Characterization of Drosophila hibris, a gene related to human nephrin

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SUMMARY

Hibris encodes a protein that is a newly identified member of the immunoglobulin superfamily and has homology to vertebrate Nephrins and Drosophila Sticks-and-Stones. The Hibris protein has eight Ig-like domains, a fibronectin domain and a 160 amino acid cytoplasmic tail. The hibris transcript is expressed in a broad range of tissues and across life stages. In the embryo, hibris transcript is present in the mesectoderm, then in a group of cells at the developing CNS midline and in a subset of glia. In the periphery, hibris is expressed by fusion competent myoblasts and the epidermal muscle attachment site cells. Deletion analyses show that loss of hibris does not visibly affect embryonic CNS or somatic muscle development. However overexpressing hibris in the somatic mesoderm disrupts myoblast fusion. Furthermore, when overexpressed in the epidermis, Hibris causes comprehensive derangement of muscle insertion locations. A similar myoblast fusion defect is observed when the Drosophila homologs of DM-GRASP/BEN/SCI (irregular chiasm-roughest and dumbfounded) are deleted together. Our S2 cell aggregation assays have revealed a heterotypic interaction between Hibris and Dumbfounded, but not between Hibris and Irregular Chiasm-Roughest. We propose that Hibris is an extracellular partner for Dumbfounded and potentially mediates the response of myoblasts to this attractant.

Key words: Drosophila, hibris, Nephrin, Mesoderm, Myoblast, Myotube, Muscle attachment sites, Mesectoderm, Dumbfounded, DM-GRASP, BEN, SCI

INTRODUCTION

Proteins of the immunoglobulin superfamily (IgSF) have been implicated in the development and maintenance of a diverse range of tissue types across a broad spectrum of organisms. In their roles as receptors and ligands, IgSF proteins underpin cell-cell adhesion and cell-cell signaling functions (Goodman, 1996; Simmons et al., 1997; Bracco et al., 2000; Kamiguchi and Lemmon, 2000). They can act both homotypically and/or heterotypically, and often interact with other IgSF members (Goodman, 1996; Brummendorf and Rathjen, 1996; Walsh and Doherty, 1997; Kamiguchi and Lemmon, 2000). Owing to both the diverse and overlapping activities of IgSF proteins, the result of the loss of function of an IgSF protein can range from catastrophic to negligible. Similarly, altered levels of expression or localization can have severe or minor effects on development and survival.

In the present study, we have examined the action of Drosophila Hibris (Hbs). Hbs is a new member of the recently identified Nephrin subfamily of the IgSF. The Nephrins are represented in organisms ranging from humans to worms (Kestila et al., 1998; Holzmann et al., 1999; Ahola et al., 1999; Teichmann and Chothia, 2000). In the Drosophila embryo, Hbs is now implicated in myoblast fusion and myotube guidance during somatic mesoderm development, influencing contact between mesodermal-mesodermal and mesodermal-epidermal cells. Until very recently, there has been extremely limited information on the extracellular molecules that facilitate these processes. With the gradually increasing identification of proteins that are involved in these events, several are now from the IgSF with two being from the Nephrin subfamily (Bour et al., 2000) (present study) and two from the DM-GRASP/BEN/SCI subfamily (Ruiz-Gomez et al., 2000) (H. A. D. and H. S., unpublished).

During myoblast fusion in Drosophila, there are two identifiable populations of myoblasts – founder cells and fusion-competent myoblasts. The founder cells are specialized mesodermal cells that serve as a scaffold to which fusion competent myoblasts fuse (Bate, 1990). In order for fusion to occur, the fusion-competent myoblasts must find and recognize the founder cells. In Drosophila another Nephrin-like protein, Sticks and Stones (Sns), is present on the fusion competent myoblasts but not on the founder cells (Bour et al., 2000). In the absence of Sns, there is a comprehensive failure of myoblast fusion and the somatic musculature does not form (Paululat et al., 1999; Bour et al., 2000). This suggests that Sns has a role in the fusion-competent myoblasts-founder cell recognition and/or fusion initiation processes. Interestingly a phenotype identical to that for sns was reported for a deletion that affected the IgSF proteins Dumbfounded (Duf) and IrreC-Rst. In the absence of both duf and irreC-rst, the fusion competent myoblasts and founder cells fail to fuse. Returning duf expression to the mesoderm can largely rescue this combinatorial phenotype, and when ectopically expressed, Duf
can attract fusion competent myoblasts to novel locations (Ruiz-Gomez et al., 2000), which illustrates the attractant activity of Duf. While the role of irreC-rst in the deletion phenotype remains to be analyzed, overexpression in the mesoderm can block myoblast fusion (H. A. D. and H. S., unpublished). The IgSF proteins Sns, Duf and IrreC-Rst represented the first extracellular proteins involved in the myoblast-founder cell recognition/fusion process (Frasch and Leptin, 2000).

