Crumbs interacts with moesin and $\beta_{\text{Heavy}}$-spectrin in the apical membrane skeleton of Drosophila

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The apical transmembrane protein Crumbs is necessary for both cell polarization and the assembly of the zonula adherens (ZA) in Drosophila epithelia. The apical spectrin-based membrane skeleton (SBMS) is a protein network that is essential for epithelial morphogenesis and ZA integrity, and exhibits close colocalization with Crumbs and the ZA in fly epithelia. These observations suggest that Crumbs may stabilize the ZA by recruiting the SBMS to the junctional region. Consistent with this hypothesis, we report that Crumbs is necessary for the organization of the apical SBMS in embryos and Schneider 2 cells, whereas the localization of Crumbs is not affected in karst mutants that eliminate the apical SBMS. Our data indicate that it is specifically the 4.1 protein/ezrin/radixin/moesin (FERM) domain binding consensus, and in particular, an arginine at position 7 in the cytoplasmic tail of Crumbs that is essential to efficiently recruit both the apical SBMS and the FERM domain protein, D.Moesin. Crumbs, Discs lost, $\beta_{\text{Heavy}}$-spectrin, and D.Moesin are all coimmunoprecipitated from embryos, confirming the existence of a multimolecular complex. We propose that Crumbs stabilizes the apical SBMS via D.Moesin and actin, leading to reinforcement of the ZA and effectively coupling epithelial morphogenesis and cell polarity.

Introduction

The functions of an epithelium depend on the polarized organization of its individual epithelial cells. The acquisition of a fully polarized phenotype involves a cascade of complex events including cell–cell adhesion, assembly of a lateral cortical complex, reorganization of the cytoskeleton, and polarized targeting of transport vesicles to the apical and basolateral membranes (Yeaman et al., 1999). Genetic studies in Drosophila have further revealed evidence for apical, lateral, and basal cues for epithelial polarization (Knust, 2000; Tanentzapf et al., 2000). Crumbs is an apical transmembrane protein that is responsible for organizing the apical pole in the fly and is expressed in all primary epithelia of Drosophila where it is concentrated just above the zonula adherens (ZA)* at the apical–lateral domain boundary (Tepass et al., 1990). Crumbs overexpression results in expansion of the apical domain (Wodarz et al., 1995), whereas loss of Crumbs disrupts the polarity of epithelial cells causing the breakdown of epithelial tissues (Tepass et al., 1990). In crumbs mutants, the ZA fail to coalesce at the apicolateral border, suggesting that Crumbs is involved in organizing this junctional structure, and thus in determining the location of the border between the apical and the lateral domains (Grawe et al., 1996; Tepass, 1996).

Surprisingly, most of the polarity functions in crumbs mutants are rescued by expression of its transmembrane and short cytoplasmic domains, suggesting that the major interactions regulating cell polarity and shape in the embryo are mediated by the 37 intracellular amino acids of this large (2,139 amino acids) protein (Wodarz et al., 1993). This hypothesis is reinforced by the observation that a nonsense mutation in the crumbs$^{\text{fido}}$ allele, preventing the translation of the last 23 amino acids of the cytoplasmic tail, produces a severe loss of function phenotype (Wodarz et al., 1993). Furthermore, it has been shown that the MAGUK family protein that is responsible for organizing the apical pole in the fly is expressed in all primary epithelia of Drosophila where it is concentrated just above the zonula adherens (ZA)* at the apical–lateral domain boundary (Tepass et al., 1990).

*Abbreviations used in this paper: B16, $\beta_{\text{Heavy}}$-spectrin; Crb: crumbs; DE, Drosophila epithelial; Dlt, discs lost; FERM, 4.1 protein/ezrin/radixin/moesin; mAb, monoclonal antibody; PDZ, PSD-95/DLG/ZO-1; ROK, Rho-associated kinase; S2, Schneider 2; SBMS, spectrin-based membrane skeleton; Sdt, stardust; VSV-G, vesicular stomatitis virus–protein G; ZA, zonula adherens.

Key words: epithelial polarity; zonula adherens; Drosophila; spectrin; D.Moesin

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member Stardust (Sdt) binds to the last four amino acid resi-
dues (ERL1) of the cytoplasmic tail of Crumbs, along with the
PSD-95/DLG/ZO-1 (PDZ) domain protein Discs-lost
(Dlt) (Bhat et al., 1999; Klebes and Knust, 2000; Bachmann
et al., 2001; Hong et al., 2001). These two proteins are both
required for epithelial polarity, and thus Crumbs, together
with Dlt and Sdt, defines a membrane–associated complex
in the apical cytocortex of epithelial cells that is necessary for
the proper generation of the polarized phenotype. Addi-
tional contributions from lateral proteins below the ZA,
such as Scribble, are also involved in maintaining polarity
and the integrity of the ZA. Because loss of scribble function
results in a phenotype reminiscent of Crumbs overexpres-
sion, it has been suggested that the position and integrity of
the ZA arises from a balance between the Crumbs-Dlt/Sdt
complex at the apical border and the Scribble network on its
basal side (Bilder and Perrimon, 2000).

