Grainy head promotes expression of septate junction proteins and influences epithelial morphogenesis

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Summary

Transcription factors of the Grainy head (Grh) family are required in epithelia to generate the impermeable apical layer that protects against the external environment. This function is conserved in vertebrates and invertebrates, despite the differing molecular composition of the protective barrier. Epithelial cells also have junctions that create a paracellular diffusion barrier (tight or septate junctions). To examine whether Grh has a role in regulating such characteristics, we used an epidermal layer in the *Drosophila* embryo that has no endogenous Grh and lacks septate junctions, the amnioserosa. Expression of Grh in the amnioserosa caused severe defects in dorsal closure, a process similar to wound closure, and induced robust expression of the septate junction proteins Coracle, Fasciclin 3 and Sinuous. Grh-binding sites are present within the genes encoding these proteins, consistent with them being direct targets. Removal of Grh from imaginal disc cells caused a reduction in Fasciclin 3 and Coracle levels, suggesting that Grh normally fine tunes their epithelial expression and hence contributes to barrier properties. The fact that ectopic Grh arrests dorsal closure also suggests that this dynamic process relies on epithelia having distinct adhesive properties conferred by differential deployment of Grh.

Key words: *Drosophila*, Grainy head, Septate junctions

Introduction

During *Drosophila* development, the Grainy head (Grh) transcription factor is expressed in the epidermis and a subset of other epithelia that form strongly adhesive layers exposed to the external environment (e.g. trachea) (Bray and Kafatos, 1991; Hemphala et al., 2003; Uv et al., 1994). In the absence of *grh*, these epithelial cells have altered morphology and lose expression of enzymes that cross-link the apical extracellular matrix (cuticle) (Bray and Kafatos, 1991; Hemphala et al., 2003; Mace et al., 2005; Ostrowski et al., 2002). Similarly, in mice lacking the Grh-related gene GRHL3, the outer protective layer of the skin, the stratum corneum, is defective (Ting et al., 2005; Yu et al., 2006), and in *Xenopus* embryos, GRHL3 and GRHL1 are expressed in the outer cells and regulate the expression of keratins (Chalmers et al., 2006; Tao et al., 2005). Thus, Grh proteins have highly conserved roles in regulating terminal differentiation of robust protective epithelia.

In addition to regulating terminal differentiation per se, Grh might also have other functions in these epithelia. For example, at stages before the stratum corneum is formed, *Grhl3* mutant mice have defects in re-epithelialisation following wounding (Stramer and Martin, 2005; Ting et al., 2005). They also have altered levels of many tight-junction-associated proteins, including occludins and claudins (Yu et al., 2006). Likewise, in *Drosophila*, *grh* expression commences prior to cuticle secretion and correlates with stages at which these epithelia acquire occluding junctions (septate junctions) (Tepass and Hartenstein, 1994). Nevertheless, Grh is dispensable for the establishment of basic barrier properties, because septate junctions are still present in *grh*-mutant tracheal cells (Hemphala et al., 2003). However, because the barrier characteristics of occluding junctions vary between epithelia (Furuse and Tsukita, 2006), the conserved expression of *grh* family proteins in the highly impermeable surface epithelia led us to investigate further whether Grh could directly regulate expression of epithelial junction components in *Drosophila*.

We began our investigations by expressing Grh ectopically in the amnioserosa (AS), a single-layered epithelium that has no septate junctions (Tepass and Hartenstein, 1994; Gorfinkiel and Martinez Arias, 2007), to determine whether Grh could convert this tissue into one with barrier epithelia characteristics. The AS is normally devoid of Grh expression and plays an important role in co-ordinating the fusion between the epidermal sheets during dorsal closure (Fig. 1A,B). From these studies, we uncovered a role for Grh in regulating expression of septate junction proteins, which we have further confirmed using loss-of-function mutations and by showing that the genes contain Grh-binding sites. Thus, in addition to co-ordinating expression of matrix proteins, Grh also regulates the intrinsic barrier properties of epithelia through its effects on components of cell junctions.