As fusion-competent myoblasts fuse to founder cells, the ends of the developing myotubes form structures highly reminiscent of the filopodia that are found on axonal growth cones (Bate, 1990). Just as growth cone filopodia search for guidance cues, so too do the filopodia-like structures on myotubes are used to search for epidermal cells that will serve as sites for muscle attachment (Bate, 1990). An understanding of the molecular mechanisms that facilitate myotube guidance is still very much in its infancy. To date, the only extracellular molecules implicated in the guidance of myotubes are the ligand/receptor pair, Slit/Roundabout (Robo – an IgSF protein) (Kidd et al., 1998; Kidd et al., 1999), and the receptor tyrosine kinase Derailed (Callahan et al., 1996). In slit mutant embryos, a subset of muscle fibers is no longer attracted to its appropriate attachment sites, while overexpressing the Robo receptor in a myotube subset causes them to target attachment sites with high Slit levels (Kramer et al., 2001). Mutations that disrupt the derailed receptor tyrosine kinase gene also cause aberrant attachment site targeting by a small subset of myotubes (Callahan et al., 1996).

Clearly the small number of extracellular molecules involved in myoblast fusion or myotube guidance identified to date is insufficient to explain fully the molecular basis of coordinated fiber formation and correct localization of attachments. In the present study, we show that hbs is expressed in a diverse range of tissues, including fusion-competent myoblasts and the epidermal cells where the protein. Overexpression of the duf gene in adult tissues, Duf represented the first extracellular proteins involved in the fusion competence of myoblasts and the epidermal cells where the expression of the Duf gene occurs in a diverse range of tissues, including fusion-competent myoblasts and ectopic expression experiments were carried out as previously described (Bieber, 1994). For mixed aggregation assays, cells were pre-labeled with Dil or DiO by adding 5 μl/ml of 2.5 mg dye/ml ethanol to the cells after transfection, shaking for 3 hours, then washing before induction.

MATERIALS AND METHODS

Genetic stocks
Oregon R wild-type and white1118 flies were obtained from Corey Goodman. P-element lines l(2)k08015, l(2)k02064, l(2)k04218 and sch01238 are from the Bloomington Stock Center. Lines used for ectopic expression and overexpression experiments were elan-GAL4 (Luo et al., 1994); GMR-GAL4 (Freeman, 1996); pmr-GAL4 (Calleja et al., 1996); omb-GAL4 (Apidianakis et al., 1999); da-GAL4 (Wodarz et al., 1995); en-GAL4 (A. Brand, Wellcome/CRC Institute, Cambridge); sca-GAL4 (Klaes et al., 1994); twi-GAL4 on the third chromosome (Greig and Akam, 1993; Hacker and Perrimon, 1998); UAS-sim (Xiao et al., 1996); and sim-GAL4 (Steve Crews). Mutant lines used were sim10Ikar/TM3 fzs-lacZ (Nambu et al., 1990); Nsr (Bour et al., 1995); Df(1)w67cm (Ruiz-Gomez et al., 2000); rscCT and irecUB883 (Ramos et al., 1993); sns1/4CyO;genlacz (Susan Abmayr); Df(3)Re7D i56/TM3 fzs-lacZ (Jagla et al., 1997); and Df(3)Re7F/TM3 fzs-lacZ (Mohler and Pardue, 1984).

Cloning and molecular analysis of hbris
Primers were designed to PCR amplify a genomic DNA fragment that encodes part of the hbs ORF (sense strand primer: 5'-CCGTACTCTGTAAGTAGATCGC-3', antisense primer 5'-GATGTAGATGTTGCACC-'). The PCR fragment was amplified from adult fly genomic DNA and used to screen a Drosophila embryonic 9-12 hour cDNA library (Zinn et al., 1988). cDNA inserts from positive plaques were sequenced (by Jackson Laboratories) and the data were organized and translated using Lasergene software (DNASTAR). BLAST searches of public databases were conducted to identify homologs in other organisms. To determine intron/exon boundaries, cDNA4 sequence was aligned with the genomic sequence for the region (Berkeley Drosophila Genome Project). cDNA4 (longest cDNA) was used to probe a northern blot using standard Church conditions (Sambrook et al., 1989).

Immunohistochemistry, in situ hybridization and eye sectioning
Oregon-R wild-type embryos were collected over a 24 hour period for analyzing the hbs wild-type expression pattern, and over 18 hours for analyzing expression in the sim loss- and gain-of-function experiments. Staging was in accordance with Hartenstein (Hartenstein, 1993). A non-radioactive antisense RNA probe was generated using cDNA4 as the template, and in situ hybridization for embryos followed the protocol of Kopczynski et al. (Kopczynski et al., 1998). For double-labeling with antibodies, embryos were washed when the desired intensity of the in situ expression pattern was attained, then stained in accordance with published protocols. In situ hybridization of wild-type third instar larvae imaginal discs followed the protocol available at http://www-bier.ucsd.edu/imagdisc.html. Adult eyes were fixed and sectioned as described (Tomlinson and Ready, 1987).

S2 cell aggregation assay
cDNA4 was subcloned into the EcoRI site of the RnHa3 vector (Bunch et al., 1988). Other constructs used included Sns-RmHa3 and Ha-Duf-RmHa3 (kind gifts from Malabika Chakravarti and Susan Abmayr); IrreC-RmHa3 (Schneider et al., 1995); Fasciclin II-RmHa3 (Fambrough and Goodman, 1996); and Side-RmHa3 (Sink et al., 2001). S2 cells were transfected using Superfect (Qiagen) in accordance with the manufacturer’s directions. Induction of protein expression with copper sulfate and assaying of cell aggregation were carried out as previously described (Bieber, 1994). Cells were immunohistochemically processed to check for protein expression (Bieber, 1994). For mixed aggregation assays, cells were pre-labeled with Dil or DiO by adding 5 μl/ml of 2.5 mg dye/ml ethanol to the cells after transfection, shaking for 3 hours, then washing before induction.

