Basigin (EMMPRIN/CD147) interacts with integrin to affect cellular architecture

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Summary
Basigin, an IgG family glycoprotein found on the surface of human metastatic tumors, stimulates fibroblasts to secrete matrix metalloproteases that remodel the extracellular matrix. Using Drosophila melanogaster we identify intracellular, matrix metalloprotease-independent, roles for basigin. Specifically, we found that basigin, interacting with integrin, is required for normal cell architecture in some cell types. Basigin promotes cytoskeletal rearrangements and the formation of lamellipodia in cultured insect cells. Loss of basigin from photoreceptors leads to misplaced nuclei, rough ER and mitochondria, as well as to swollen axon terminals. These changes in intracellular structure suggest cytoskeletal disruptions. These defects can be rescued by either fly or mouse basigin. Basigin and integrin colocalize to cultured cells and to the visual system. Basigin-mediated changes in the architecture of cultured cells require integrin binding activity. Basigin and integrin interact genetically to affect cell structure in the animal, possibly by forming complexes at cell contacts that help organize internal cell structure.

Key words: Basigin, EMMPRIN, CD147, Integrin, Cell structure, Drosophila, Geled

Introduction
Basigin or EMMPRIN (extracellular matrix metalloproteinase inducer, CD147, OX47, 5A11) is a cell-surface glycoprotein of the IgG superfamily, found on a variety of metastatic tumors (Biswas et al., 1995; Muraoka et al., 1993; Polette et al., 1997; Bordador et al., 2000; Kanekura et al., 2002). Basigin can promote matrix metalloprotease (MMP) secretion from fibroblasts (Kataoka et al., 1993; Li et al., 2001) and also from tumor cells (Sun and Hemler, 2001). The extracellular domain alone can stimulate fibroblasts to secrete MMPs by interacting with an unidentified receptor (Guo et al., 1997). Basigin oligomerizes with itself (Fadool and Linser, 1996; Yoshida et al., 2000) and it also interacts physically with α3β1 integrin at points of cell-cell contact, but not at focal adhesions (Berditchevski et al., 1997). Integrins are cell-surface dimers composed of one α and one β subunit that bind to a variety of extracellular matrix molecules, as well as to some cell-surface receptors (Arnaout et al., 2002). They play roles in cell attachment, cell migration and cell-cell interactions. The gene for Drosophila basigin (previously known as gel) has been shown to interact genetically with integrins in the Drosophila embryo (Reed et al., 2004).

Several lines of evidence suggest a role for basigin in metastasis. First, basigin expression and metastasis correlate in human melanoma (Kanekura et al., 2002). Second, basigin stimulates MMPs from fibroblasts adjacent to tumors (Zucker et al., 2001) and MMPs remodel the ECM, allowing tumor invasion (Nabeshima et al., 2002). Third, co-culture of basigin-expressing melanoma cells with fibroblasts results in MMP induction and migration of tumor cells through a reconstituted basement membrane; anti-basigin antibodies block both of these activities (Kanekura et al., 2002). Fourth, expression of basigin in slow-growing breast cancer cells lines that are then injected into mouse mammary tissue, leads to larger and more invasive tumors than controls (Zucker et al., 2001). Lastly, basigin promotes adhesion-independent cell growth and this may contribute to secondary tumor formation (Mariët al., 2004).

Basigin is one of a three-member family in mammals that includes embigin and neuroplastin (SDR1, gp55/gp65). In vertebrates, basigin is expressed in a variety of tissues including the developing retina, blood-brain barrier, CNS, thymus, epithelial tissues and a variety of immune cells (Fadool and Linser, 1994; Fan et al., 1998b). Embigin is expressed in mouse embryos and many tissues in the adult (Huang et al., 1990; Fan et al., 1998a). Neuroplastin is expressed in the nervous system in the cortex, cerebellum and hippocampus (Langnaese et al., 1997), and in some non-neural tissues.

In mammals, the basigin gene encodes two nearly identical protein isoforms both with two IgG-C2 domains (Kanekura et al., 1991), as well as an isoform with three IgG-C2 domains (Ochrietor et al., 2003). The neuroplastin gene also encodes two isoforms, a two-IgG protein (gp55) and a three-IgG protein (gp65). Both forms are expressed in the brain, but the gp65 protein is brain specific (Langnaese et al., 1997). The gp55 form is expressed along the axon whereas the gp65 protein is concentrated at postsynaptic densities and may play a role in long-term potentiation (Smalla et al., 2000).
Here we identify previously unknown functions for basigin family proteins by examining the *Drosophila* homologue, D-basigin. In particular, we find that D-basigin has dramatic effects on internal cell architecture, both in culture and in vivo, and that it mediates these effects through interactions with integrins. This function appears to be independent of MMPs.