Results and Discussion

To test the role of Grh in regulating epithelial characteristics, we specifically expressed the epidermal splice forms (N/K) in the amnioserosa, an epithelial tissue normally devoid of Grh (using *c381::Gal4* and *G332::Gal4*; Fig. 1C,F-H). This was sufficient to block dorsal closure (Fig. 1G,H), an effect previously seen with ubiquitous Grh overexpression (Attardi et al., 1993). The effects were most penetrant with *c381::Gal4* (hereafter referred to as AS^{c381}>*grh*) which resulted in 100% of embryos having dorsal holes at stage 17/hatching, when all wild-type embryos had completed dorsal closure (Fig. 1H; AS^{G332}>*grh* resulted in >50%

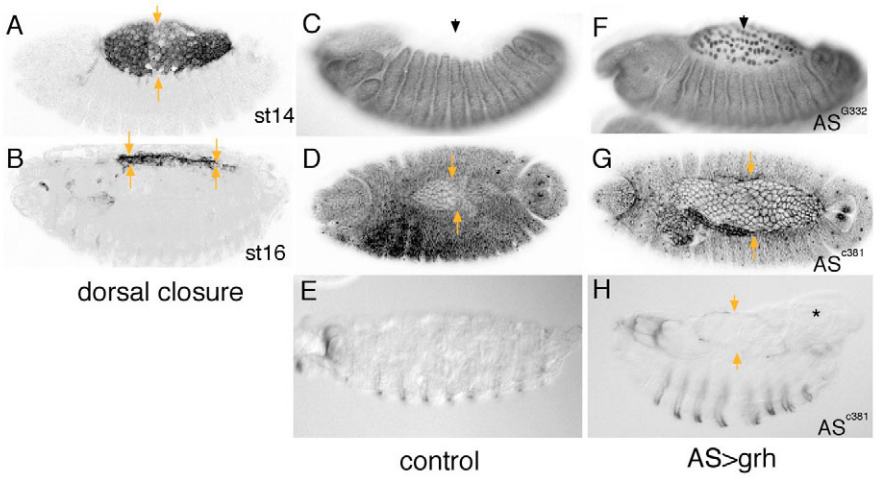

Fig. 1. Expression of Grh in the amnioserosa perturbs the progression and outcome of dorsal closure. (A,B) Dorsal closure: a membrane-associated Myc-tag labels amnioserosa at the stages indicated (lateral view; *c381::Gal4*). Arrows mark the edge of the epidermal sheets. (C,F) Grh expression in wild-type (C) and $AS^{G332}>grh$ (F) embryos (arrows indicate amnioserosa; stage 14, lateral view). (D,G) Phalloidin staining marks the cell outlines in dorsal view of wild-type (D) and $AS^{c381}>grh$ (G) stage-15 embryos; zippering has commenced at the poles in control but not in $AS^{c381}>grh$ embryos. Arrows mark the edge of the epidermal sheets. (E,H) Cuticles from hatching stage. $AS^{c381}>grh$ embryos (H) have a dorsal hole (arrows) and internal organs are extruded (asterisk).

with dorsal holes). Defects were already evident earlier (stage 14-16). In $AS^{c381}>grh$ embryos, the amnioserosa was less contracted than wild type and contained cells with abnormal morphology. In addition, the epidermal edges failed to meet at the poles (Fig. 1G). Thus, expression of Grh disrupted the ability of amnioserosa cells to function in dorsal closure, suggesting that it altered their fundamental properties and/or perturbed their interactions with the epidermis.