Spectrins are long, tetrameric, F-actin–crosslinking pro-
teincomplexes comprised of two α and two β subunits (for review
see Bennett and Baines, 2001). The spectrin-based mem-
brane skeleton (SBMS) is a branching cytoskeletal network
of spectrin-crosslinked F-actin associated with the various
membrane compartments in the cell. Each SBMS is bound
to the membrane via interaction with integral membrane
proteins and phospholipids (De Matteis and Morrow,
2000). At the plasma membrane, spectrin, in conjunction
with cortical F-actin, provides a structural basis for modula-
tion of cell shape and membrane stability in both epithelial and
nepithelial cells. In Drosophila, a single α-spectrin isoform
combines with either of two, structurally distinct β-isomers
(β-spectrin and βheavy-spectrin [βH]) to produce (αβ)2 and
(αβH)2 tetramers, respectively. In epithelial cells of Drosoph-
ila, (αβ)2 tetramers are restricted to the basolateral mem-
brane, while the (αβH)2 tetramers localize to the apical
membrane and the ZA (Dubreuil et al., 1997; Lee et al.,
1997; Thomas et al., 1998; Thomas and Williams, 1999).

All three spectrin subunits are essential for normal devel-
opment. βH, encoded by the karst locus, is an essential pro-
tin that is required for epithelial morphogenesis (Thomas
et al., 1998). karst mutant cells exhibit altered shapes and dis-
ruption of the ZA indicating that (αβH)2 contributes to the
integrity of the latter, but is not necessary for apico basal
polarity per se (Zarnescu and Thomas, 1999). Similarly, com-
plex phenotypes are caused by mutations in the fly α– and
β-spectrin genes as well as in the orthologous genes in Caen-
norhabditis elegans (Lee et al., 1993; de Cuevas et al., 1996;
Dubreuil et al., 1998; McKeown et al., 1998; Dubreuil et
al., 2000; Moorthy et al., 2000). Together, these studies in-
dicate that the SBMS has an essential role in cell structure
and morphogenesis (for review see Thomas, 2001), making
the identification of proteins that recruit and/or organize
this structure of considerable interest.

Spectrins are generally recruited to the membrane via
adapter proteins that link the SBMS to integral membrane
proteins (Bennett and Baines, 2001). Two families of such
adapter proteins have been well characterized: ankyrins and
protein 4.1 family members. The former binds to the midre-
gion of the β-spectrin spectrin repeat array (Lombardo et al.,
1994), whereas the latter forms a ternary complex between
the actin-binding domain of β-spectrin and F-actin itself (Marfata
et al., 1997). Protein 4.1 is part of a larger superfamily of pro-
teins containing protein 4.1/ezrin/radixin/moesin (FERM) do-
 mains (Chishiti et al., 1998) that function to attach cortical
F-actin to a variety of integral membrane proteins (Tsukita
and Yonemura, 1999). The existence of multiple adapter protein
genescan, as well as alternatively spliced isoforms, generates great
diversity in the number of proteins to which an SBMS can be
attached (see De Matteis and Morrow, 2000 for a list of almost
50 spectrin associated proteins). The recruitment of conven-
tional β-spectrins by adapter proteins is well characterized
(e.g., Jenkins and Bennett, 2001), however, the cues recruiting
spectrin to the apical domain are currently uncharacterized, as
are the adapter proteins that associate with the βH isoform.

Overexpression of Crumbs in the embryonic ectoderm
causes an enlargement of the apical membrane and a concom-
itant expansion in the distribution of βH staining (Wodarz
et al., 1995). This result suggested that this apical polarity cue
might also be responsible for recruiting and/or organizing
the apical SBMS. To investigate this possibility, we looked for
ge netic and physical interactions between βH and Crumbs. In
this paper, we report that at least one allele of crumbs is a
dominant enhancer of the karst phenotype, and that whereas
the Crumbs distribution is unaffected in karst mutants, βH is
mislocalized in the epithelial cells of crumbsβH500 mutants.
Furthermore, overexpression of Crumbs led to redistribution
of βH, DMoesin, and actin, indicating that Crumbs acts up-
stream of βH in organizing the apical SBMS. We also de-
scribe that clustering of a chimeric-tagged form of Crumbs in
Schneider 2 (S2) cells induces cocapping of βH and DMoesin.
This provides evidence for a relationship between Crumbs
and these two proteins under physiological conditions. This
interaction is dependent on a consensus motif for the bind-
ing of proteins of the FERM family in the cytoplasmic tail
of Crumbs. Finally, we show that Dlt, Crumbs, βH, and
DMoesin coimmunoprecipitate, indicating that a multipro-
tein complex is recruited by Crumbs. These results indicate
that Crumbs mediates a novel coordination between cell po-
larity, junctional stabilization, and morphogenesis.