Generation of polyclonal antibody and use
The Sali/Pst fragment was subcloned into the Sal/HindIII sites of the pQE31 expression vector (Qiagen) to generate a six-histidine tagged fusion protein. Five times at 21 day intervals, two adult rabbits were immunized with 250 μg of fusion protein (at Covance Research Products). The antibody was concentrated over Protein A beads (Sigma) (Sambrook et al., 1989). We routinely preabsorbed the
antibody against 2 hours embryos overnight before use at a final concentration of 1:500.

**Generation of X-ray deletions in the hibris region**

Male EP(2)2590 (Rorth et al., 1998) or l(2)h04218 (Kania et al., 1995) flies were irradiated with 5000 rad for 10 minutes, then crossed to w; ScO/CyO virgins. Progeny were screened for the removal of the P-element, indicated by reversion to white eye color. In situ hybridization with cDNA4 was used to screen for the absence of embryonic hbs expression.

**Generation of UAS-hibris transformants**

cDNA4 was subcloned into the EcoRI site of pUAST (Brand and Perrimon, 1993). The transformant line was generated in a w1118 background and mapped to the 2nd chromosome. For the UAS-secreted Hbs construct cDNA4 was partially digested with EcoRI and completely digested with EcoNI, generating a new stop codon 5' to the transmembrane domain. Six independent transformant lines were isolated.

**RESULTS**

**Isolation of the hibris gene**

*hbs* was initially identified in a search of the Berkeley Drosophila Genome Project (BDGP) database using another IgSF member – sidestep (Sink et al., 2001). PCR amplification of a genomic fragment containing part of the putative hbs ORF permitted the isolation of several cDNAs. Northern analysis revealed that the hbs transcript is a single band approximately 6.5 kb in size (data not shown), however our longest cDNA, cDNA4, is 4712 bp. Sequencing in from the ends of this cDNA revealed a poly-A+ tail and part of the 5' UTR.

**Hibris is a member of the Nephrin subfamily of the immunoglobulin superfamily**

BLAST searches of public databases with the cDNA4 sequence revealed it was in the GenBank database as hibris (*hbs*) (AF210316) (Artero and Baylies, 1999). The *hbs* gene encodes protein domains that are characteristic of members of the immunoglobulin superfamily (IgSF). Translation of the sequence (Figs 1, 2A) uncovered a characteristic conserved start site (Cavener, 1987) followed by a stretch of hydrophobic amino acids typical of signal peptides (von Heijne, 1990). Following the signal sequence are six consecutive immunoglobulin-like (Ig) domains. The next region has the tryptophan and potential 'second' cysteine characteristic of Ig domains, yet lacks the first conserved cysteine. This modified Ig domain is followed by two complete Ig domains, then a single fibronectin type-III (FN) domain. There are 12 potential sites for asparagine-linked glycosylation on the ectodomain. Following the FN domain there are the 26 hydrophobic amino acids of the transmembrane domain, then a cytoplasmic tail consisting of 160 amino acids (Figs 1, 2A) that contains 11 tyrosine residues. Analysis of the cytoplasmic tail with the PEST Sequence Utility program on the ExPASy Molecular Biology Server predicts a PEST sequence (Rogers et al., 1986) in the amino acids KSQSEAEPSNDDVYSK starting at amino acid 1078.

The type, number and order of the Hbs extracellular domains are conserved in the Nephrin protein subfamily (Fig. 1). Nephrins have been found in human (Kestila et al., 1998), mouse (Holzman et al., 1999), rat (Ahola et al., 1999) and *C. elegans* (Teichmann and Chothia, 2000). Another Nephrin subfamily protein, Sticks and Stones (Sns), was recently identified in *Drosophila* (Bour et al., 2000). The vertebrate Nephrin forms share close to ~80% amino acid identity with each other. By comparison, Hbs shares ~23% amino acid identity with the ectodomain of human Nephrin, 20% with the ectodomain of *C. elegans* Nephrin and 46% overall with *Drosophila* Sns. While the endodomain of the Nephrins is highly conserved between the vertebrate forms (~80% identity between human and mouse), the fly forms differ greatly from the vertebrate forms (Hbs and vertebrates, ~10% identity), the worm (Hbs and worm, ~10% identity) and even each other (Hbs and Sns, ~27% identity).

Based on information from the BDGP (Kimmerly et al., 1998), *hbs* maps to 51D11-E1 on the right arm of the second chromosome. 51D11-E1 is covered by a contig of sequenced P1s. Alignment analyses showed that cDNA4 straddled a >40 kb region in P1s DS00087 and DS04940, with the bulk of the *hbs* ORF lying in a region of approximately 5 kb (Fig. 2B). Exons range from 172 bp to 647 bp. While most introns are small (23 bp to 130 bp), the intron between the first and second exon is almost 40 kb in size (Fig. 2B).