### Materials and Methods

#### Immunohistochemistry

Adult fly heads were fixed in 3% paraformaldehyde in 5× phosphate buffer for 5 hours, washed three times for 10 minutes in PBS, incubated overnight in PBS plus 20% sucrose and subsequently mounted in TissueTek (Fischer Scientific) and quick frozen in liquid nitrogen. Sections 10-15 µm thick were collected onto slides pretreated with poly-L-lysine (Sigma). Samples were blocked for 1 hour in PBS plus 1% Triton X-100 and either 2% BSA or normal goat serum (Vector Labs), then incubated overnight in primary antibody en bloc. Slides were washed three times for 30 minutes in PBS plus 0.5% Tween 20. Secondary antibody was applied for 1 hour. Biotinylated goat anti-rat, anti-mouse or anti-chicken secondary antibodies (Vector Labs) were resuspended in 1 ml PBS and diluted 1:200 in blocking solution. Slides were washed as before. The ABC HRP kit (Vector Labs, PK-6100) was used according to the manufacturer’s instructions. Visualization was via the Vector VIP stain (SK4600). Slides were mounted with Permount and photographed with a digital camera. Immunofluorescence images were prepared from material fixed and treated the same way, but using fluorescent secondary antibodies (described below). These samples were visualized by fluorescence microscopy and the images converted to black and white in Adobe Photoshop.

#### Cell culture and labeling

S2 cells were grown in Schneider’s medium with 10% FBS (Invitrogen). High Five cells were grown in HyQ serum-free media supplemented with 10% calf serum (Invitrogen). Between 2×10^6 and 5×10^6 cells were plated on 25-mm-square coverslips pre-treated with 0.1 mg/ml poly-L-lysine (Sigma). They were allowed to attach for 18 to 36 hours before fixation for 20 minutes in 4% paraformaldehyde. Fixative was washed out with four rinses in PBS. Cells were permeabilized for 10-20 minutes with PBS plus 0.3% Triton X-100. Cells were fixed for 15 minutes in blocking buffer (PBS containing 0.3% Triton X-100 and 2% BSA, Sigma). Primary and secondary antibodies were diluted in blocking buffer and applied for 1 hour each. Each antibody incubation was followed by three washes with 0.3% Triton X-100 in PBS. Coverslips were dried and mounted in glycerol gelatin (Sigma) with 1 mg/ml p-phenylene diamine (Sigma).

S2 cells with and without integrin genes were obtained from Daniel L. Brower (University of Arizona). High Five cells and the pIZT expression vector with the V5 tag were obtained from Invitrogen and M. L. Brower (University of Arizona). High Five cells were grown in HyQ serum-free media supplemented with 10% calf serum (Invitrogen). Between 2×10^6 and 5×10^6 cells were plated on 25-mm-square coverslips pre-treated with 0.1 mg/ml poly-L-lysine (Sigma). They were allowed to attach for 18 to 36 hours before fixation for 20 minutes in 4% paraformaldehyde. Fixative was washed out with four rinses in PBS. Cells were permeabilized for 10-20 minutes with PBS plus 0.3% Triton X-100. Cells were fixed for 15 minutes in blocking buffer (PBS containing 0.3% Triton X-100 and 2% BSA, Sigma). Primary and secondary antibodies were diluted in blocking buffer and applied for 1 hour each. Each antibody incubation was followed by three washes with 0.3% Triton X-100 in PBS. Coverslips were dried and mounted in glycerol gelatin (Sigma) with 1 mg/ml p-phenylene diamine (Sigma).

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### Antibodies and labels

D-basigin peptide antibody was raised in chickens (Alpha Diagnostics) to LIADENKFIIDKTDNTDGKYSC, a peptide uniquely found in D-basigin. Other antibodies were obtained from the following sources: Anti-β-gal, Promega (#Z378A) used 1:1000; anti-V5 (Invitrogen) used 1:500; anti-cPS1 (monoclonal DK.1A4), anti-βPS integrin (monoclonal CF.6G11) and anti-μPS2 (CF.2C7) were obtained from Daniel L. Brower (University of Arizona); anti-elav (mouse and rat monoclonal antibodies), anti-repo and 24B10 from the Developmental Studies Hybridoma Bank (University of Iowa); antitubulin (Sigma T4026, used according to the manufacturer’s instructions); biotinylated secondary antibodies were from Vector Labs. Alexa-568 anti-chicken, Alexa-488 anti-mouse and Alexa-568 phalloidin were all from Molecular Probes.