Grh promotes expression of septate junction proteins

One explanation for the defects caused by Grh expression in amnioserosa cells is that these cells acquire epidermis-like characteristics, such as septate junctions (SJs) characteristic of conventional barrier epithelia. Proteins that localise to SJs include the FERM-domain protein Coracle, the immunoglobulin-family adhesion protein Fasciclin 3 (Fas3), the transmembrane protein Neurexin (Nrx), and the claudin-related proteins Sinuous (Sinu) and Megatrachea (Behr et al., 2003; Genova and Fehon, 2003; Lamb et al., 1998; Schulte et al., 2003; Tepass et al., 2001; Wu et al., 2004). In addition, the Discs large (Dlg)-Scribble-l(2)gal (Lgl) complex initially localises basolaterally and becomes incorporated into SJs (Knust and Bossinger, 2002). To investigate whether Grh regulates such components we examined their expression in $AS^{c381}>grh$ embryos. Expression of Fas3, Coracle and Sinu was strikingly upregulated in the amnioserosa of $AS^{c381}>grh$ embryos in comparison to control embryos (Fig. 2A-F,I-J′,K-M,O). Nrx and Atpα (Na/K-ATPase subunit) were more weakly upregulated (Fig. 2H,K and data not shown), and Dlg was upregulated in a patchy manner, although this effect was less penetrant (Fig. 2G,J″,N,P). Thus, levels of several different SJ proteins are increased by Grh expression in the amnioserosa. Of these, Fas3 was the earliest that could be detected.

In conventional epithelia, junctional proteins are localised to discrete domains in the lateral membrane (Knust and Bossinger, 2002). In $AS^{c381}>grh$ embryos, the sub-cellular localisation of SJ proteins was abnormal. Fas3, Coracle and Dlg were more diffuse than in wild type and frequently expanded along the apical and/or basal surface (Fig. 2M-P and data not shown). For example, Fas3 proteins were present in a more apical plane than the adherens junction component E-cadherin (Fig. 2O,O′), and Dlg was expanded throughout basal and apical regions (Fig. 2P,P′). Because E-cadherin itself was still localised at apical junctions in *AS>grh* embryos (Fig. 2N,P), the underlying apical/basal polarity appears unaffected. Thus, the altered distribution of Fas3, Coracle and Dlg suggests that Grh is sufficient to promote expression of SJ proteins, but not to ensure the correct organisation of these proteins within the apical-basal axis.

SJ proteins are reduced in *grh*-mutant cells

The upregulation of SJ proteins caused by ectopic Grh expression is complementary to the apical membrane expansion detected in *grh* loss-of-function mutants (Hemphala et al., 2003). However, SJ proteins (e.g. Coracle) are still present in the mutant tracheal and epidermal cells (Hemphala et al., 2003) (data not shown). Thus, Grh is apparently not essential for expression of SJ proteins, although it can clearly promote their expression ectopically. One way to reconcile these differences is if Grh fine-tunes the expression of such proteins to increase or strengthen lateral junctions in mature epithelia. We tested this by generating clones of *grh*-mutant cells in the wing imaginal disc, in which the juxtaposition of wild-type and mutant cells aids detection of subtle changes in expression levels. SJ proteins were still present in mutant wing disc cells, as they were in mutant tracheal cells. However, using this approach it was possible to detect a reduction in the levels of Fas3 and Coracle in cells lacking *grh* (Fig. 3A,B). This was most consistent for Fas3: the majority (11/16) of clones scored had a detectable reduction in Fas3. With Coracle, the effects were more variable, but 5/16 *grh*-mutant clones had subtle decreases in its levels. The fact that the effects were subtle and variable could be a consequence of timing, because we assayed the consequences of removing Grh at a relatively early stage in the maturation of these epithelia. Nevertheless, removal of Grh was not sufficient to compromise the barrier properties of the tracheal epithelia in the embryo, as measured by dextran exclusion experiments (Fig. 3E). Fluorescent dextrans injected into wild-type and *grh*-mutant embryos failed to enter the lumen of the trachea, indicating that they are unable to pass through the junctions. By contrast, when injected into mutant embryos in which SJs were compromised, dextran rapidly spread throughout the tracheal lumen. Thus, Grh is not essential for the establishment of SJs, although it can influence the levels of SJ proteins (at least in the wing disc) and is sufficient to promote their expression ectopically. These data suggest a model in which Grh in *Drosophila* elevates the expression of SJ proteins in a similar manner to the effects of GRHL3 in mice on claudins and occludins, proteins found in the analogous tight junctions (Yu et al., 2006). In neither animal is