**Testing Hibris binding ability**

Proteins belonging to the IgSF are frequently implicated in cell-cell adhesion (Goodman, 1996; Brummendorf and Rathjen, 1996; Walsh and Doherty, 1997). The ability of Hbs, Sns, Duf, IrreC-Rst and Side to bind homotypically was tested with the S2 cell aggregation assay (Bieber, 1994). As a negative control, S2 cells were transfected with RmHa3 vector (Bunch et al., 1988), and as a positive control, S2 cells were transfected with Fasciclin II-RmHa3 (Fambrough and Goodman, 1996). Homotypic aggregation was observed for Fasciclin II and Duf. To test for heterotypic interactions, the S2 cells were labeled with either DIl (red) or Dio (green), and the aggregates were examined using confocal microscopy. When Fasciclin II-transfected cells (red) were mixed with RmHa3-transfected cells (green), all aggregates formed contained only red cells. Similarly, when Duf-transfected cells (red) were mixed with RmHa3-transfected cells (green), aggregates were again all comprised of only red cells (Fig. 2C). When Duf-transfected cells (red) were mixed with Hbs- (Fig. 2C) or Sns-transfected cells (green), the resultant aggregates all had both red and green cells, but when Duf-transfected cells (red) were mixed RmHa3- or Irrc-transfected (green) cells, all the resultant aggregates contained only red fluorescent cells. A result matrix is presented in Fig. 2D.

**hbs is expressed in a temporally and spatially complex manner**

*hbs* is expressed in a highly dynamic manner across tissue types and life stages. At stage 5, along the dorsal surface of the cellularized embryo, a strong narrow band of *hbs* expression is present that extends along approximately two-thirds the length of the embryo. This band of expression broadens laterally, decreases in length, and becomes confined to the dorsal furrows (data not shown). By stage 8, dorsal expression is still present at the furrows, and *hbs* expression also begins ventrally, where it is associated with the mesectodermal cells. Expression strengthens in the mesectodermal cells as they move into close juxtaposition with one another at the ventral midline, forming
neighboring columns (across stages 9 and 10) (Fig. 3A,B). Expression continues during stage 11 as the mesectodermal cells intermingle, divide and move internally. By stage 12, as the midline axonal scaffold is forming (Klambt et al., 1991), a subset of midline cells posterior to the developing posterior commissure continue to express \textit{hbs} (3C). The number of \textit{hbs}-expressing cells at the midline decreases so that by late stage 14 there are two to three \textit{hbs}-expressing cells below the posterior commissure (Fig. 3D). Expression in these cells is absent by stage 15. Double-labeling of glial cells with anti-Repo mAb revealed that a subset of the exit glia at the edge of the CNS were \textit{hbs} positive from stages 12 to 15.

In the periphery during stage 10, laterally located clusters of cells begin expressing \textit{hbs} and are distinct patches at stage 11 (Fig. 3B). These cells are visceral mesoderm, as their nuclei co-label with the anti-myocyte enhancer factor 2 polyclonal antibody (anti-MEF2) (Lilly et al., 1995). By late stage 11, the somatic and visceral mesoderm expresses \textit{hbs}. During stage 12, \textit{hbs} is clearly present in the fusion competent myoblasts (Fig. 3E). This expression is truly restricted to fusion competent myoblasts. In \textit{Notch} (\textit{N\textsuperscript{AK11}}) mutant embryos, all myoblasts adopt a founder cell fate (Corbin et al., 1991; Bour et al., 2000) and in \textit{N\textsuperscript{AK11}} mutant embryos, there are no \textit{hbs}-positive myoblasts (Fig. 3F). Similarly, co-labeling with anti-Kruppel antibody revealed that the fusion-competent myoblasts, but not the founder cells, were \textit{hbs} positive (Fig. 3G). By stage 14, expression in the somatic mesoderm has ceased. As mesodermal expression decreases, epidermal expression begins. At stage 12, this expression is several cells broad and occurs at the segment boundary and in lateral patches. It becomes confined to the muscle attachment sites by stage 14 (Fig. 3H). Along the dorsal edges of the embryo lie some \textit{hbs}-positive cells. These cells are MEF2 negative, identifying them as the pericardial cells (Ward and Skeath, 2000).

In third instar larvae, in the eye/antennal disc, \textit{hbs} expression is strong behind the morphogenetic furrow, and also as clusters within the furrow (Fig. 3I). In the larval brain there is \textit{hbs} expression in the optic lobes (Fig. 3J). Expression in the larval wing disc consists of a striking cruciform pattern (Fig. 3K), corresponding to the regions that abut the presumptive wing margin, and those areas destined to be wing veins L3 and L4. More proximally, is expression in the region destined to be wing veins L0 and L1. There is also light expression in the presumptive notum region. In leg discs expression is seen in concentric circles (Fig. 3L).

\textit{hbs} mutant embryos do not display overt phenotypes

In order to determine the function of Hbs during embryonic development, a series of deletions were generated by irradiation of nearby P-elements (Fig. 4A). For \textit{EP(2)2590}, over 50 \textit{w\textsuperscript{−}} flies were isolated from the 71,700 progeny screened and 13 stable lines were successfully established, while for \textit{l(2)k04218} 55,600 progeny were screened and 29 lines established. Deletion 12 removes \textit{hbs} expression as assayed by in situ hybridization with cDNA4, and is lethal over...
l(2)k06403 but not l(2)k04218. As our smallest deletion removing hbs, this line has a phenotype where the ventral muscle pattern is abnormal in two to three hemisegments per mutant embryo. The abnormality consists of a loss of some muscle fibers from the ventral muscle group (Fig. 4B). In hemisegments where the muscle patterning is normal, motor innervation is also normal (Fig. 5D). Deletion 11 does not remove or disrupt hbs expression, yet also shows the ventral muscle defect that was seen in Deletion 12 (as does Deletion 6). Furthermore, in deletions 11 and 12 transheterozygotes, the muscle phenotype is present (Fig. 4C). As such, the ventral muscle phenotype maps to a gene(s) in the region other than

