermal cells, although elevated levels of the isoforms containing the spectrin repeats were already apparent in tendon cells (Fig. 5, G–I and K–M). We did not detect any staining with the plakin repeat antibody that did not colocalize with staining with the spectrin repeat antibody (Fig. 5, and not depicted), indicating that there is not a prevalent form of Shot analogous to BPAG1e, which has the plakin repeats at the COOH terminus and no spectrin repeats.

To determine the subcellular structure labeled by the plakin repeat antibody, the staining was compared with markers of junctional areas. The large salivary gland cells had the clearest separation of the different subcellular compartments, as seen by labeling adherens junctions with an-

tiphosphotyrosine (PY; Fig. 5 Q), and labeling of septate junctions with the anti-Discs large antibody (Fig. 5 R). The plakin repeat-containing Shot isoforms were found concentrated at the adherens junctions (Fig. 5 P). This suggests that these isoforms have a distinct function that differs from the role of the shorter isoforms that are concentrated at the apical and basal surfaces of the tendon cells.

To test whether the signals for targeting to lateral junctions are found in the plakin repeat region, we constructed fusions between segments of the plakin repeat region and GFP (Fig. 1). These were expressed in stripes in the epidermis using the GAL4 system (Brand and Perrimon, 1993). The more NH₂-terminal segment was sufficient to target GFP to the lateral junctions, whereas the more COOH-terminal segment was uniform in the cytoplasm (Fig. 5, N and O). This suggests that first, the NH₂-terminal segment of the plakin repeat region contained targeting information for adherens junction localization, and second, this targeting is not provided by plakin repeats per se as both fusion proteins contained plakin repeats but only one was targeted to adherens junctions.

Different alleles of *shot* abolish different protein isoforms

We examined alleles of *shot* by immunofluorescence labeling of mutant embryos to assess whether they affected the ex-