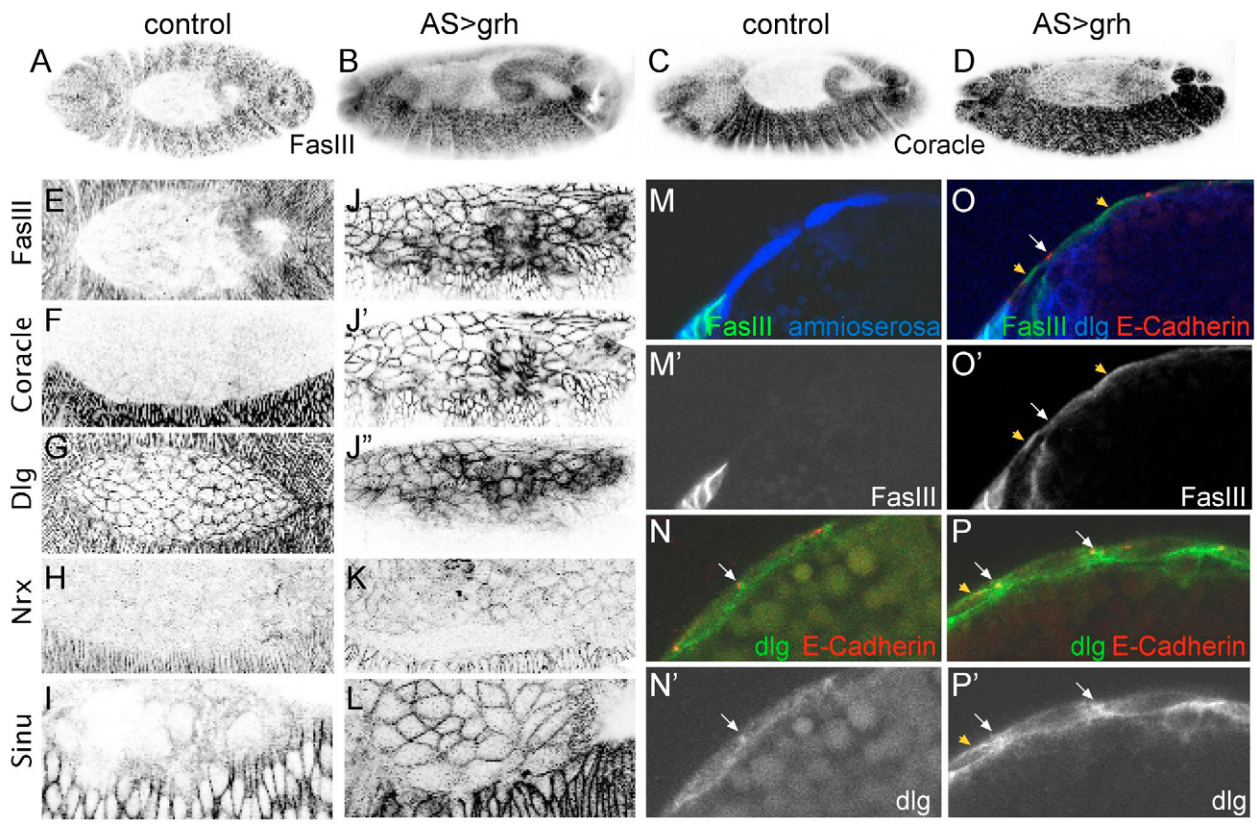

Fig. 2. Grh promotes expression of SJ components in the amnioserosa. Stage-14 control (A,C,E-I,M,N) and AS^{c381}>*grh* (B,D,J-L,O,P) embryos stained as indicated. Dorsal views of whole embryos (A-D) or of amnioserosa (E-L, single confocal sections; J-J″ are individual channels from one embryo). (M-O) Transverse sections stained as indicated to reveal the apical/basal distributions. (M,O) Upregulated Fas3 (FasIII) in AS^{c381}>*grh* embryos accumulates apically (green in O, white in O′; e.g. orange arrowheads). (N,P) Dlg is also upregulated in AS^{c381}>*grh* embryos (green, P; white P′) and spreads along the apical surface (e.g. orange arrowheads). (M-P) White arrows mark lateral junction sites.

there complete loss of these proteins in the *grh*/*Grhl3* mutants, but their levels and distribution are altered in a manner that could alter the robustness of an epithelial barrier.