Results

The membrane organization of βH depends on Crumbs

Previously, we have shown that βH exhibits a very close colo-
calization with the adhesion protein Drosophila epithelial
(DE)-cadherin at the light microscope level throughout ZA
formation and at the mature junction (Thomas et al., 1998;
Thomas and Williams, 1999; Fig. 1 A for a schematic dia-
gram of the organization of junctions in Drosophila epithe-
lia). Not surprisingly, βH and Crumbs exhibit a similarly
close apposition at this level of resolution (Fig. 1 B, left), as
Crumbs is located at the apical margin of the ZA (marginal
zone; Tepass, 1996). βH clearly colocalizes with DE-cadherin
at times when Crumbs is not present (e.g., during early cellu-
larization; see Thomas and Williams, 1999) and exhibits reg-
ulatory changes reflecting the area of the ZA itself in early
eye development (Thomas et al., 1998). However, strenuous
efforts to localize βH at the ultrastructural level have been
unsuccessful for some time, leaving unresolved the issue of
whether the βH domain only lies at the ZA or in the mar-
ginal zone, or encompasses both in mature epithelia.
Overexpression of the Crumbs cytoplasmic tail causes a concomitant expansion in the distribution of \( \beta_H \) (Wodarz et al., 1995), suggesting that Crumbs might be responsible for recruiting and/or organizing \( \beta_H \) in normal cells. Therefore, we looked to see if the distribution of \( \beta_H \) was perturbed in \( \text{crumbs}^{8F105} \) mutant embryos. Crumbs itself is mislocalized to the cytoplasm and to a lesser extent the apical domain of the cell.

### crumbs is a dominant enhancer of karst

Given the close functional and spatial relationship between \( \beta_H \) and the ZA (Thomas and Williams, 1999; Zarnescu and Thomas, 1999), we looked for a genetic interaction between \( \text{karst} \) and \( \text{crumbs} \). Such an interaction is likely to be modest due to the existence of multiple pathways for recruiting \( \beta_H \) (see Discussion). Furthermore, all \( \text{karst} \) alleles isolated to date exhibit variable expressivity necessitating a statistical approach. The interaction test was thus limited to the most readily quantified feature of the pleiotropic \( \text{karst} \) phenotype, the degree of lethality. Comparison of viability rates between \( \text{karst crumbs} / \text{karst} \) and \( \text{karst} / \text{karst} \) genotypes reveals a statistically significant enhancement of lethality in the presence of one mutant \( \text{crumbs}^{11A22} \) allele (\( \text{crb}^{2} \)) significantly increased lethality in all genotypic combinations (\( * = P < 0.05; ** = P < 0.01 \)). See Materials and methods for details on the statistical analysis of these data. Error bars represent 95% confidence intervals.

### A Crumbs/D\text{Moesin}/\beta_H complex in embryos

In order to test for a physical interaction between Crumbs and \( \beta_H \), we performed coimmunoprecipitations using an affinity-purified antibody to \( \beta_H \) and embryo extracts (Fig. 3).
The βII immunoprecipitates were probed for α-spectrin, a known partner of βII, or for Crumbs. Both proteins were present in βII immune complexes. As expected, Dlt also coimmunoprecipitated Crumbs under the same conditions. Control immunoprecipitations with a polyclonal rabbit anti–mouse (Fig. 3) or an irrelevant antiserum against Bub3 (unpublished data) did not bring down Crumbs, indicating that this interaction between Crumbs and βII was specific.

Spectrin is known to bind indirectly to transmembrane proteins via adapter proteins such as ankyrin and protein 4.1 family members (Introduction). Moreover, it has been speculated that the juxtamembrane region of the Crumbs cytoplasmic domain contains a consensus binding motif for a FERM domain protein (Klebes and Knust, 2000). The Drosophila FERM domain protein, DMoesin, localizes in the apical region of epithelial cells (McCartney and Fehon, 1996), and has been associated with the regulation of cell shape changes (Edwards et al., 1997). Double staining with DMoesin and Crumbs on WT embryos indicates that these two proteins are in close proximity (Fig. 4 A), suggesting that these two proteins could interact. Therefore, we immunoprecipitated DMoesin from WT embryos and probed the immunoprecipitates for the presence of Crumbs by immunoblotting (Fig. 4 B, left). The presence of Crumbs in the immune complex indicates that the two proteins are in a common protein complex. This was confirmed by the fact that immunoprecipitation of Dlt from embryonic extracts also brought down DMoesin (Fig. 4 B, right). It was not possible to test whether both DMoesin and βII coimmunoprecipitated with Crumbs, because the only available antibody against the extracellular domain of Crumbs fails to immunoprecipitate it (unpublished data).