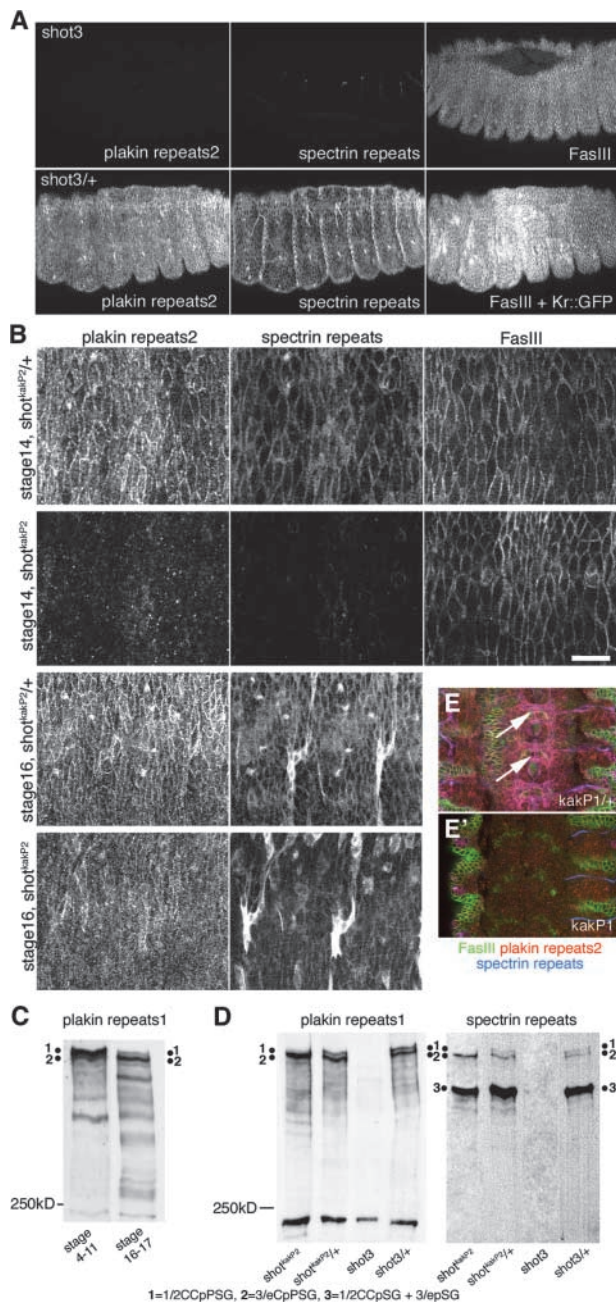


Figure 6. Analysis of Shot isoform expression in *shot* alleles. (A) Staining with both anti-Shot antibodies is lost in *shot*³ mutant embryos, compared with heterozygous siblings, whereas they still labeled with the control antibody against Fasciclin III (FasIII). The mutant embryos were distinguished by the absence of Kr::GFP on the balancer chromosome (in the same channel as FasIII). (B) In *shot*^{kakP2} embryos early (stage 14) expression of Shot is strongly reduced, whereas late (stage 16) expression is not affected, stained as in A. Bar, 20 μ m. (C) WB analysis shows that the majority of Shot isoforms in early wild-type embryos were the higher molecular mass, ABD containing isoforms (1), relative to later when the shorter isoform predominated (2). (D) Western analysis of extracts from *shot* mutant embryos and heterozygous siblings probed with anti-plakin repeat 1 and anti-spectrin repeat antibodies. The *shot*³ allele resulted in the absence of all shot isoforms, whereas *shot*^{kakP2} eliminates the highest molecular mass band (1) and some of the spectrin repeat only band (3). (E and E') The *shot*^{kakP1} allele eliminates the expression of Shot containing plakin and/or spectrin repeats from the nervous system (E') though not the epidermis (as shown for *shot*^{kakP2} in B,

pression of different Shot protein isoforms. The allele *shot*³, which behaves genetically as an amorphic/null allele (Lee et al., 2000), abolished labeling with plakin repeat antibody 2 and the spectrin repeat antibody (Fig. 6 A). This confirms that these antibodies are specific for the products of the *shot* gene, and that *shot*³ is a null allele. We also examined two alleles containing an identical insertion of a P-element after transcription start sites 2 and 1, but before 3 and e, *shot*^{kakP1} and *shot*^{kakP2} (Fig. 1; Gregory and Brown, 1998). The insertion is predicted to hinder transcription from the first two start sites, blocking production of Shot forms containing the full ABD, but not affect transcription from the second two start sites. Consistent with this, these alleles eliminated staining with the anti-ABD antibody (Gregory and Brown, 1998), which also suggests that the anti-ABD antibody does not recognize the partial ABD encoded by transcripts starting at the third promoter. At stage 14, *shot*^{kakP2} mutant embryos showed reduced epidermal staining with both plakin repeat and spectrin repeat Shot antibodies, compared with a control lateral membrane marker, Fasciclin III (FasIII), but by stage 16 staining appeared close to normal (Fig. 6 B). This suggests that earlier in development most Shot protein contains the full ABD in conjunction with the plakin and spectrin repeat domains, whereas later the forms containing plakin and spectrin repeats but lacking the full ABD will be made. This temporal change in expression pattern was confirmed by Western analysis (Fig. 6 C), as was the elimination of all isoforms in *shot*³, and just the ABD containing isoforms, one of which being the largest isoform containing all domains, in *shot*^{kakP2} (Fig. 6 D). Previous in situ analysis (Lee et al., 2000) indicated that isoforms lacking the full ABD were only expressed in the epidermis, whereas forms containing the ABD were expressed strongly in the nervous system and the epidermis. Therefore, we predicted that nervous system expression of Shot should be eliminated in the *shot*^{kakP1} allele, and this proved to be the case (Fig. 6, E and E'). Shot alleles that lack the largest plakin repeat isoform in mid-embryogenesis (stage 14), e.g., the P-insertion alleles *shot*^{kakP1} and *shot*^{kakP2}, have a weakly penetrant zygotic mutant phenotype consisting of rips in the epidermis (Gregory and Brown, 1998). This suggests that the function of the Shot isoforms containing the plakin repeats at the adherens junctions is to maintain epithelial integrity.