### Files and mosaics

Mosaics were prepared by the method of Stowers and Schwarz (Stowers and Schwarz, 1999). P-element insertion P1096 and P1478 were obtained from the Bloomington *Drosophila* Stock Center, as were EGUF/hid lines for FRT40A. The bsg<sup>265</sup> excision allele was created by crossing P1478 to a fly line containing transposase and selecting for a loss of the eye color marker encoded within the engineered P-element. Integral alleles and integrin monoclonal antibodies were obtained from Daniel L. Brower (University of Arizona).

### Electron microscopy

The lamina, innervated by either control or bsg<sup>265</sup> mutant photoreceptors, was prepared for electron microscopy (EM) using previously reported methods (Meinertzhagen, 1996; Meinertzhagen and O’Neel, 1991). Single sections containing cartridge profiles cut in cross-section were examined and digital montages collected from images obtained with a Philips Tecnai 12 operated at 80 kV, using a Kodak Megaview II camera with software (AnalySIS, Soft Imaging System, Münster).

### Northern blots

Total mRNA was isolated from animals at different developmental stages using a kit (Qiagen) and RNA was quantified by running samples on a gel and estimating the relative intensity of rRNA bands. Blotting was done by standard techniques (Sambrook and Russell, 2000). A radioactive probe was made to the cloning region of basigin by isolating the basigin gene from an agarose gel and using the gene as a template in a random primer reaction made using a random primer kit (New England Biolabs). The probe was labeled with [32P]dCTP and used to probe the mRNA, as described (Sambrook and Russell, 2000).

### BLAST analysis

BLAST analyses of mammalian genes using the *Drosophila* genome as a database for comparison were carried out at the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/blast/blast_form.html.) using full protein sequences for neuroplastin, basigin and embigin. Default settings for the site were used. An amino acid-based search was chosen and the database chosen included all predicted proteins for the genome. Predicted proteins that had basigin homology were examined by following available links to see if these proteins were of similar length to basigin and had characteristic features of the basigin protein family, including transmembrane domains, IgG domains and high sequence homology in or near the transmembrane domain.

### Results

#### Structure of the *Drosophila basigin* gene

In a screen for genes that act in the eye we discovered the gene for the *Drosophila* homologue of mammalian basigin. This gene maps to cytological band 28E3, spans 25 kb and had been previously designated gelled (gel) (Castrillon et al., 1993), but has been renamed basigin (bsg). We used all three mammalian basigin protein sequences to search the *Drosophila* genome for predicted proteins related to basigin and found that bsg is the
only Drosophila gene that encodes a basigin family member in flies. According to data from the Drosophila genome project (available at Flybase, http://flybase.bio.indiana.edu/), the bsg gene encodes nine distinct transcripts that appear to fall into three structural classes (Fig. 1). These transcripts encode two predicted protein isoforms of D-basigin, 265 and 298 amino acids, each with two IgG-C2 domains, a transmembrane domain and a short internal tail (Fig. 2A). Class 1 and 2 bsg transcripts contain distinct 5’ non-coding exons that splice onto common coding exons to code for D-basigin 265. The class 3 bsg transcript encodes D-basigin 298. The two isoforms are identical over 240 amino acids, differing only at the N- and C-termini.

A northern blot showed that bsg was expressed in all stages of Drosophila development tested (Fig. 1B). Only two sizes of bsg message were seen. The larger, more diffuse band corresponded to class 1 and 2 transcripts that have a nearly identical predicted size of ~2.5 kb (Fig. 1B). Around embryonic stage 5 a slightly smaller transcript was expressed that corresponded more closely to the predicted size of ~2 kb for the class 3 transcript. As no other transcript sizes were seen, we concluded that the Drosophila bsg gene probably encodes only the two predicted isoforms of D-basigin. The expression of this smaller transcript at very early stages may mean that it is maternally contributed.