To determine if this complex exists in vivo, we examined embryos expressing the chimeric Crumbs protein, Crumbs\textsuperscript{myc-intra} (Wodarz et al., 1995) under the regulation of the Gal4 binary expression system (Brand and Perrimon, 1993). Crumbs\textsuperscript{myc-intra} is widely distributed on the cell membrane outside of the normal Crumbs domain when overexpressed in ectodermal cells (Wodarz et al., 1995). To provide a side-by-side comparison with normal cells we used the engrailed-Gal4 driver to limit expression to cells at the posterior border of each segment in the ectoderm of the embryo. We find that expression of this protein causes an identical mis-distribution of DMoesin, βII and actin (Fig. 5) indicating that this complex forms in vivo and that Crumbs is linked to the actin cytoskeleton.

The Crumbs cytoplasmic domain induces accumulation of Discs lost, DMoesin, and βII in transfected S2 cells

Next, we turned to S2 cells in culture in order to try and understand how Crumbs, βII, and DMoesin interact in vivo. S2 cells do not express Crumbs (Wodarz et al., 1993), but have been shown to express βII (Dubreuil et al., 1997) and DMoesin (McCartney and Fehon, 1996). Because Dlt is a key component of the Crumbs pathway (Bhat et al.,...
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We used anti-Dlt antibodies to probe S2 homogenates by immunoblotting and found that Dlt is expressed at significant levels in S2 cells (unpublished data). Thus, S2 cells offer a suitable model to understand some aspects of Crumbs’ molecular networks using transfected Crumbs constructs.

A Crumbs construct in which most of the extracellular domain was replaced by an epitope of vesicular stomatitis virus–protein G (VSV-G) (Fig. 6 A; recognized by the monoclonal antibody [mAb] P5D4), was stably expressed in S2 cells. The encoded crumbs (CRB)–VSV-G WT protein is transported to the cell surface where it is recognized by both the P5D4 mAb and a polyclonal antibody raised against the cytoplasmic domain of Crumbs (Fig. 6 B; see Materials and methods). This protein is equivalent to the Crumbsmyc-intra protein that we used in the overexpression experiments in embryos (Fig. 5).

H accumulates at the plasma membrane in adherent S2 cells (Dubreuil et al., 1997), but not when these cells are grown in suspension (Fig. 7, untransfected cells), whereas Dmoesin is always associated with the plasma membrane. CRB–VSV-G WT expression caused no conspicuous change in the distribution of Dmoesin or H in adherent cells with both proteins colocalizing at the plasma membrane (unpublished data).

We further investigated the possibility of a molecular association between Crumbs, Dmoesin, and H using the technique of capping. CRB–VSV-G WT was concentrated in patches on the surface of transfected S2 cells growing in suspension by treatment with the P5D4 mAb and a polyclonal anti-mouse antibody coupled to FITC. Capping with the secondary antibody was allowed to proceed for 5 minutes at room temperature (Fig. 7). Not only was Dlt recruited to the patches containing CRB–VSV-G WT as expected (Fig. 7 A, top), but both H and Dmoesin were also concentrated at such regions, indicating that there is indeed a link between Crumbs and these proteins in vivo (Fig. 7, B and C, top). Typically, a background level of ~25% nonspecific

![Figure 5](image)

**Figure 5.** Overexpression of Myc-intraWT leads to the redistribution of βH, Dmoesin, and actin. Confocal micrographs of part of the epidermis of a stage 13/14 embryo. Cells at the posterior margin of each segment are expressing Myc-intraWT driven by an engrailed-Gal4 driver. Embryos were stained for Myc and βH, Dmoesin, (Dmoesin), or actin (Act) as indicated. βH, Dmoesin, and actin are all redistributed along with Myc-intraWT (arrows). Bar, 5 μm.

![Figure 6](image)

**Figure 6.** Expression of CRB–VSV-G WT in S2 cells. (A) Sequences of the CRB–VSV-G fusion proteins expressed in this study. CRB–VSV-G WT is a fusion of the VSV-G epitope with the stalk region, transmembrane domain and the intracellular domain of Crumbs. Stop mutations in position 6 or 15 truncate the intracellular domain of Crumbs resulting in a cytoplasmic domain of 5 and 14 amino acids in variants CRB–VSV-G S6 and CRB–VSV-G 8F105, respectively.

CRB–VSV-G 8F105 Y10A and R7A are CRB–VSV-G 8F105 constructs with point mutations (asterisk) replacing Tyr10 and Arg7 with an alanine, respectively. (B) CRB–VSV-G WT expression was induced in stably transfected S2 cells and cells were fixed and double labeled with a mouse anti–VSV-G and a rabbit anticytoplasmic domain of Crumbs antibodies followed by FITC-conjugated anti–mouse (left) and TRITC-conjugated anti–rabbit (middle) antibodies. The two antibodies stained the same subcellular structures and in particular the plasma membrane (right). Bar, 10 μm.
capping was observed with this assay, and therefore was subtracted from all the percentages reported below. Using this procedure, a robust average of ~50% capping was seen with CRB–VSV-G WT after removal of the background, despite the fact that S2 cells exhibit some heterogeneity in their level of expression of Dmoesin and βII.