Loss of Shot causes a double-layering phenotype in the follicle epithelium

To test Shot's involvement in maintaining epithelial integrity, we analyzed a range of markers of epithelial junctions in embryos homozygous for the alleles *shot*³ and *shot*^{kakP1/2}, but did not observe any obvious defects for these markers (unpublished data). To remove any maternal contribution, we made *shot*³ germline clones, but due to a defect in oogenesis, no eggs were produced. Therefore, we turned our attention to another well-characterized epithelium in which Shot is expressed, the

and is also found for *shot*^{kakP1} compared with heterozygous control embryos (E). Three segments of the embryonic nerve cord are shown, arrows point to labeled axonal commissures, anterior is up, and staining with FasIII is used as a control.

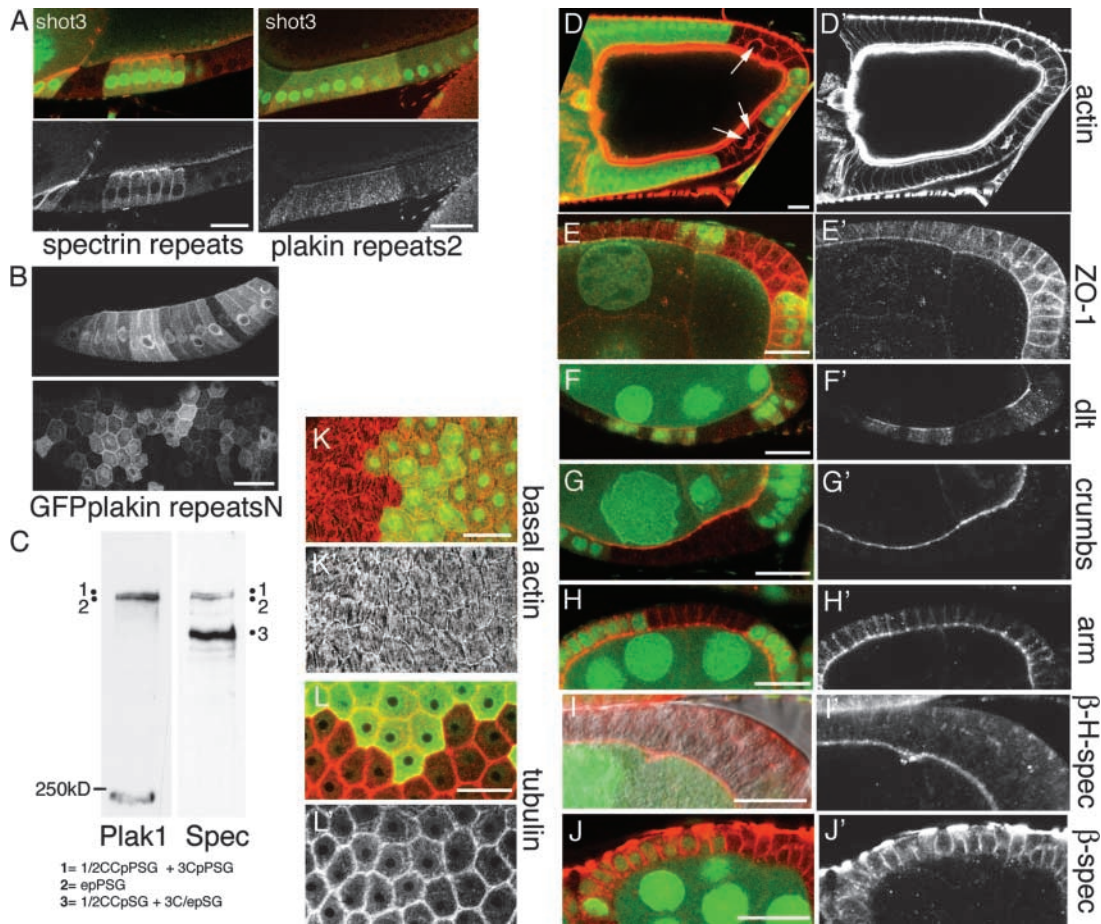


Figure 7. Shot isoforms containing the plakins repeats are expressed cortically in the follicle epithelium and are required for cell–cell adhesion. A and D–L show analysis of the follicle epithelium in mosaic egg chambers containing clones of cells mutant for *shot* that are marked by the absence of GFP. (A) Shot isoforms containing the plakins and spectrin repeats are expressed in follicle cells and localize to the cortex. Shown are wild-type cells (GFP positive) and mutant clones of the genotype *shot*³. The individual channels for the plakins and spectrin repeat staining are shown. Note that the plakins repeat antibody does not penetrate the egg chamber well, a common problem in this tissue. (B) The NH₂-terminal GFP fusion of the plakins repeats is localized cortically in the follicle epithelium. The top panel is a cross section of the follicle cells, whereas the bottom panel is scanned at the apical end of the lateral surfaces of the cells. The nuclear appearance of GFP may be due to unspecific targeting of GFP. (C) WB of ovaries showing that similar isoforms of Shot are expressed in this tissue as in the embryo (Figs. 4 and 6). (D–L) Analysis of different markers in *shot*³ mutant clones. (D and D') Loss of Shot leads to two layers of cells in the follicle epithelium, instead of the normal monolayer, as revealed by staining filamentous actin. Note that actin accumulated where mutant cells of the two layers contact each other (arrows). (E and E') ZO-1, a marker of adherens junctions in *Drosophila*, accumulates in a similar position to actin. (F and F') The apical polarity complex