Homology between mouse and fly basigin

Mouse basigin showed 26% identity and 34% similarity with Drosophila basigin protein. The extracellular domains showed 20% identical residues and 28% similar residues, whereas there was 80% identity in or near the transmembrane domains (Fig. 2). Indeed, the transmembrane domains of basigin, neuropilin and embigin from many different species show very high identity (Fig. 2) (Ochrietor et al, 2003), including spaced leucines, as well as conserved proline and glutamic acid residues. The presence of a charged residue in the transmembrane domain is consistent with the fact that basigin forms complexes (Fadool and Linser, 1996), possibly within the plane of the membrane. There was no homology in the short internal tail between mouse and D-basigin with the exception of the first five cytoplasmic residues (Fig. 2). D-basigin showed 30% similarity to both rat neuropilin and rat basigin.

D-basigin promotes cytoskeletal rearrangement in cultured cells

The bsg265 transgene that codes for D-basigin 265 was introduced permanently into insect High Five cells. These cells are derived from the embryo of the cabbage looper (Trichoplusia ni) and used as a baculovirus expression system. High Five cells permanently transfected either with empty vector or with bsg265 transgene were labeled with Alexa 568-phalloidin to visualize actin microfilaments (Fig. 3A), or with anti-tubulin antibody to visualize microtubules (Fig. 3B). Two classes of cells were seen showing two clearly distinct cytoskeletal arrangements. One class of cells showed actin filaments in an almost exclusively cortical pattern (Fig. 3A, Fig. 4F). These cells invariably showed a nuclear concentration of tubulin (Fig. 3B) and were spherical (not flattened to the dish). The second class of cells showed elaborated microfilaments (Fig. 3C, Fig. 4F) and microtubules (Fig. 3B) throughout the cytoplasm. These cells appeared flattened to the dish in light microscopy.

When D-basigin protein was expressed in these cells, the number of each cell type changed noticeably. About 85% of control High Five cells showed cortical actin microfilaments (Fig. 3C and similar to Fig. 4F) and a round morphology with a nuclear concentration of tubulin (Fig. 3B), whereas only 15% of cells showed elaborate microfilaments and microtubules and a flattened appearance by light microscopy. By contrast 80% of D-basigin-expressing cells showed an elaboration of microfilaments (Fig. 3A, Fig. 4E) and microtubules (Fig. 3B) whereas only 20% showed a rounded morphology with cortical actin and a nuclear concentration of tubulin. Thus basigin expression in High Five cells led to a fivefold increase in the number of cells showing elaborated microfilaments and microtubules and a flattened appearance. This change in cytoskeletal rearrangement seemed to result from the cell-autonomous expression of D-basigin. First, these changes were independent of cell contact, as physically isolated basigin-expressing cells were just as likely to show the altered cytoskeletal arrangement as cells that were touching. Second, these changes in cell architecture were not due solely to

**Fig. 1.** Basigin gene structure and developmental northern blots. (A) Structure of the bsg gene encodes nine transcripts according to data from the Drosophila genome project. Transcribed portions of the gene are shown as blue boxes. Transcripts fall into classes 1a-d, 2a-d and 3. Transcripts 1a-d and 2a-d encode the same protein, D-basigin 265 (sequence shown in Fig. 2). Transcript 3 encodes a slightly longer protein, D-basigin 298. The P elements, P1096 and P1478, inserted at precisely the same location (marked by arrow) 1145 bp from the ATG for D-basigin 265 and 981 bp from the start of the first coding exon. The location of the start codon for transcript 3 is also indicated. The excision line 19 (black bar) contains a 4 kb deletion that removes the first coding exon. (B) Northern blot showing expression of bsg in embryos at stages 5, 9, 13, and 16, first-, second- and third-instar larvae and adult heads (A). The major band of ~2.5 kb is consistent with the length of class 1 and 2 bsg transcripts.
We therefore tested whether D-basigin-mediated integrins can promote cell attachment and cause cells to spread. The D-basigin protein expressed in High Five cells had a V5 sequence (Arnaout et al., 2002), the peptide GRGDS is commonly used as a competitive inhibitor for such integrin binding (Huang et al., 1993). When D-basigin-expressing cells were cultured in the presence of a GRGDS peptide (Fig. 4F), the cells looked indistinguishable from control High Five cells, showing a rounded morphology with cortical actin filaments. By contrast, D-basigin-expressing cells grown without peptide (Fig. 4E) had elaborated microfilaments and a flattened appearance. D-basigin-expressing cells were much less affected by a control peptide, GRGES at the same concentration of 200 µg/ml (not shown). Cells incubated with GRGES showed that 65% of the cells spread compared to 80% of control cells.

