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

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Accepted 9 December 2007
Journal of Cell Science 121, 747-752 Published by The Company of Biologists 2008
doi:10.1242/jcs.019422

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 the 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 ASc381>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; ASG332>grh resulted in >50%
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 $A^{S^{381}}$ > $grh$ embryos. Expression of Fas3, Coracle and Sinu was strikingly upregulated in the amnioserosa of $A^{S^{381}}$ > $grh$ embryos in comparison to control embryos (Fig. 2A-F, I-J). Nrx and Atnp (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 $A^{S^{381}}$ > $grh$ 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 with 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 dextran 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
Grh regulates septate junction proteins

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 *ASc381>grh* embryos and the distribution of other adhesion complexes. In *ASc381>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). 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 *ASc381>grh* embryos was accompanied by altered distribution of β-position-specific (βPS)
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 Asc381>grh cells, βPS integrin 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
Grh regulates septate junction proteins

Electrophoretic mobility-shift assays (EMSAs) were carried out as described previously (Nagel 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'-CTAGGCTTTGACCCCCGTGCCTGCGT-3'; underlined oligonucleotides correspond to putative Grh-binding sites) and 100 fM (femtograms) or 1 pM (picomoles) of the following cold competitors where indicated: fos/J-CGGTTGGCT-3'; fosA3B-5'-CTAGAGGGGACCGTTTTGCTC-3'; coraA, 5'-CTAGACAGCTTGACTGAG-3'; coraB, 5'-CTAGAAAAACCGGTGTTTG-3'; and N-box, 5'-GATCGCCACGAGCAGCAAGAGTTG-3'. For luciferase assays, fragments encompassing the Grh-binding sites were assayed in an assay to test genomic DNA by PCR and subcloned into a pG3-L3-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-Grh). Transfection conditions and luciferase assays (Promega) were carried out as described previously (Nagel et al., 2005).

We thank Greg Beitel, Manzoor Bhat, Peter Bryant, Rick Fehon and Hiroki Oda for antibodies, and members of our labs for discussions. This work was supported by grants from the Medical Research Council (S.J.B.), the Wellcome Trust (N.H.B.) the Swedish Research Council (A.U.) and TIFR (M.N.).

**References**