component Dlt is strongly reduced in *shot*³ mutant clones in late egg chambers. In contrast Crumbs, a member of the same complex is not affected (G and G'). (H and H') The adherens junction component β-catenin (Armadillo) is slightly reduced by loss of Shot. Epithelial polarity is maintained in the absence of Shot because β-spectrin (J and J') and β-heavy-spectrin (I and I') as markers of basal and apical domain integrity, respectively, are localized normally. Note that I shows the fluorescent channels overlaid with the transmission image to indicate that a double-layered clone is shown. In the middle of the figure, K and K' show the unaltered basal network of parallel actin fibers in the follicle epithelium, and L and L' show normal microtubule appearance in *shot*³ mutant clones. Bars, 20 μm.

follicle epithelium. During early stages of oogenesis, the follicle cells form a columnar epithelium that surrounds all 16 germ cells. As oocyte development progresses the follicle cells covering the oocyte remain columnar, whereas those overlying the 15 nurse cells become squamous. Antibodies against both spectrin and plakins repeats detected Shot at lateral and apical membranes of the follicle cells, the apical surface contacting the germ cells (Fig. 7 A). As in the embryonic epidermis, the NH₂-terminal GFP fusion of the plakins repeats localized to cell circumferences and lateral cell outlines (Fig. 7 B). WB analyses of ovary lysates revealed that the Shot isoforms expressed in the ovary resemble those in the embryo (Fig. 7 C; see Fig. 4 A for comparison).

To analyze the function of Shot in the follicle epithelium, clones of cells homozygous for the *shot*³ allele were generated using the FLP-FRT system (Xu and Rubin, 1993). By marking the wild-type allele of Shot with GFP, the mutant cells could be distinguished by the absence of GFP. *shot*³ clones of cells were often double-layered in egg chambers from stage 6–7 onwards (Fig. 7, D and D') and frequently showed actin accumulated at the contacts between the two layers (Fig. 7, D and D', arrows). Double-layered clones were only detected in cells overlying the oocyte, and at stage 10 of oogenesis were usually found in the posterior half of the follicle cells covering the oocyte. This phenotype was also observed in mutant clones generated from the allele *shot*^{hakPI} that abol-

ishes expression of isoforms containing the full ABD, including the largest isoform observed (unpublished data).