D-basigin partially colocalizes with integrin in integrin-transfected S2 cells

Previous work indicated that basigin colocalizes with some integrins at cell-cell contacts (Berditchevski et al., 1997). To examine if D-basigin and integrin colocalize within the cell, we generated antibody to a peptide in the extracellular domain of D-basigin. This antibody did not label control High Five cells normally contain many vesicles even when D-basigin is not expressed. Lastly, a subset of D-basigin immunolabeling colocalized to the actin cytoskeleton, especially at points of cell-cell contact (Fig. 4A,B) and near cell edges (Fig. 4C,D). The degree of colocalization in isolated cells varied. However, in cells that were in physical contact, D-basigin-actin colocalization at cell-cell contacts was invariable (Fig. 4A,B).

D-basigin-mediated changes in cell architecture require integrin binding

Integrins can promote cell attachment and cause cells to spread out in culture. We therefore tested whether D-basigin-mediated changes in cell architecture depended on integrin binding. Because many integrins bind to ECM molecules, such as collagen and fibronectin, at an Arg, Gly, Asp (RGD) target sequence (Arnaout et al., 2002), the peptide GRGDS is commonly used as a competitive inhibitor for such integrin binding.
Basigin and integrin affect cell structure

D-basigin partially colocalizes with integrin in the retina

We next looked for colocalization between D-basigin and integrin in the Drosophila visual system because we had originally identified bsg in a visual system screen. Adult head sections were double-labeled with anti-D-basigin (Fig. 5B) and monoclonal antibodies against βPS integrin (Fig. 5C), which are expressed in the retina (Brower et al., 1995).

D-basigin antibody (Fig. 5B) revealed lines of immunofluorescent puncta in the retina. Labeling the same sections with anti-αPS1 integrin antibodies (Fig. 5C) or anti-βPS antibodies (not shown) revealed multiple points of colocalization at these puncta, the positions of which did not correspond to ECM and were therefore probably points of cell-cell contact. Integrin-specific antibodies also showed a clear line of expression at the basement membrane (arrows, Fig. 5C) whereas D-basigin antibody did not strongly label the membrane (Fig. 5B) in most samples. Integrins are expressed in retinal pigment cells, and this line may represent the focal adhesions that the cells make with the basement membrane (Longley and Ready, 1995). D-basigin was not expressed in these pigment cells (see below).

D-basigin is expressed in photoreceptors and in basal glia

The above labeling did not allow us to identify the specific retinal cell types that express D-basigin protein. To identify these, we examined expression from an enhancer trap line in the gene for D-basigin, bsg. Two P-element insertions in bsg (P1096 and P1478, insertion point indicated in Fig. 1A) were obtained from the Bloomington Drosophila Stock Center. Both contain a bacterial lacZ gene encoding a nuclear form of β-galactosidase. This lacZ gene contains no regulatory sequences and thus the bsg regulatory elements should drive expression (i.e. it should act as an ‘enhancer trap’). Anti-β-gal revealed expression in photoreceptors and basal glia in adult head-sections from both lines (Fig. 5A). Basigin expression was examined in the larval eye disc, using both the enhancer trap line and in-situ hybridization, and no exception was seen in either of these cell types at this stage.

Basigin gene mutations

The two P-element insertions in bsg mentioned previously, labeled P in Fig. 1A, are located 1145 bp from the start of transcription for the D-basigin 265 protein isoform (Fig. 1A). Homozygous mutant animals from both lines died after the second larval instar with only 3% of mutant larvae living to the third instar. The insertions failed to complement each other. Because this P-insertion did not interrupt the coding portion of the gene, animals carrying this mutation may have produced some functioning protein. To generate a more severe allele, the P-element (P1478) was mobilized; such mobilization occasionally caused loss of genetic material near the insertion site. We established 200 excision lines in which the P-element was missing; 182 were viable, indicating a clean excision of the P-element, whereas 18 were homozygous lethal and failed to complement the original P-element allele. By DNA blot analysis, two excision lines, bsg6265 and excision number 64, were shown to be missing ~4 kb, including the first coding exon for the D-basigin 265 protein. Both lines showed high embryonic lethality with 75-80% of the animals dying as embryos. Those embryos that did hatch died within the first day and were small, lethargic and uncoordinated.