hbs-positive cells lie posterior and internal to the posterior commissure (pc) as it and the anterior (ac) are developing during stage 12. (D) By stage 14, only a subset of cells below the pc are still Hbs positive (arrowheads). (E) Stage 13 embryo. hbs is expressed by fusion competent myoblasts. (F) hbs is not expressed in muscle founder cells as shown in the age matched Notch mutant embryo. (G) At stage 11 hbs is expressed in fusion competent myoblasts (arrowheads) but not the Kruppel-positive founder cell (arrow). (H) Expression of hbs in the muscle attachment sites of a stage 14 embryo. (I) hbs is expressed in cells within and behind the morphogenetic furrow of the larval eye/antennal disc. (J) In the larval brain, hbs is expressed in the optic lobes in the locations where the photoreceptors terminate (arrows). In the wing disc (K), hbs expression is present in stripes that bound the presumptive wing margin (arrowhead), the stripes corresponding to wing veins L3 and L4 (downward arrows) and L0 and L1 (horizontal arrow). (L) In the leg disc, there are broad concentric circles of hbs.

Fig. 4. (A) Map showing location of genes and P-elements in the region of hbs, and the regions removed by x-ray-induced deletion of EP(2)2590. Deletion 12 embryos show perturbed ventral musculature in a subset of hemisegments (B, arrow), and this phenotype is also observed in Deletion 11/Deletion 12 embryos (C, arrow). (D) Innervation of muscles is normal (arrows show innervation at muscle 12) in hemisegments without defects, and also in the muscles present in the disrupted regions.
Characterization of *Drosophila* Hibris

It appears that *hbs* mutant embryos do not have an overt muscle phenotype as: (1) the muscle phenotype in deletion 12 is no different from that in the transheterozygote deletions 11 and 12 embryos; (2) muscle number, insertion sites and innervation are normal in the unaffected hemisegments; and (3) unfused myoblasts are barely evident in late stage 16. We also assayed the nervous system across stages 12-17 with mAbs BP102, 1D4, 22C10, anti-wrapper and anti-Repo, and did not find any defects (data not shown). As such, Hbs appears functionally redundant in the development of the embryonic somatic mesoderm and central nervous system. Deletion 12 in trans with *sns*, irreC-rst or the *duf irreC-rst* deletion did not produce phenotypes in the embryonic muscles or the adult.

**hbs misexpression gives robust embryonic and adult phenotypes**

In the embryo, overexpression of *hbs* in the CNS using the *sim-GAL4*, *elav-GAL4*, *da-GAL4* or *sca-GAL4* drivers did not disrupt axonal pathfinding (assessed with mAbs 1D4, BP102 and 22C10) or glial cell placement and number (analyzed with anti-Wrapper and anti-Repo mAbs) (data not shown). By contrast, overexpression of *hbs* in the mesoderm in homozygous *UAS-hbs;twi-GAL4* embryos partially disrupts myoblast fusion, but not muscle fiber number or sites of muscle attachment (Fig. 5B). This phenotype is evident in all hemisegments of all embryos. When *hbs* is misexpressed using the *da-GAL4* driver, unfused myoblasts are again present, and in all hemisegments, some muscle fibers are inserted at inappropriate attachment locations (Fig. 5C). *twi-GAL4* expression is restricted to mesoderm, while *da-GAL4* is expressed in all tissues, suggesting that aberrant muscle fiber attachments may be due to *hbs* misexpression in the epidermis. To further test this idea, we misexpressed *hbs* in the epidermis with additional GAL4 drivers. The expression patterns of drivers are consistent with the embryonic expression patterns described for the associated genes (H. A. D and H. S., unpublished). Driving in the engrailed pattern (Kornberg et al., 1985) in the epidermis with *en-GAL4* disrupted attachments made by several lateral muscle fibers in most hemisegments of all embryos (Fig. 6B). This was strongly exacerbated, occurring in all hemisegments of all embryos, by overexpressing more broadly and strongly in the lateral epidermis with *sca-GAL4* (Fig. 6C), a *GAL4* line that drives in the *sca* pattern (Mlodzik et al., 1990) in the epidermis and CNS. Driving with *pnr-GAL4* in the dorsal ectoderm (Winick et al., 1993; Heitzler et al., 1996), dorsal muscle attachments sites are radically disrupted, with the muscle fibers often failing to cross the segment and instead aligning with the segment boundary (Fig. 6D). Unfused myoblasts were also seen in the epidermal gain-of-function embryos.

The effects of increased *hbs* expression in imaginal discs were assessed using drivers giving general or specific domains of expression. The distal wing margin was abnormal in *omb-GAL4*/*;UAS-hbs/+ and *UAS-hbs*/*;da-GAL4*/+ flies. There

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**Fig. 5.** Misexpression of *hbs* in the embryonic mesoderm disrupts myoblast fusion. (A) Wild type pattern of the ventral musculature. (B) *UAS-hbs;twi-GAL4* embryo showing partial failure of myoblast fusion. Arrowheads indicate unfused myoblasts. (C) *UAS-hbs/+;da-GAL4/+* showing both partial failure of myoblast fusion (arrowheads), and absence of a muscle fiber in the ventral muscle group (arrow). Preparations are stained with mAb FMM5.