The perturbation of the integrity of the epithelial layer in the absence of Shot prompted us to analyze the localization of components of the adhesion and polarity complexes that are required for epithelial integrity (for reviews see Johnson and Wodarz, 2003; Perez-Moreno et al., 2003). These include components of the adherens junction, and the apical complex, which has recently been implicated in the assembly, positioning and maintenance of the adherens junction, and consists of the transmembrane protein Crumbs and the two cytoplasmic scaffolding proteins Stardust and Discs Lost (Dlt), (Bilder et al., 2003; Tanentzapf and Tepass, 2003). The adherens junction component β -catenin/Armadillo appeared slightly reduced in most, but not all *shot³/shot³* clones (Fig. 7, H and H'). In addition, ZO-1, a PDZ-protein associated with adherens junctions in *Drosophila* (Takahisa et al., 1996; Takahashi et al., 1998) accumulated aberrantly, concentrating at the contacts between the double-layered mutant cells, as actin did (Fig. 7, E and E'). Apical staining for Dlt was strongly reduced (Fig. 7, F and F'), although the localization of Crumbs (Fig. 7, G and G') and Stardust (not depicted) were not altered by the absence of Shot. These results demonstrate that Shot is essential for the stable association of the proteins Armadillo to adherens junctions and Dlt to the apical complex. As these proteins are important for cell polarity, it was possible that the double layering was due to loss of epithelial polarity rather than a loss of cell adhesion. However, cell polarity appeared normal in cells lacking Shot, as judged by the normal apical distribution of β -heavy-spectrin (Fig. 7, I and I') and lateral distribution of β -spectrin (Fig. 7, J and J').

Previous work demonstrated that the shorter forms of Shot lacking the plakin repeats have a role in epithelial cells in linking integrin adhesive junctions to the cytoskeleton (Gregory and Brown, 1998; Prokop et al., 1998). Therefore, we tested whether the double-layering phenotype was due to the loss of a similar integrin dependent process in the follicle epithelium. Integrins are expressed on the basal surface of the follicle epithelium and are needed to align parallel actin fibers at the basal side of all follicle cells to allow oocyte elongation (Bateman et al., 2001). Loss of integrins perturbs the arrangement of the basal actin fibers, but this was not observed in the absence of Shot (Fig. 7, K and K'). Microtubule organization and levels (Fig. 7, L and L'), integrin localization and oocyte elongation (not depicted) were normal in *shot³* mutant cells. In addition, Shot did not colocalize with integrins at the basal surface of follicle cells, but rather was found only at cell–cell contacts. This indicates that the loss of epithelial integrity observed in the absence of Shot is due to the loss of cell–cell adhesion, and not cell–matrix adhesion.

In summary, Shot is localized to adherens junctions in both the embryonic and follicular epithelia. Defects in epidermal integrity were observed as rips in the epidermis in *shot^{kakP1}* mutant embryos (Gregory and Brown, 1998), and as double layering of follicular epithelial cells mutant for *shot³* or *shot^{kakP1}*. The altered distribution of actin and ZO-1 and the reduction of Armadillo and apical Dlt in the absence of Shot demonstrates that Shot is essential for the organization of the apical adhesion belt protein complexes.

Discussion

The spectraplakin protein Shot was initially identified by our group and others as an important player in mediating integrin adhesion in *Drosophila* (Gregory and Brown, 1998; Strumpf and Volk, 1998). In this paper, we report on an integrin-independent function for spectraplakins that appears to be mediated by the largest protein isoforms: the maintenance of epithelial integrity. We provide biochemical evidence that giant spectraplakins proteins exist that contain the ABD, plakin repeats and spectrin repeats. This makes the largest isoforms of Shot (8,846 aa) the third largest protein in flies after dumpy and kettin (Kolmerer et al., 2000; Wilkin et al., 2000). The expression data are supported by EST and cDNA sequences (Fig. 1). The existence of similar mammalian isoforms encoded by the *MACF1* and *BPAG1* spectraplakin genes has been predicted from the analysis of mRNAs and cDNAs (Gong et al., 2001; Leung et al., 2001b). The Shot isoforms containing both plakin and spectrin repeats have a novel intracellular localization in the embryonic epidermis and the follicle epithelium: at cell–cell junctions in the zonula adherens. We have identified a portion of the plakin repeat domain that is sufficient to target GFP to adherens junctions indicating that the plakin domain is responsible for targeting these Shot isoforms to junctions. We are currently trying to identify the interacting proteins that lead to this localization.

In the epidermis of the early embryo, reduction of the largest isoform of Shot, which is the most abundant form at this stage (with the P-insertion alleles *shot^{kakP1}* and *shot^{kakP2}*) caused tears in the epidermis (Gregory and Brown, 1998), suggesting that the giant form containing all domains is required at adherens junctions to maintain cell adhesion. The low penetrance of this phenotype suggests that this function of Shot can in most cells be compensated for by other proteins of the junction. This redundancy was not found in the follicular epithelium, where loss of Shot caused the normal single layer of cells to become disorganized and double-layered in a majority of mutant clones. This double-layering phenotype is consistent with loss of lateral adhesion.