D-basigin affects the subcellular structure of photoreceptor neurons

Given that D-basigin affects cell architecture in culture, we were interested to know if it affected cell structure in the animal. To address this, we looked for the effects of D-basigin on placement of internal cellular organelles in photoreceptors. Because the mutations are embryonic lethal, we made mosaic animals in which D-basigin protein expression was missing...
only in the eye and invariably missing from photoreceptor neurons. We generated such mosaics by the method of Stowers and Schwarz (Stowers and Schwarz, 1999) in which FLP recombinase is expressed from the eye-specific promoter of the eyeless gene (ey). Eyeless-FLP mediates recombination in the eye between chromosome arms bearing engineered copies of the FLP binding sites (FRTs) near their centromeres. We recombined a chromosome arm bearing a bsg mutation with a chromosome arm bearing the cell death gene hid expressed specifically in all photoreceptors. After recombination and chromosome segregation, only photoreceptors that inherit two copies of mutant bsg survive to repopulate the eye; bsg eyes were almost normal in size.

Photoreceptor nuclei were visualized with an antibody against elav, a neuron-specific nuclear protein. Normally, photoreceptor nuclei lie in tight rows across the eye (e.g. Fig. 6C), so that any mislocalization is readily detected. The nuclei of the R1-R6 photoreceptors lie in the apical region of the retina (Fig. 6C). The nuclei of the R7 photoreceptors are just proximal to those of R1-R6 and the R8 nuclei lie near the basement membrane of the retina (Fig. 6C).

Photoreceptor nuclei of mosaic flies mutant in the eye for the hypomorphic P1096 allele, which encodes a nuclear β-gal, were visualized with anti-β-gal (Fig. 5A). Most nuclei were properly located, although a few nuclei were misplaced (Fig. 6A). We saw similar results for these mosaics with anti-elav (not shown). In mosaics that are mutant in the eye for the bsg<sup>δ265</sup> excision allele, elav immunolabeling revealed that 16-50% of photoreceptor nuclei were mislocalized (Fig. 6B). Nuclei were counted as misplaced only if they were obviously located between the normal position for R7 and the normal position for R8, in the region of the eye where no nuclei are usually located (see Fig. 6C). Thus nuclei that were slightly displaced were not counted. Sections from a total of 18 animals were counted (10,250 nuclei). Although the range of nuclear misplacements per fly was 16-50%, most animals fell within the lower end of this range, the average number of misplaced nuclei, pooling data from all animals, being 22%.

The nuclear placement defect was rescued by expressing D-basigin 265 (Fig. 6C). We counted nuclear placement in 12 animals that were mutant in the eye for bsg<sup>δ265</sup>, but also contained a bsg<sup>δ265</sup> transgene that expressed D-basigin 265 in photoreceptors and found only 1% of misplaced nuclei. Expression of the mouse basigin gene in photoreceptors also rescued the nuclear misplacement (Fig. 6D) with only 1.5% of nuclei misplaced in a total of 12 animals counted (7300 nuclei counted). Thus despite limited sequence homology, mouse basigin can promote the formation of normal cell architecture in flies.

Photoreceptors R1-R6 terminate in the lamina, or first optic neuropile. We examined laminas in which only the photoreceptors are mutant for bsg<sup>δ265</sup> (i.e. the postsynaptic lamina neurons and glia are wild type). Rough endoplasmic reticulum (rER) was found misplaced into the mutant 

**Fig. 4.** D-basigin partially colocalizes with actin and integrin. (A-F) High Five cells expressing D-basigin with a C-terminal V5 tag. (A and C) Cells labeled with anti-V5 antibody and visualized with Alexa 488-conjugated secondary antibody. (B,D-F) Cells labeled with Alexa 568-phalloidin to visualize F-actin. (A) D-basigin is expressed diffusely and in numerous vesicles within the cell. There is strong D-basigin expression at cell-cell contacts (arrow). (B) Actin colocalizes with D-basigin at cell-cell contacts (arrow). (C-D) High magnification view of the edge of a basigin-expressing cell (ca. 15 μm total width). There is significant colocalization of D-basigin (C) and actin filaments (D) as shown for marked filaments (asterisks). Some actin filaments do not colocalize with D-basigin (red arrowhead in D). D-basigin-mediated cell spreading requires integrin activity. (E) D-basigin-expressing High Five cells cultured under normal conditions. (F) Cells cultured in the presence of 200 μg/ml GRGDS peptide which competes for integrin binding sites. (G) S2 cell expressing Drosophila αPS1βPS integrins labeled with antibody against D-basigin and visualized with Alexa 568-conjugated secondary. (H) The same cell as in G labeled with a mixture of antibodies to αPS1 and βPS integrin and visualized with Alexa 488-conjugated secondary antibody. D-basigin and integrin colocalize at many sites. Bar, 20 μm (A,B); 60 μm (E,F); 10 μm (G,H).
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To look for genetic interactions between bsgΔ265 and integrin genes, we made double mutants by creating males that carried the mysb45 allele (coding a mutant βPS integrin), but were also homozygous mutant only in the retina for the P1096 bsg allele. These animals showed obvious misplacement of nuclei (Fig. 6F). The average number of misplaced photoreceptor nuclei per head section, after examining at least 12 animals of each genotype, was three times higher in the double mutants than that predicted from the summed effect of the two single mutations. Mosaics doubly mutant for mysb45 and bsgΔ265 also showed a more severe photoreceptor nuclear misplacement phenotype than the sum of the two single mutations would predict; 80% of nuclei were misplaced (not shown) compared with an average of 24% for bsgΔ265 and 1-2% for mysb45.