To investigate the role of the cytoplasmic domain of Crumbs for the interaction with Dmoesin or βII, we expressed the truncated construct CRB–VSV-G S6 in which only the first five amino acids of the cytoplasmic domain remained (Fig. 6 A). This mutation prevented binding to Dlt in a GST pulldown assay (unpublished data) and essentially eliminated the ability to cluster Dlt as expected (Fig. 7 A, bottom). CRB–VSV-G S6 was also unable to efficiently recruit Dmoesin and βII, indicating that the cytoplasmic domain of Crumbs was also necessary for the interaction with βII and Dmoesin (Fig. 7 B and C, bottom).

The Crumbs FERM domain binding site is required to efficiently recruit both Dmoesin and βII.

The capping technique provides a readily quantifiable assay for interactions between the Crumbs cytoplasmic domain and other proteins. Thus, we next used it to determine which part of the cytoplasmic domain of Crumbs is necessary for the interaction with Dmoesin and βII. A second truncation mutant, CRB–VSV-G 8F105 (Fig. 6 A), that mimics the crumbs8F105 allele with a stop codon at position 15 of the cytoplasmic domain (Wodarz et al., 1993), was able to recruit βII and Dmoesin just as efficiently as CRB–VSV-G WT, but could no longer bind to Dlt as predicted (Fig. 8). These results indicate that the distal part of the Crumbs cytoplasmic domain is not crucial for the Dmoesin/βII–Crumbs interaction, in contrast to the interaction between Crumbs and Dlt (Bhat et al., 1999; Klebes and Knust, 2000) and Crumbs and Sdt (Bachmann et al., 2001; Hong et al., 2001).

Two motifs in the NH2-terminal and the COOH-terminal regions of the cytoplasmic domain of Crumbs are necessary for the rescue of a normal polarized phenotype in

Figure 7. Dlt, βII, and Dmoesin colocalize with capped CRB–VSV-G WT in transfected S2 cells. CRB–VSV-G WT (A–C, top) or S6 (A–C, bottom) were transiently expressed in S2 cells, followed by capping and staining with mouse anti–VSV-G antibody and fluorescein-conjugated anti–mouse antibody before fixation. After fixation and permeabilization, cells were additionally stained for either Dlt (A, Dlt), βII (B, βII), or Dmoesin (C, Moe). Dlt, βII, and Dmoesin were all redistributed to capped sites with CRB–VSV-G WT indicating a connection between these proteins and the cytoplasmic domain of Crumbs in S2 cells (arrows highlight specific examples). Bar, 10 μm.

Figure 8. Quantitative analysis of βII recruitment to CRB–VSV-G cap sites. S2 cells expressing the different CRB–VSV-G constructs were scored for Dlt, βII, or Dmoesin (Moe) colocalization after capping with anti–VSV-G and secondary antibodies. Results are expressed as the mean percentage of cocapping seen for each protein from three independent experiments (except for Moe 8F105, Y10A, and S6, which were performed twice, and Dlt 8F105 which was done once).
**Discussion**

The *crumbs–Dlt–Sdt* pathway is essential for polarity and has been shown to be a major apical signal for establishing the ZA at the apical–lateral boundary (for reviews see Knust, 2000; Muller, 2000; Bilder, 2001). The observation that mutations affecting βH and Crumbs both cause a junctional phenotype, along with the close colocalization of both proteins in the marginal zone of epithelial cells, suggested a possible connection between the activities of these two proteins. Here we report evidence that Crumbs can recruit apical βH, together with the FERM domain protein DMoesin and βH. Our data are in good agreement with the hypothesis that polarity cues are used to organize the SBMS (Yeaman et al., 1999). Together, the above data are consistent with the multifunctional nature of spectrin membrane skeletons and with the idea that specific pathways recruit the SBMS to establish spatially distinct polarized membrane domains, whereas general COOH-terminal membrane association domains permit tight membrane association and network integration (Lombardo et al., 1994; Bennett and Baines, 2001).

**The cytoplasmic domain of Crumbs recruits DMoesin and βH**

The previously reported partial rescue of crumbs mutants by the *crumbs*+/myc-intra construct (Wodarz et al., 1995) suggested that the transmembrane and cytoplasmic domains of Crumbs might be sufficient to concentrate βH to some areas of the apical membrane. We have confirmed and extended this result, showing that the critical region for recruiting βH is just 9 amino acids from position 6 through 14 of the cytoplasmic domain in the putative FERM domain binding site (Klebes and Knust, 2000). Within this region, a conserved tyrosine residue at cytoplasmic domain position 10 (crucial for Crumbs function in vivo; Klebes and Knust, 2000) and an arginine at position 7 are both required for this activity. It is worth noting that all Crumbs genes cloned so far contain a charged amino acid residue at position 7 in the cytoplasmic domain (see Klebes and Knust, 2000), suggesting that this is an evolutionarily conserved interaction site.