How do Shot isoforms containing the plakin repeats contribute to the integrity of cell adhesion? The loss of adhesion could arise from defects in establishing apical–basal polarity, the initial establishment of cell adhesion, or the maintenance of cell adhesion. The absence of Shot caused a slight reduction in junctional Armadillo and a stronger reduction of the apical complex component Dlt (Fig. 7), suggesting that Shot plays a role in their recruitment or maintenance. The Dlt–Crumbs complex is not only important for adherens junction assembly but helps to establish epithelial polarity (Medina et al., 2002; Roh et al., 2002; Bilder et al., 2003; Tanentzapf and Tepass, 2003). However, Shot does not seem to be involved in the establishment of polarity as the markers β -spectrin and β -heavy-spectrin were distributed normally (Fig. 7). Nor is Shot required for the initial assembly of adherens junctions because the phenotype in *shot* mutant clones did not appear until late during oogenesis, after adherens junctions have been established. Therefore, the loss of adhesion and the double layering observed in *shot* mutant clones is unlikely to be a secondary effect of a loss of apico–basal polarity or failure in the formation of adhesive junctions.

Crumbs and Dlt are linked together via Stardust and are only partially interdependent for their apical localization, so that a role for Shot in the stabilization of Dlt localization is fully consistent with previous results (Tanentzapf et al., 2000; Tepass, 2002; Bilder et al., 2003; Tanentzapf and Tepass, 2003). In some *dlt* mutant clones Crumbs can be retained in the apical membrane, suggesting a second mechanism to localize Crumbs, and the apical Dlt localization is only slightly reduced in *crumbs* clones. Reduction or absence of Armadillo does not abolish Dlt localization (Tanentzapf et al., 2000). Whether the loss of apical Dlt accumulation is cause or effect of the loss of adhesion in *shot* mutant clones remains to be elucidated.

Taking these findings into account, we propose that Shot aids in formation of the link between the adherens junction and the associated belt of actin filaments. Because the genetic evidence shows that the ABD is needed for function in the follicle epithelium (*shot*³ and *shot*^{kakP1} show the same phenotype), Shot could stabilize the adherens junction associated actin cytoskeleton by helping to link it to the membrane and/or cross-link it to microtubules that are associated with adherens junctions (Chausovsky et al., 2000; Waterman-Storer et al., 2000; Ligon et al., 2001). Our ability to visualize actin associated with adherens junctions is hindered by the high level of actin generally at the cortex and in the apical microvilli, thus, the normal appearance of actin in the absence of Shot does not rule out this proposed function. The association of part of the Shot plakin repeat domain with adherens junctions suggests that this could be the key region involved in attaching Shot to the membrane, leaving the ABD and GAS2 domain free for other interactions. Loss of Shot function may then cause a weaker link between the junction and the actin-based adhesion belt. During stage 9 of oogenesis the follicle cells undergo a rearrangement, when the cuboidal follicle cells that have surrounded the whole egg chamber up until that point start to concentrate over the oocyte and become columnar, whereas only a few anterior cells become squamous and cover the nurse cells (for review see Dobens and Raftery, 2000). The forces that occur during this follicle cell reorganization could lead to a rupture of weakened adherens junctions in the *shot* mutants, causing the observed double layering. The accumulation of actin could be due to the fact that the basal cell in a double layer tries to reestablish an apical surface, which is supported by the weak β -heavy-spectrin staining in between layers. ZO-1, a component of adherens junctions, accumulates with the actin, but at higher levels than in wild-type junctions. This may be a combination of these components in both cells of the bilayer or just abnormally elevated levels at the apical surface of the basal cell. The proposed role of Shot in stabilizing adherens junctions after their initial establishment is consistent with in vitro data analyzing the vertebrate Shot orthologue MACF1/ACF7 (Karakesisoglou et al., 2000). After induction of cell–cell contact in tissue culture cells, part of MACF1 localizes to sites of cell–cell contact, but with slower dynamics than integral components of the adherens junctions and desmosomes, suggesting that it is associated with preformed junctions.