Some integrin gene allelic combinations also showed nuclear misplacement. Animals heterozygous for mewM6, a null allele for αPS1 integrin (Brower et al., 1995) showed normal placement of photoreceptor nuclei. Animals heterozygous for mysb45, a βPS1 allele, showed normal nuclear placement, similar to the mysb45 hemizygous males (Fig. 6E) just discussed. However, animals heterozygous for both mewM6 and mysb45 showed 3% misplaced nuclei (Fig. 6G; >600 nuclei from three different animals counted).

MMP2 is not required in the eye for photoreceptor architecture

Because mammalian basigin stimulates secretion of MMPs, we examined the role of MMPs in the fly visual system. Drosophila has two MMP genes, Mmp1 and Mmp2, both required for viability. Only Mmp2 is expressed in the developing eye (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003). If D-basigin were acting primarily through MMP-2, then flies lacking MMP-2 in the retina should have the same phenotypes as those found in bsgΔ265 mutant retina. Using the same method previously described to make bsgΔ265 eye mosaics, we made flies that were mutant in the eye for a null Mmp2 allele, Mmp20.3072 (Page-McCaw et al., 2003). We saw no misplaced photoreceptor cell nuclei (Fig. 6H). In case MMP1 functionally replaces MMP-2, we made mosaics that were mutant in the eye for both genes. These also showed no misplaced nuclei (not shown). Finally,
we saw no effect on nuclear placement when we drove expression of *Drosophila* TIMP (tissue specific inhibitors of MMPs) in the eye (not shown), even though this TIMP gene has previously been reported to block biological activity of *Drosophila* MMPs (Page-McCaw et al., 2003).

**Discussion**

Basigin is found on a wide variety of metastatic tumors and has been shown to enhance tumor growth and invasiveness (Muraoka et al., 1993; Polette et al., 1997; Bordador et al., 2000; Kanekura et al., 2002; Zucker et al., 2001). In mammalian systems, basigin stimulates secretion of MMPs (Li et al., 2001; Kanekura et al., 2002). MMPs, in turn, promote tumor cell invasion by breaking down the ECM (Kataoka et al., 1993; Li et al., 2001; Sun and Hemler, 2001). Thus basigin has been thought to act outside the cell, primarily though MMPs, even though basigin has also been shown to interact with several different molecules suggesting that at a molecular level it may be multifunctional (reviewed by Toole, 2003).

In this study we identify previously uncharacterized roles for basigin. We show that D-basigin affects the intracellular architecture of the cells in which it is expressed, possibly by complexing with integrins and actin. This function appears not to require MMPs.

**D-basigin alters cell structure**

In High Five cells, D-basigin expression promotes the rearrangement of both the actin and tubulin cytoskeleton with consequent formation of lamellipodia. Likewise, in *Drosophila* photoreceptors, D-basigin is required for normal cell architecture. In mosaics in which D-basigin expression is missing from the photoreceptors, the nuclei, rough endoplasmic reticulum and mitochondria in these cells are all misplaced. Photoreceptor terminals mutant for *bsg* are also larger than wild-type terminals. All these defects suggest disruption of the cytoskeleton.

**D-basigin acts through integrins**

There are many reasons to believe that D-basigin affects cell structure by interacting with integrins. First, D-basigin-mediated cell spreading is blocked by peptides that block integrin binding sites. Second, D-basigin and integrin colocalize in the *Drosophila* retina. Third, D-basigin and integrin colocalize to sites within the cell in integrin-expressing *Drosophila* S2, as well as in human cells (Berditchevski et al., 1997), in which the two proteins also co-immunoprecipitate. This suggests they may form a complex in the membrane. Fourth, allelic combinations of integrin gene mutations show disruption of retinal cell structure, e.g. misplaced nuclei, similar to those in *bsg* mosaics. Fifth, *bsg* and integrin gene mutations interact to affect nuclear placement. Sixth, *bsg* and integrin genes have been shown to interact genetically to affect dorsal closure and germ band retraction in the *Drosophila* embryo (Reed et al., 2004).