Grh binds to target sites in *fas3* and *coracle* genes

To investigate whether genes encoding SJ proteins could be direct targets of Grh, the *coracle* and *fas3* genes were analysed for sequences that had good matches to a weighted matrix derived from known Grh-binding sites (Almeida and Bray, 2005) and that were conserved in the cognate genes from highly diverged drosophilids (*D. pseudoobscura*, *D. virilis*, *D. mojavensis*). There were two conserved matches to the Grh-binding-site consensus in the first intron of *fas3* (*fas3A*, 5′-ACCGGTTT-3′; *fas3B*, 5′-ACCAGTTT-3′) and in the first intron of *coracle* [*coraA*, 5′-ACCAGTTT-3′ (–strand); *coraB*, 5′-ACCGGTTT-3′ (–strand)]. These four sites were recognised by Grh in vitro in a competition assay in which their binding affinities were compared with a high-affinity Grh target site, Gbe2, from the *dopa decarboxylase* gene (Uv et al., 1994) (Fig. 3C). Putative sites from *fas3* and *coracle* significantly reduced binding to the labelled Gbe2 probe, and were even more effective than a similar excess of the cognate Gbe2 site, demonstrating that they are high-affinity binding sites for Grh. Thus, both *fas3* and *coracle* have the potential to be direct targets of Grh.

To further test their potential for regulation by Grh, fragments encompassing the Grh-binding sites were inserted upstream of a minimal promoter fused to luciferase and expression was assayed in the presence and absence of Grh in transient transfection assays (Fig. 3D). In total, 3/4 fragments conferred Grh responsiveness (>2.5×) on the reporter. In addition, two fragments from *sinu* that encompassed putative Grh-binding sites [*sinu1*, 5′-ACCTGTTC-3′ (–strand); *sinu2*, 5′-TCCGGTTT-3′] were tested in the same assay, and *sinu2* also showed a response to Grh. Together, these data suggest that the effect of Grh on SJs involves direct regulation of component-encoding genes.

Effects of Grh expression on adhesive properties of the amnioserosa and epidermis

Because ectopic expression of Grh has a profound effect on dorsal closure, we looked more closely at the morphology of AS^{c381}>*grh* cells and the distribution of other adhesion complexes. In AS^{c381}>*grh* embryos there were large variations in shape and size of amnioserosa cells and the contacts with the adjacent dorsal epidermis were dramatically different (Fig. 4C-F and data not shown). A subset of epidermal cells had expanded contact with an amnioserosa cell at the expense of their neighbours, which became bunched together (Fig. 4C″,D″,F). It appeared, therefore, that many Grh-expressing amnioserosa cells had maximised the contact with a single epidermal cell, rather than making contact with five to six cells, as in wild type. Ultimately, some amnioserosa cells appeared to lose contact with the epidermal cells.

The change in morphology in AS^{c381}>*grh* embryos was accompanied by altered distribution of β-position-specific (βPS)