**Fig. 6.** Misexpression of *hbs* in the embryonic epidermis disrupts muscle attachment. (A) Wild-type embryonic somatic musculature in three hemisegments. Arrows indicate a segmentally reiterated group of three muscle fibers in the lateral musculature, while arrowheads indicate the external ventral muscles. (B) *UAS-hbs/en-GAL4* embryo showing occasional failure of the three muscle fibers (shorter arrows) to insert at their dorsal attachment sites. More ventral muscles fibers are also occasionally defective (arrowhead). (C) The ability of the three lateral muscle fibers to insert at their dorsal attachment sites is comprehensively overturned in the *UAS-hbs/sca-GAL4* embryo (shorter arrows). Ventral muscles fibers sometimes no longer span the hemisegment correctly (arrowheads). (D) Overexpression of *hbs* in the dorsal epidermis with the *pnr-GAL4* driver results in dorsal muscle fibers failing to extend across the segment (arrows), or sending a thinned process (arrowhead). Many fibers remain aligned with the segment boundary. Preparations are stained with mAb FMM5.
Table 1. Misexpression of \( hbs \) in the wing disc does not alter microchaete number

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was a blistered appearance at the distal wing edge in all \( \text{omb-GAL4/+;UAS-hbs/+} \) flies (Fig. 7B), and blistered or notched distal wing margins in the \( \text{UAS-hbs/+;da-GAL4/+} \) flies (Fig. 7C). On the dorsal thorax (notum) the large anterior section (scutum) has two mechanosensory bristle populations: macrochaetae (large bristles) and microchaetae (small bristles) (Fig. 7D). Microchaetae on the central scutum are in 10 longitudinal rows and fairly constant numbers. Misexpression of \( hbs \) in the wing discs with \( \text{pnr-GAL4, sca-GAL4 or da-GAL4} \) (Fig. 7E) perturbed the linear arrangement of the microchaetae, but their number was unaltered (Table 1).

Driving \( \text{UAS-hbs with GMR-GAL4 or sca-GAL4} \) in the eye-antennal disc gave a rough eye phenotype (Fig. 7G,H) with disorganization of the ommatidia and bristles. Driving with the \( \text{da-GAL4} \) driver gave the strongest rough eye phenotype with occasional fusion of ommatidia (Fig. 7I). Examination with anti-Elav antibody showed that the photoreceptor clusters were irregularly placed (Fig. 7K-M), and pigment cells were absent at sites of ommatidia fusions (Fig. 7O-Q). Larval photoreceptor pathfinding and targeting appeared normal when examined with mAb 24B10 (data not shown).

Overexpressing a secreted form of Hbs with all of the aforementioned GAL4 drivers did not generate gain-of-function phenotypes (data not shown). In addition, the adult \( hbs \) gain-of-function phenotypes were not suppressed as transheterozygotes with \( \text{sns, irreC-rst or the duftirreC-rst deletion.} \)

Correct \( hbs \) expression depends on \( \text{sim, Notch and bap, but not mef2} \)

\( hbs \) expression in the mesoderm and developing CNS midline partially overlaps with the expression of the transcription factor \( \text{single minded (sim)} \) (Crews et al., 1988). In \( \text{sim} \) embryos, the mesodermal progeny survive but fail to differentiate or migrate to appropriate locations (Nambu et al., 1990). In \( \text{sim} \) mutant embryos, \( hbs \) expression was abolished at the CNS midline (Fig. 8B). When \( \text{sim} \) was misexpressed in all neuroblasts with the \( \text{sca-GAL4} \) driver (Klaes et al., 1994), the domain of \( hbs \) expression at the CNS midline was expanded (Fig. 8C). Yet when \( \text{sim} \) was misexpressed in all post-mitotic neurons using \( \text{elav-GAL4} \) drivers (Luo et al., 1994) \( hbs \) expression was unaltered (data not shown).

Notch is pivotal in the development of multiple tissue types (Hoppe and Greenspan, 1986; Corbin et al., 1991; Hartenstein et al., 1992). At the developing ventral midline, Notch activity is essential for establishing \( \text{sim} \) expression (Morel and Schweisguth, 2000). Consequently, in \( \text{N\alpha K11} \) mutant embryos \( hbs \) expression is also lost at the CNS midline (Fig. 8D) at stage 12 and onwards, after depletion of the Notch maternal contribution. In addition, Notch is crucial for the development of fusion competent myoblasts (Corbin et al., 1991). In \( \text{N\alpha K11} \) mutant embryos, where myoblasts are transformed to founder cells (Corbin et al., 1991; Bour et al., 2000), \( hbs \) expression is absent in the mesoderm (Fig. 3F). We also examined whether \( hbs \) is downstream of two mesoderm-specific transcription factors, \( \text{bap} \) (Azpiazu and Frasch, 1993) and \( \text{mef2} \) (Bour et al., 1995; Lilly et al., 1995). \( hbs \) expression in visceral mesoderm was greatly decreased in \( \text{bap} \) mutant embryos (Fig. 8F), but unaffected in the \( \text{mef2} \) mutant embryos (Fig. 8E).