FERM domains are found in the protein 4.1 family of proteins which link the SBMS to cell-surface receptors (Hoover and Bryant, 2000) as well as several other proteins which organize the cortical actin (ezrin/radixin/moesin; Bretscher, 1999; Tsukita and Yamamura, 1999). The founding member of this group, protein 4.1, was originally identi-
fied as a major component of the erythrocyte SBMS where it facilitates the interaction of spectrin with actin and the transmembrane protein Glycophorin C (Marfatia et al., 1997). Therefore, the presence of a conserved FERM binding domain in the Crumbs cytoplasmic domain suggests that Crumbs may bind to βH via a FERM domain protein.

In *Drosophila*, the FERM domain family includes the proteins Coracle, DMerlin, DMoesin, and Expanded (McCarty and Fehon, 1996). Of these four proteins, Coracle is an unlikely candidate to bind to the Crumbs juxtamembrane domain since it is localized to the septate junctions basal to the ZA (Fehon et al., 1994). However, the DMerlin, DMoesin, and expanded proteins are localized in part or in whole at the ZA region in epithelia (McCarty and Fehon, 1996; Boedigheimer et al., 1997), and could thus be involved in the interaction between Crumbs and βH. The fact that none of protein 4.1 family members known in *Drosophila* contains a spectrin-binding domain as defined by the archetypal protein 4.1 does not necessarily abrogate this hypothesis. βH-spectrin is clearly recruited to the membrane by different mechanisms than its basolateral counterpart (Dubreuil and Grushko, 1999), and this specificity would likely be reflected in divergent interaction domains. In this work, we have found that βH and DMoesin can both coimmunoprecipitate Crumbs. Furthermore, our capping assay and embryo expression evidence provide in vivo support for this result. Not only will DMoesin cocap with the Crumbs cytoplasmic domain, it is dependent on exactly the same sequences that recruit βH. These results, together with the existence of the consensus binding site for a FERM domain protein in Crumbs, strongly support the hypothesis that DMoesin forms a bridge between Crumbs and the SBMS (see model in Fig. 9). A functional test of this relationship must await mutations in the DMoesin locus become available. Thus, the current data, although highly suggestive, do not formally distinguish between the possibility of a DMoesin bridge between Crumbs and the SBMS, and the existence of two separate complexes with direct interaction between Crumbs and βH or DMoesin in each. Significantly, actin did not cap consistently with Crumbs in S2 cells and was not present in our immunoprecipitates (unpublished data). This suggests that other components present in epithelial cells are necessary for stabilization of the actin skeleton around the Crumbs complex. It also indicates that βH is specifically recruited to the proposed complex and is not merely a passive arrival along with bulk actin.

Our results indicate that Crumbs interacts with at least two different protein networks, a DMoesin/Spectrin/actin-based network and a PDZ protein scaffold (Dlt/Sdt). However, it is unclear at present whether Dlt/Sdt and DMoesin/βH/actin coexist in the same complex with Crumbs. In the erythrocyte model, glycophorin C is linked to spectrin via a ternary complex containing protein 4.1 and the PDZ domain protein p55 bound to a topologically similar pair of binding sites to the two functional regions identified in the Crumbs cytoplasmic domain (Marfatia et al., 1997). If such a ternary complex forms in association with Crumbs, then the observation that the Sdt-binding domain of Crumbs is not required for the interaction between Crumbs and βH, would indicate that the latter cannot be dependent on Dlt/

![Figure 9. Model for the protein interactions in the Crumbs complex. A model summarizing all the published data and those presented in this study is drawn to show the interactions occurring inside the Crumbs complex. The amino acids playing a crucial role for the interactions are indicated. See Discussion for details.](image)

Sdt for association with Crumbs in such a complex or that both interactions can coexist.

**A model for Crumbs action in apical network formation**

Because both the *crumbs* and *karst* phenotypes disrupt the ZA (Grawe et al., 1996; Zarnescu and Thomas, 1999), we hypothesize that Crumbs promotes the accumulation of βH to the apicolateral region during gastrulation to orchestrate the fusion of spot adherens junctions and/or to stabilize the ZA. Moreover, the observation that *karst* mutants exhibit morphogenetic defects without any loss of epithelial polarity (Zarnescu and Thomas, 1999), whereas *dlt* mutants exhibit a strong polarity phenotype (Bhat et al., 1999), suggests that the polarization and junction building functions of Crumbs are separate and parallel pathways. In support of this hypothesis, a paper appeared while this manuscript was under review indicating that the FERM domain binding region of Crumbs is indeed required for correct organization of the ZA (Izaddoost et al., 2002).