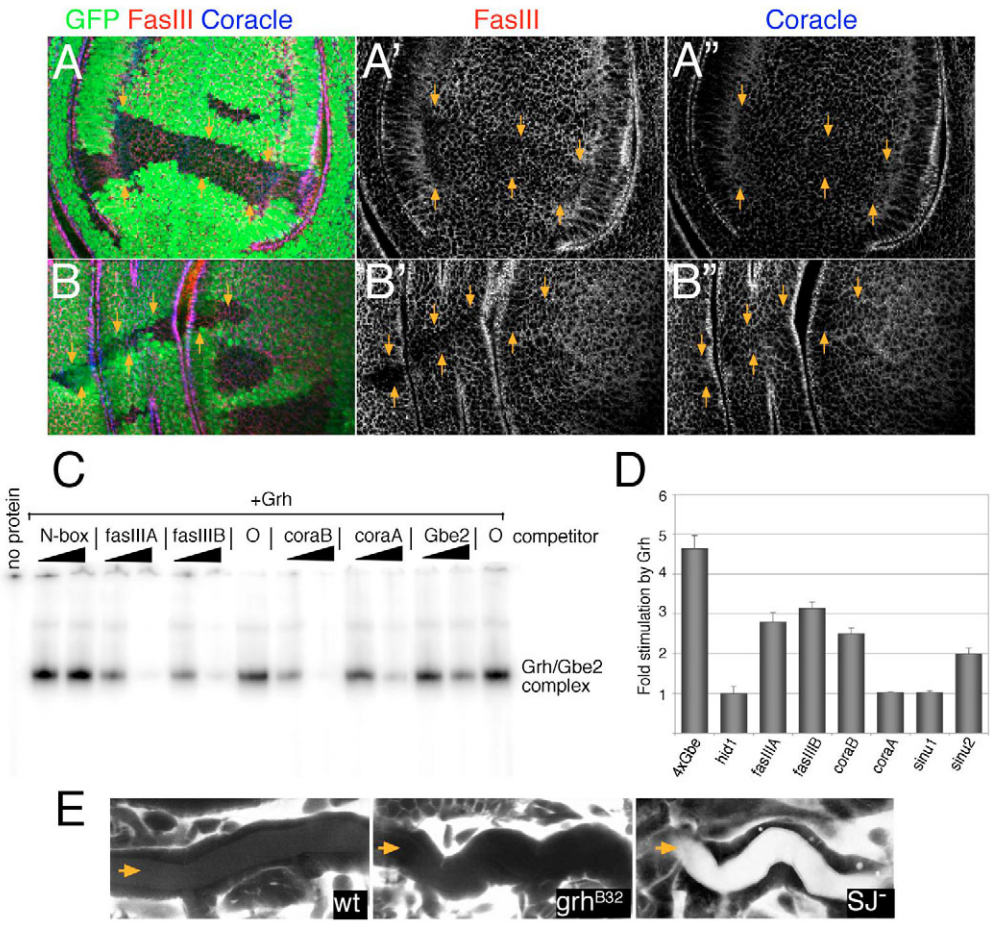

Fig. 3. *fas3* and *coracle* are potential targets of Grh. (A,B) Levels of Fas3 (FasIII; red, A,B; white, A′,B′) and Coracle (blue, A,B; white, A″, B‴) are subtly reduced in *grh*-mutant clones (identified by absence of GFP, green, in A,B). Clone boundaries are indicated by arrows. (C) Grh binds to sites from *fas3* and *coracle* genes. Amount of Grh complexed with labelled Gbe2-binding sites in the absence (O) or presence of the indicated competitors (5× molar excess and 50× molar excess). N-box is an unrelated *E(spl)* target site. Lane 1 has no added protein; all other lanes contain GST-Grh bacterial extract. (D) Fold stimulation by Grh of luciferase reporter genes containing fragments spanning the indicated sites. 4×Gbe, positive control with oligomerised Gbe2 sites; hid1, negative control. (E) Rhodamine-conjugated dextran is excluded from the tracheal lumen (arrows) of wild-type (wt) and *grh*-mutant (grh^{B32}/grh^{B32}) embryos but not from SJ mutants (*Atpα*; SJ^{-}).

integrins and E-cadherin (Fig. 4). In wild-type embryos, these co-localise to prominent dots at the interface between amnioserosa and epidermal cells and are present in overlapping domains associated with amnioserosa cell-cell contacts (Narasimha and Brown, 2004) (Fig. 4A,B). In AS^{c381}>*grh* cells, βPS integrins appeared diffuse and frequently spread across the apical surface (Fig. 4C,C′). Furthermore, less βPS integrin accumulated at the interface between epidermal and AS cells (Fig. 4C′,D′); instead, it was concentrated in regions with bunched epidermal cell contacts and no longer localised with E-cadherin (Fig. 4D-D″). Thus, Grh