**DISCUSSION**

**Hibris is a member of the Nephrin subfamily of IgSF proteins**

Hibris (Hbs) is a new member of the Nephrin subfamily of the immunoglobulin protein superfamily. In vertebrates, Nephrin is a component of the slit diaphragm, the molecular sieve that facilitates ultrafiltration in the kidney glomeruli (Somlo and Mundel, 2000). In *Drosophila* the Nephrin-like protein Sns has a pivotal role in somatic myoblast fusion (Bour et al., 2000), mediating association between fusion competent myoblasts and founder cells. The present study shows that Hbs, the most recently identified member of the Nephrin subfamily, can be implicated in both myoblast fusion and myotube guidance during somatic muscle formation in the *Drosophila* embryo. In addition, several adult tissues are highly responsive to excess presentation of Hbs.

It was postulated that vertebrate Nephrins might bind homotypically (Tryggvason, 1999), as IgSF proteins often do (Goodman, 1996; Brunnendorf and Rathjen, 1996; Walsh and Doherty, 1997). In the S2 cell aggregation assay, neither Hbs nor Sns mediated homotypic adhesion. Owing to the structural similarity between Hbs and Sns and their expression patterns they were also tested in S2 cells for ability to bind in trans and did not. This is the first evidence suggesting that Nephrin proteins interact heterophilically in *trans* with other potentially non-Nephrin extracellular partners.

**IgSF proteins and myoblast fusion**

During normal development fusion competent myoblasts fuse to a specialized subset of myoblasts called ‘founder cells’. These founder cells act as scaffold for muscle formation (Bate, 1990). A crucial issue is the identity of the proteins that mediate the recognition/fusion event. The *Drosophila* homologs of the IgSF Nephrin subfamily and the DMGRASP/BEN/SC1 subfamily provide the first putative links for the molecular pathways.

Both *Drosophila* Nephrin-like proteins, Sns and Hbs, are present on the fusion competent myoblasts but not the founder cells (Bour et al., 2000) (present study). In the *sns* mutant, fusion-competent myoblasts do not fuse to founder cells, and consequently normal muscle fibers fails to form (Bour et al., 2000). Potential roles for Sns are: (1) to recognize a founder cell derived attractant and facilitate fusion competent myoblast movement towards the founder cell; (2) to help form adhesive junctions at sites where fusion will be initiated; or (3) to function as the receptor/ligand that initiates myoblast fusion (Bour et al., 2000; Frasch and Leptin, 2000). In contrast to *sns*, in the absence of *hbs* myoblast fusion and muscle formation...
appear relatively normal when assayed with anti-muscle myosin antibody. Additionally, motor axon response to the muscles is normal, as judged by the pattern of connectivity. One interpretation of the failure to produce a loss-of-function phenotype is that Hbs is redundant. Alternatively, the function of Hbs may not be completely duplicated, but its absence may give a phenotype that is subtler than we can detect given our reagents and/or criteria.

With the identification of Sns and Hbs on fusion-competent myoblasts, the question arises as to what the corresponding extracellular partners may be on the founder cells/forming myotubes. IrreC-rst (Ramos et al., 1993) and Duf (Ruiz-Gomez et al., 2000) are the Drosophila members of the DM-GRASP/BEN/SC1 (Burns et al., 1991; Tanaka et al., 1991; Pourquie et al., 1992) subfamily of the IgSF. duf is expressed by the founder cells but not the fusion-competent myoblasts (Ruiz-Gomez et al., 2000), and irreC-rst is expressed in the embryonic mesoderm but the identity of the cells was not specified (Ramos et al., 1993). Myoblast fusion fails in the combined absence of duf and irreC-rst (Ruiz-Gomez et al., 2000). The return of duf expression to the mesoderm can rescue the phenotype; however, rescue with irreC-rst was not attempted. So the respective contribution of the two proteins to the fusion phenotype is uncertain. However, as Duf misexpression can guide myoblasts to novel locations, it is considered a founder cell-derived attractant (Ruiz-Gomez et al., 2000).

The similar fusion phenotype for the sns mutant and the duf/irreC-rst deletion and the attractive properties of Duf suggest Sns and Duf underscore a fusion-competent myoblast-founder cell attraction mechanism (Frasch and Leptin, 2000). Meanwhile, hbs overexpression in the somatic mesoderm partially phenocopies the sns loss-of-function mutant and the irreC-rst/duf deletion, suggesting reduced attraction of myoblasts to the myotubes. However myoblast fusion is also partially blocked when IrreC-Rst is overexpressed in the mesoderm (H. A. D. and H. S., unpublished), and myoblasts go to ectopic locations when Duf is presented in the epidermis (Ruiz-Gomez et al., 2000). So the Hbs gain-of-function phenotype could also be interpreted as the response of myoblasts to an imbalance of attractive forces.

Support for Hbs mediating an attractive function comes from the S2 cell assays. Under the given assaying conditions, neither Sns nor Hbs interacts homotypically, and Hbs does not bind to Sns in trans. These observations go against a model where Hbs might block in trans an Sns-mediated attraction between fusion competent myoblasts and bias the interaction towards the Duf-expressing founder cells. In the S2 cell aggregation assay,
both Hbs and Sns show an interaction with Duf, mediating heterophilic adhesion between S2 cells. But neither Sns nor Hbs induced aggregates in combination with IrreC-Rst or Side (other 5 Ig domain proteins in muscles) (Ruiz-Gomez et al., 2000; Sink et al., 2001). These results support a model where both Hbs and Sns facilitate the Duf-induced attraction of fusion competent myoblasts to founder cells. But the results do not rule out other interaction combinations between these different proteins. Further experiments are required to determine if they act in cis or in complexes, or whether they require different conditions for binding to one another in trans in the S2 cell assay (Bieber et al., 1994).