It is important to note that an additional function for Shot in cell–cell contacts in the trachea has been described

previously (Lee and Kolodziej, 2002), but that in this case Shot functions in remodelling the cytoskeleton rather than cell–cell adhesion. In the absence of Shot, the tracheal cells fail to fuse and the specialized actin fibers and apical bundles of microtubules associated with cadherin contacts do not form normally. Shot isoforms containing either the ABD or the microtubule-binding GAS2 domain, but not the plakin repeats, can rescue these defects, showing that the largest isoforms containing the plakin repeats are not required. The role of Shot in the tracheal cells may, therefore, be more similar to its role in connecting microtubules to the plasma membrane in the tendon cells, rather than the function we have described in mediating the integrity of epithelial sheets.

How is the function of the largest Shot protein isoforms linked to the presence of the plakin repeats? Plakin repeat-containing isoforms of the *Drosophila* spectraplakins Shot seem to behave in a peculiar way. In their proposed function, i.e., maintenance of epithelial integrity, they rather resemble members of the spectrin family of proteins that have been shown to be important in organizing cortical domains at sites of adhesion (Belkin and Burridge, 1995; Pradhan et al., 2001). In contrast, the previously described Shot isoforms that lack the plakin repeats are involved in the link between integrin receptors and the cytoskeleton, a “classical” plakin protein function. The difference in usage of these isoforms may have arisen in flies because the usual plakin repeat binding partner, cytoplasmic intermediate filaments, is missing, freeing this domain to adopt a new function. Alternatively, stabilizing adherens junctions through the plakin repeat-containing largest isoforms may be a conserved intermediate filament-independent function of all spectraplakins.

The adherens junction recruitment signal comprises only part of the plakin repeat domain, leaving the other part of the domain available for additional functions. The plakin repeats in *Drosophila* appear to be always incorporated into the middle of the protein, whereas EST and cDNA data from BPAG1 in mouse show two different ways of incorporation of the repeats: in the middle in BPAG1a/b, and at the very end in BPAG1e (with no spectrin repeats or GAS2 domain being incorporated; Leung et al., 2001b). In *C. elegans* the orthologue of Shot, vab-10, is expressed in two distinct protein isoforms (Bosher et al., 2003 and unpublished data): one resembles the initially described dystrophin-like isoform of Shot and the other one resembles BPAG1e and ends with the plakin repeat domain. For all isoforms ending with plakin repeats, the plakin repeat region either has been shown to bind intermediate filaments, or be required for the link to intermediate filaments. We would speculate that plakin repeats have alternative binding partners if found in the middle of a protein. It will be interesting to see if the interaction of the internal plakin domains with proteins at the adherens junctions is conserved in the mammalian spectraplakins. Demonstrating that a domain makes different protein interactions depending on whether it is in the middle versus the end of a protein would provide new insight into how molecular interactions can be regulated through differential splicing of highly modular proteins.

Materials and methods

Fly strains

The Shot alleles stocks used were: *FRTG13 shot³/Cyo Kr::GFP*, *shot^{k03010}/Cyo Kr::GFP* (kakP1), and *shot^{k034050}/Cyo Kr::GFP* (kakP2). The UAS-GFP plakin repeats/N/C constructs were expressed with the following Gal4 lines: *patched-Gal4* in the embryo and *Cy2-Gal4* in the follicle epithelium. Mutant clones were induced in flies of the genotypes: *hsFLP; FRTG13 shot³/FRTG13 2xUbq::GFP* or *hsFLP; FRTG13 shot^{k03010}/FRTG13 2xUbq::GFP*. To induce mitotic recombination, larvae were heat shocked for 2 h at 37°C at the end of second instar and analyzed after 10 d, or adults were heat shocked for 2 h at 37°C and analyzed after 5 d, or both treatments were combined.

In situ hybridization

In situ hybridization of whole-mount embryos was performed as described previously by Tautz and Pfeifle (1989). Images were obtained by photography on a microscope (model DMR; Leica) with a Spot digital camera (Diagnostic Instruments) and composites were assembled using Photoshop (Adobe Systems).