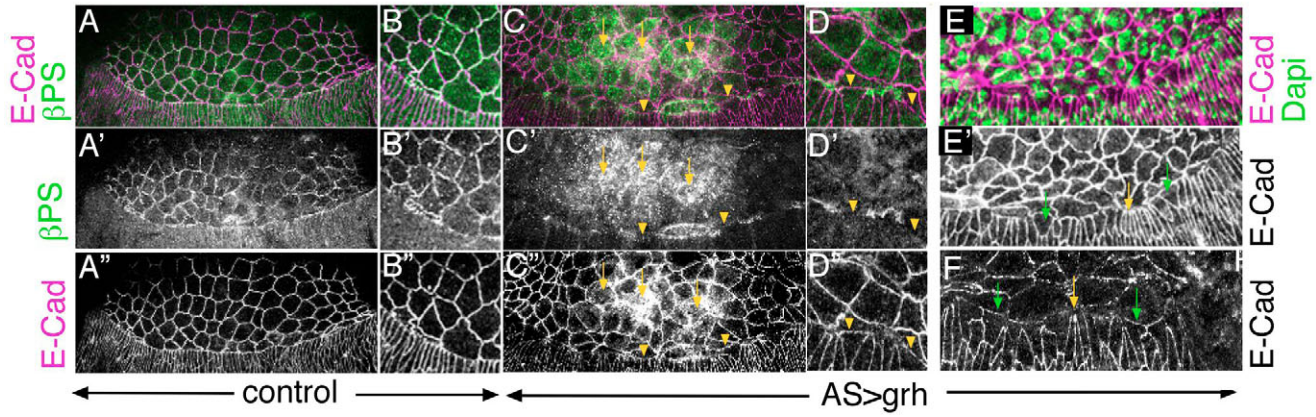

Fig. 4. Grh expression results in altered cellular morphology and disrupts the distribution of integrins and cadherin. Dorsal views of control (A,B) and AS^{c381}>*grh* (C-F) embryos stained with antibodies against βPS integrin (green, A-D; white, A′-D′) and E-cadherin (magenta, A-E; white, A″-D″,E′,F). (A-D) AS^{c381}>*grh* cells have poor localisation and patchy accumulation of βPS integrin (e.g. arrows, C,C′), reduced βPS integrin levels at epidermal interface (arrowheads, C,C′,D,D′), reduced co-localisation between βPS integrin and E-cadherin (compare B with D), and some mislocalisation of E-cadherin (e.g. arrows C″). (E,F) Defects in the epidermis include bunching (orange arrows) and splaying (green arrows) of epidermal cells, and a detachment between the epidermis and the amnioserosa. (E) Nuclei are visualised with DAPI.

perturbs other adhesive characteristics of amnioserosa cells. This could be an indirect consequence of the increase in SJ proteins, causing altered distribution of apical and basal adhesion receptors, or Grh could additionally regulate the expression levels of cadherin and integrins (Almeida and Bray, 2005). Whichever the mechanism, amnioserosa cells acquire altered adhesion properties with neighbouring epidermal cells, which could explain why dorsal closure is perturbed.

Concluding comments

The results we obtained from ectopic Grh expression have helped uncover functions that are not easily evident from loss-of-function experiments and suggest that Grh is normally involved in fine-tuning the expression levels of proteins, such as Fas3, Coracle, Sinu, Nrx and Dlg, which are involved in conferring robust barrier function on the epidermis. Given the observation that GRHL3 also fine-tunes the levels of junction proteins in mice (Yu et al., 2006), it appears that this represents a highly conserved aspect of Grh function. In addition, Grh might be intrinsic to the observed cross-talk between the extracellular matrix and junctional complexes (Tonning et al., 2005; Wang et al., 2006), because it plays a role in regulating both elements.

Grh transcription factors are also components in a conserved mechanism for wound healing, in part via their effect on extracellular matrix deposition/synthesis (Mace et al., 2005; Stramer and Martin, 2005). Our results suggest that regulation of cell junctions might also be important for epidermal 'sealing'. They further suggest that differences in Grh levels or activity could regulate morphogenesis within an epithelium, as well as the ability of epithelia to adhere to one another, by influencing the levels and distribution of septate/tight junction proteins and other adhesion molecules. This could also explain the role of GRHL3 during neural tube closure in mice (Ting et al., 2003), an epithelial fusion event that shares features with dorsal closure.