The loss of βH function causes defects in cell shape change that are associated with apical contraction driven by an apically located actomyosin contractile ring (McKeown et al., 1998; Zarnescu and Thomas, 1999; for review see Thomas, 2001). In this context the discovery that this spectrin isoform is complexed with DMoesin is particularly provocative, as the activity of the latter is strongly correlated with modulation of cell shape and the actin cytoskeleton (Edwards et al., 1997; Tsukita and Yonemura, 1999). Furthermore, the activity of moesin is modulated by phosphorylation in response to activation of Rho-associated kinase (ROK) in par-
allel with myosin II. Both Moesin and myosin light chain are activated by ROK phosphorylation and by ROK mediated inhibition of the myosin/moesin phosphatase (e.g., Fukata et al., 1998; Eto et al., 2000). Therefore, we speculate that β4H is part of the cytoskeletal network that facilitates such cell shape changes, and that in organizing spectrin at the membrane, Crumbs would appear to be acting as a molecular coordinator of polarity and morphogenesis. Furthermore, the finding that in human, mutations in CRB1 lead to pathologies such as retinitis pigmentosa (RP12) (den Hollander et al., 1999) emphasizes the importance of deciphering the molecular networks associated with Crumbs in Drosophila. The human orthologue of β4H, βV-spectrin, is strongly expressed in photoreceptor cells (Staback and Morrow, 2000). This raises the exciting possibility that a similar interaction between CRB1 and βV-spectrin exists in these cells. This will be examined in future work.

Materials and methods

Fly stocks

The crumbs8105, crumbs1A227, and P(UAS-Myc-IntraWt)38.14a (Wodarz et al., 1995) strains were provided by Dr. E. Knust (Heinrich Heine University, Düsseldorf, Germany); the karst alleles 1, 2, and 14.1 were originally described in Thomas et al. (1998). These alleles have now been sequenced and the specific lesions are as follows: karst is a nonsense mutation in codon 1919 producing a protein truncated near the end of segment 16 (see Thomas et al. (1997) for an explanation of the segment nomenclature); karst is a nonsense mutation in codon 1656 causing truncation of the protein in the middle of segment 14; karst is a small deletion that removes 22 bp from the third position of codon 1659–1666, inclusive. The resulting frameshift results in termination 5 amino acids downstream. This produces a protein truncated in the middle of segment 14 that is very similar in length to that of the karst allele. Therefore, all three alleles produce proteins of about half the size of native β4H, and lack both the tetramerization site and COOH-terminal PH domain region. Recombinant karst crumbs cDNAs were generated and verified by standard techniques. Oregon-R was used as WT stock. In the overexpression experiments the engramed GAL4 driver line was used to activate expression of UAS-Myc-IntraWt.

Statistical analysis

The karst phenotype exhibits variable expressivity (Thomas et al., 1998; Zarensuc and Thomas, 1999), and thus enhancer/suppressor interactions must be characterized in replicate experiments with appropriate statistical comparisons. In this paper, viability to adulthood is expressed as a lethal fraction of the Mendelian expectation estimated using a maximum likelihood model to determine the cost of each allelic combination of karst. Because karst cannot be maintained for many generations over the TM3 chromosome (because the 6CD region is not effectively balanced and the karst2 allele is a small deletion that removes 22 bp from the third position of codon 1659–1666, inclusive. The resulting frameshift results in termination 5 amino acids downstream. This produces a protein truncated in the middle of segment 14 that is very similar in length to that of the karst allele. Therefore, all three alleles produce proteins of about half the size of native β4H, and lack both the tetramerization site and COOH-terminal PH domain region. Recombinant karst crumbs cDNAs were generated and verified by standard techniques. Oregon-R was used as WT stock. In the overexpression experiments the engramed GAL4 driver line was used to activate expression of UAS-Myc-IntraWt.

Antibodies

A serum raised against the cytoplasmic domain of Crumbs was affinity purified and used at a dilution of 1:50 for immunofluorescence. A mouse monoclonal anti-Crubby antibody MabCaq4 (provided by Dr. E. Knust) was used at a dilution of 1:2 for immunostaining and immunoblotting. A rabbit polyclonal anti Dil antibody provided by Dr. M. Bhat (Mount Sinai School of Medicine, New York, NY) (Bhat et al., 1999) was used at a dilution of 1:3,000 for immunoblotting and 1:300 for immunoprecipitation and immunofluorescence, respectively, and at 1:400 for capping experiments. Affinity-purified anti β4H serum (243) was prepared as previously described (Thomas and Kiehart, 1994) and used at 1:1,000 or 1:500 for immunoblots and immunofluorescence, respectively. Antibodies against DMOesin were provided by D. Kiehart (Duke University, Durham, NC), prepared as described (Edwards et al., 1997), and used at 1:500 for immunoprecipitations, 1:2,000 for immunofluorescence, and 1:20,000 for immunostaining. The anti-myc monoclonal monoclonal antibody 9E10 (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:50 and TRITC-phalloidin (Sigma-Aldrich) was used at a dilution of 1:100.