Production of polyclonal antibodies and GFP fusion proteins

To generate polyclonal antisera against the plakin repeat domain of Shot, antigens were prepared from DNA fragments consisting of nucleotides 900–1,845 (plakin repeats 1) and 7,755–8,850 (plakin repeats 2) of the large plakin repeat exon expressed in bacteria using the pGEX system. GFP fusion proteins in inducible UAS vectors were generated by subcloning nucleotides 1,400–4,100 (GFPplakin repeats N) and nucleotides 6,000–9,000 (GFPplakin repeats C) of the large plakin repeat exon into pUASp with GFP at the NH₂ terminus.

Embryo and ovary extracts, IP, and Western blotting

Embryos were collected overnight or at the stages indicated, dechorionated in 50% bleach for 3 min and rinsed in water. Embryos were homogenized in at least five times their volume of solubilization buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) in a glass homogenizer on ice. The lysates were incubated on ice for 30 min and centrifuged for 10 min at 10,000 rpm at 4°C. The upper lipid layer formed after centrifugation was removed and the clear lysate used for immunoblotting and IPs. To obtain ovary lysates, well-fed females were dissected and the ovaries were solubilized in five volumes of solubilization buffer as described in this paragraph for embryo lysates.

Aliquots of 15 μl of embryo lysate per lane or 5 μl of ovary lysate per lane were run on a 4.2% separating gels and transferred onto PVDF membrane as described previously (Gregory and Brown, 1998). Rainbow markers (Amersham Biosciences) were used to indicate mobilities of 250 and 160 kD. Western analysis was performed as described previously in Röper et al. (2000). Anti-Shot antisera were diluted 1:200 for anti-ABD (Gregory and Brown, 1998); 1:4,000 for anti-plakin repeats 1; 1:2,000 for anti-plakin repeats 2; and 1:4,000 for anti-spectrin repeats (Strumpf and Volk, 1998). Antibodies were revealed by chemiluminescence using the Western blotting detection reagents (Amersham Biosciences).

For each IP, 400 μl of lysate diluted to 1 ml in solubilization buffer were incubated with the primary antibody (1:200 for anti-ABD; 1:200 for anti-plakin repeats 1; 1:150 for anti-plakin repeats 2; and 1:250 for anti-spectrin repeats) overnight at 4°C. Immunocomplexes were collected by incubation with protein A-sepharose. Proteins were eluted from the beads by boiling in sample buffer.

Immunofluorescence and confocal analyses

Embryos were collected at the indicated stages of development, and processed for immunofluorescence using standard procedures. Ovaries were dissected from well-fed females and processed for fluorescence using standard procedures. Primary antibodies were diluted 1:200 for anti-plakin repeats 2; 1:250 for anti-spectrin repeats (provided by T. Volk, The Weizmann Institute of Science, Israel; Strumpf and Volk, 1998); 1:500 for anti-PY (Sigma-Aldrich); 1:500 for anti-disc large (provided by R. Fehon, Duke University, Durham, NC; Fehon et al., 1994); 1:150 for ZO-1 (provided by M. Takahisa, Mitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo, Japan; Takahisa et al., 1996); 1:600 for anti-Dlt (provided by M. Bhat, Mount Sinai School of Medicine, New York, NY; Bhat et al., 1999); 1:500 for anti-Crumbs (provided by U. Tepass, University of Toronto, Toronto, Ontario, Canada; Tanentzapf et al., 2000); 1:500 for anti-Stardust (provided by E. Knust, Heinrich-Heine-Universität, Düsseldorf, Germany; Bachmann et al., 2001); 1:500 for anti-β-heavy-spectrin (provided by D.P.

Kiehart, Duke University, Durham, NC; Thomas and Kiehart, 1994); 1:200 for anti-β-spectrin (provided by L. Goldstein, University of California San Diego, La Jolla, CA; Byers et al., 1989); and 1:6 for anti-FasIII and 1:100 for antiarmadillo (both from Developmental Studies Hybridoma Bank). Confocal images were obtained using a confocal microscope (model Radiance 2000; Bio-Rad Laboratories). Confocal laser, iris, and amplification settings in experiments comparing intensities of labeling were set to identical values. Confocal pictures were assembled in Adobe Photoshop.

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