**Hibris and myotube guidance**

When hbs was globally expressed with the da-GAL4 driver, the myoblast fusion defect was enhanced, and muscle fiber insertions were also misplaced. To determine whether the latter was due to hbs misexpression in the musculature or the epidermis, we misexpressed hbs in the epidermis with several GAL4 drivers. When misexpressed in the epidermis within a hemisegment, subsets of muscles fail to traverse the hemisegment and either bunch ventrally (en-GAL4 and sca-GAL4 drivers) or align with the segment boundary (pnr-GAL4 drivers). As such, the misplaced muscle attachment phenotype observed in the da-GAL4 gain-of-function condition is attributed to epidermally rather than mesodermally misexpressed hbs.

hbs is broadly expressed in the epidermis and around at the sites where muscles will eventually attach, then becomes confined to the muscle attachment sites themselves. During normal development, Hbs may assist in slowing and constraining myotube exploration in the region where attachments must ultimately form. The data on myoblast fusion links Hbs to an attraction/adhesion mechanism. Furthermore, Duf is present in developing myotubes (Ruiz-Gomez et al., 2000) and Sns is also present at the muscle attachment sites (Bour et al., 2000). Given these expression patterns and the heterotypic interaction of Duf with Hbs and Sns, it is possible that these proteins also interact during myotube guidance, serving to direct myotubes to their epidermal attachment sites.

**Overexpression of hbs in the adult**

hbs is also expressed in the larval imaginal discs. Misexpression of hbs in the wing disc yielded abnormal distal wing margins and disorganized microchaetes on the notum. At first glance, these phenotypes look like mild loss-of-function Notch defect; however, microchaetae numbers did not deviate from normal. Hence, the microchaete phenotype appears to be due to displacement of cells, rather than changes in cell number caused by disturbance of lateral inhibition. This could arise if: (1) the cell-cell associations within proneural clusters are slightly perturbed, resulting in subtle changes in the location of the founder cells; or (2) founder cells are normally specified but subsequent alterations in cell-cell associations lead to them being slightly displaced.

Misexpression of hbs in the eye disc results in a rough eye phenotype, which is reminiscent of that seen when irreC-rst is absent or misexpressed in the eye disc (Ramos et al., 1993; Schneider et al., 1995). Yet neither gain-of-function eye phenotype was suppressed by a 50% decrease in the expression of the other gene, and no rough eye phenotype was observed in the hbs and irreC-rst transheterozygotes. As Hbs and IrreC-Rst did not give a positive result in the S2 cell aggregation assay, and the overexpression of irreC-rst in the wing imaginal disc caused a notal microchaete phenotype that differs from that for hbs (H. A. D. and H. S., unpublished), there is still no direct support that Hbs and IrreC-Rst interact directly with one another in a simple one-to-one trans binding relationship. More suitable interaction analyses await elucidation of whether Duf and Sns have roles in eye.

**Regulation of hbs expression**

The complex spatiotemporal expression pattern of hbs, added to the sensitivity of several tissues to abnormal hbs levels, brings into question the identity of the upstream regulators. We examined hbs expression in Notch, mef2, bap and sim mutant backgrounds.

Notch-mediated lateral inhibition defines which myoblasts will become founder cells (Corbin et al., 1991). hbs and sns are excluded from the founder cells and confined to fusion-competent myoblasts. As with sns, this exclusion of hbs from the founder cells occurs downstream of Notch. Thus Sns and Hbs represent two components of the molecular repertoire that distinguishes fusion competent myoblasts from founder cells.
from one another. The transcription factor Mef2 regulates expression of muscle specific genes such as myosin, MLP60A, tropomyosin I and muscleblind (Bour et al., 1995; Ranganayakulu et al., 1995; Lin et al., 1996; Lin et al., 1997; Stronach et al., 1999; Artero et al., 1998) and its absence results in a myoblast fusion defect (Lilly et al., 1995). As for sns, hbs expression also appears unperturbed in the mef2 mutant. So neither the loss of sns nor hbs expression contributes to the mef2 myoblast fusion phenotype. In contrast to Mef2, the activity of the homeobox gene bag is crucial for correct levels of expression of hbs in the early visceral mesoderm cell clusters. Similarly the Sim helix-loop-helix transcription factor (Nambu et al., 1990; Nambu et al., 1991) is essential for hbs expression in the mesectoderm and CNS midline. Whether there a direct physical regulation of hbs expression by the Sim or Bap proteins awaits analysis of the hbs promoter region.

We have presented the first evidence of a direct physical interaction between extracellular molecules that are expressed on either fusion-competent myoblasts and at muscle attachment sites (Hbs and Sns), or on muscle founder cells and developing myotubes (Duf). Our observations suggest that adhesion between these molecules may aid recognition between fusion competent myoblasts and founder cells, and between myotubes and epidermal attachment sites. Whether the large cytoplasmic domains on these proteins have signaling abilities must now be determined.

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REFERENCES