Materials and Methods

Genetics

For ectopic expression of Grh, *c381::Gal4* and *G332-Gal4* drivers were combined with *UAS::grhN/K*, and embryos from 4- to 6-hour collections were aged at 25°C to enrich for stages 13-16. *grh*-mutant clones were induced (1 hour at 37°C) in larvae of the genotype *hsFLP/w; FRT42D grh^{B32}/FRT42D PcEGFP*.

Immunochemistry

Whole-mount staining of embryos was performed according to standard procedures. Primary antibodies were monoclonal mouse anti-βPS (CF6G11, 1:3), anti-Fas3 (7G10 1:30), anti-Dlg (4F3, 1:100) and anti-Atpα (a5, 1:100), all obtained from the Developmental Studies Hybridoma Bank; rabbit anti-Dlg (1:500-1000), anti-Sinu (Wu et al., 2004); rat anti-E-cadherin [1:20 (Oda et al., 1994)]; guinea-pig anti-Coracle [1:1000 (Fehon et al., 1994)] and anti-Nrx (Baumgartner et al., 1996). Alexa-Fluor-488/568 (Molecular Probes) Cy2, Cy3 or Cy5 (Jackson Immunodiagnostics)-conjugated secondary antibodies were used at 1:200. Thick sections of fluorescently stained embryos were made as previously described (Narasimha and Brown, 2006).

Fluorescent images were acquired on a BioRad Radiance 2000 confocal microscope. Where projections are presented, sections were scanned at 0.2-0.5 micron steps. Note that, in Fig. 3A,B the GFP channel from a projection is superimposed on one optical section of the protein staining.

Target-site analysis, electrophoretic mobility-shift assays and luciferase assays

DNA sequence spanning the *fas3*, *coracle* and *sinu* genes was searched for a match to a weighted matrix derived from known Grh sites (Almeida and Bray, 2005) using Target-Explorer (Sosinsky et al., 2003). The UCSC vista browser http://pipeline.lbl.gov/cgi-bin/gateway2?bg=dm1 was used to determine conservation between *D. melanogaster* and other *Drosophila* species (Couronne et al., 2003). Positions of sites with respect to starting ATG in *D. melanogaster* are: *fas3A*, 31,097 bp downstream; *fas3B*, 34,852 bp downstream; *coraA*, 1169 bp upstream; *coraB*, 4402 bp upstream.

Electrophoretic mobility-shift assays (EMSAs) were carried out as described previously (Uv et al., 1994). Reactions contained 0.5 μl of a 1:10 dilution of bacterial extract containing Gst-P/E fusion protein, 20 femtomoles of ^{32}P-labelled *gbe2* double-stranded oligonucleotide (5′-CTAGCGATTGAACCGGTCCTGCGGT-3′; underlined oligonucleotides correspond to putative Grh-binding sites) and 100 fM (femtomoles) or 1 pM (picomole) of the following cold competitors where indicated: *fas3A*, 5′-CTAGATCGCAACCGGTTTGGGT-3′; *fas3B*, 5′-CTAGAGGGAACCAGTTTTGCCT-3′; *coraA*, 5′-CTAGAGCAAACTGGTTCAGCT-3′; *coraB*, 5′-CTAGAAAAAACCGGTTGTT-3′; and N-box, 5′-GATCAGCCACGAGCCACAAGGATTG-3′.

For luciferase assays, fragments encompassing the Grh-binding sites were amplified from genomic DNA by PCR and subcloned into a pGL3-min luciferase reporter vector containing the minimal hsp70 promoter. Details available on request. Resulting plasmids were transfected into *Drosophila* S2 cells with a renilla control plasmid in the presence or absence of a plasmids expressing Grh (pMT-Gal4 + UAS-GrhN). Transfection conditions and luciferase assays (Promega) were carried out as described previously (Nagel et al., 2005).

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