Immunofluorescent staining of embryos and cells

Immunostaining of embryos (from 2 to 14 h) was performed as described (Muller and Wieschaus, 1996) using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or rhodamine-conjugated goat anti-rabbit IgG as appropriate (Jackson Immunoresearch Laboratories, Inc.) at a dilution of 1:100. Procedures for indirect immunofluorescence of S2 cells were as described for mammalian cells (Le Bivic et al., 1989). For intracellular staining, fixed cells were permabilized with 0.05% saponin. Fluorescent secondary antibodies were used at a dilution of 1:200. For phalloidin staining, embryos were deativlized in 80% ethanol.

Immunoblots and immunoprecipitations

For immunoprecipitations, 2 to 14 h wild-type Drosophila embryos (1 g) were homogenized in 6 ml of purification buffer (10 mM Tris, pH 7.5, 0.32 M sucrose, 3 mM MgCl2) supplemented with anti-proteases (1/1,000) and orthovanadate (0.2 mM) and centrifuged for 10 min at 1,500 g. Supernatant was collected and the pellet was resuspended in 4 ml of purification buffer and centrifuged at 10,000 g for 15 min. Supernatant was ultracentrifuged for 1 h at 40,000 rpm (Ti 70 rotor; Beckman Coulter) and pellet was resuspended in 3 ml of lysis buffer (1% Igepal, 50 mM Tris, pH 7.5, 10 mM EDTA, 3 mM MgCl2) supplemented with anti-proteases and orthovanadate as described above. After incubation for 30 min at 4°C, the lysate was centrifuged for 10 min at 14,500 g, incubated for 1 h with Pan sorbin, and centrifuged at 14,500 g for 30 min. Lysates were immunoprecipitated for 2 h at 4°C using the anti-Moesin or anti-β4H, or rabbit anti–mouse antibodies (1:500; Complèigne) preabsorbed on protein A–Sepharose beads (Amersham Biosciences). Precipitates were fractionated by SDS-PAGE, electrophoretically transferred to nitrocelulose (Schleicher and Schuell GmbH), and incubated with appropriate primary and peroxidase-conjugated secondary antibodies (1:10,000; Immunotech SA). β4H was analyzed as described previously (Thomas and Kiehart, 1994).

DNA constructs, transfections and cell culture

The chimeric construct CRB–VSV-G WT was obtained by amplifying a COOH-terminal Crumbs fragment containing the stalk region, transmembrane domain and cytoplasmic domain of Crumbs (amino acid 2074–2146) using the full-length crumbs cDNA as template, a gift of Dr. E. Knust, and cloning it into the pUC19 vector containing the VSV-G tag, a gift of Dr. P. Boquet (University of Nice, Nice, France). This fusion construct was subsequently subcloned into the EcoRV-BamHI sites of the pMK33/pMtHy plasmid with a metallothionein promoter, a gift of Dr. M. Koelle (Yale University, New Haven, CT). Mutant CRB–VSV-G constructs (Fig. 5) were derived by PCR and subcloned in the same vector. All constructs were verified by sequencing (Genome Express SA).

Drosophila S2 cells were transiently or stably transfected with constructs in pMK33/pMtHy plasmid using FuGENE 6 Transfection Reagent according to the manufacturer instructions (Roche Diagnostics GmbH). Stably transfected cells were selected and maintained with Hygromycin B (Roche Diagnostics GmbH) at a concentration of 250 and 100 μg/ml, respectively. Expression of CRB–VSV-G constructs was induced by the addition of 1 mM CuCl2 to the growth medium for 17–24 h.

Capping experiments

Stably transfected S2 cells were processed as described (Jefford and Dubreuil, 2000), except that fluorescein isothiocyanate-conjugated goat antiamouse IgG (1:200 in Drosophila Ringer’s) was added for 5 min before being overlaid to polylysine-coated slides. Once overlaid, the cells were fixed and stained as above for Dlt, DMOesin, or β4H, and cells were examined with a Zeiss LSM 410 confocal microscope. Capped S2 cells expressing the CRB–VSV-G constructs were scored for the presence of fluoresceantibody-stain caps using the fluorescent channel and for Dlt, actin, DMOesin, or β4H colocalization at caps using the rhodamine channel. About 50 VSV-G–positive cells were scored for each experiment, and results are expressed as a percentage of the cocapped cells found for each protein with the CRB–VSV-G S6 construct normalized at 0% (actual capping percentage, 25% for CRB–VSV-G S6 and 75% for CRB–VSV-G WT).
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