Two additional findings support the idea that D-basigin and integrin interact to affect cell structure. First, antibodies against D-basigin can block integrin-mediated adhesion of T cells to ECM (Allain et al., 2002). Second, expression of embigin, a basigin family member, causes normally non-adherent mouse L cells to spread in an integrin-mediated fashion (Huang et al., 1993), similar to what we see with D-basigin. Given that basigin does not localize to focal adhesions (Berditchevski et al., 1997), the mechanism by which it mediates integrin-mediated cell attachment is not clear.

![Fig. 6. D-basigin expression in the retinal photoreceptor neurons is necessary for the proper placement of their nuclei. Frozen sections through the retina (brackets in B and C); la, lamina. The normal locations of the R1-8 nuclei are marked in C. (A) Section labeled with antibody to the neural nuclear protein, elav. (A) Mutant for the hypomorphic *bsg* allele, P1096. A few photoreceptor nuclei are displaced (arrow). (B) Mutant for *bsg* rescued by a GMR-*bsg* transgene that drives expression of D-basigin 265 in all photoreceptor neurons. The nuclei of the R7 photoreceptors are just proximal to those of R1-R6 and the R8 nuclei lie near the basement membrane of the retina. (D) Mutant for *bsg* rescued by a GMR-mouse basigin transgene (*Bsg*). D-basigin and integrin interact to affect nuclear placement. (E) Male carrying a viable allele of integrin, *β*PS1 (*mws*). (F) Integrin *bsg* rescued double mutant male carrying *mws* also mutant in the eye for P1096 *bsg*. Arrows indicate some of the misplaced nuclei. The number of misplaced nuclei is much greater than the sum of the two mutations independently (A and E). (G) Fly heterozygous for both the *α*PS1 integrin allele, *mew* and the BPS integrin allele *mwsb*15. Examples of misplaced nuclei are marked with arrows. (H) Fly mutant in the eye for *Mmp2*, which shows no abnormality in nuclear placement. Bar, 30 µm.
Fig. 7. R1-R6 in bsg<sup>δ265</sup> mutant terminals exhibit a mutant ultrastructural phenotype. (A) Cross section of a control cartridge innervated by non-mutant axons (control animals were generated by recombining a wild-type chromosome arm using the same basic procedure used to create mutant terminals as already described). This control exhibits a wild-type structure (Meinertzhagen and O’Neil, 1991) in which a ring of photoreceptor terminals (R) surrounds the axon profiles of lamina cells L1 and L2. The entire cartridge is surrounded by lamina epithelial glia (*). Terminals contain normal mitochondria (m) and synaptic profiles composed of synaptic vesicles, capitate projections (arrowheads) and T-bar synaptic ribbon release sites (arrow). (B) Cartridge innervated by bsg<sup>δ265</sup> photoreceptors; the terminals are of variable sizes, most are larger than controls in A, which are shown at the same magnification, although one terminal is very small (*). At this distal section plane in the lamina, close to the eye, there are more mitochondrial profiles than normal. (C) In the same lamina as B, but cut at a proximal level, R1-R6 terminals lack mitochondrial profiles. Such profiles disappear in the distal third of the lamina’s depth, with a cut-off that is sharply localized. At that level, cartridge cross-sections, as here, have some terminals with (m) and some without (*) mitochondrial profiles. (D) Individual R1-R6 terminal from C, exhibits misplaced rough ER (long arrows), never normally seen in either control or wild-type terminals and pleomorphic profiles of synaptic vesicles (short arrow); capitate projections are mostly lacking, except those that are shallow (arrowhead), whereas T-bar ribbons are normal (double arrowhead). Bar, 1 μm.
D-basigin and the cytoskeleton

D-basigin partially colocalizes with actin in High Five cells and this was especially evident at cell-cell contacts and cell edges. Partial colocalization was also previously reported in chicken retinal pigment epithelium (Schlosshauer et al., 1995). D-basigin and actin colocalization may occur as an indirect consequence of the interaction of D-basigin with integrins. The D-basigin/actin colocalization seen here was similar to that seen for D-basigin/integrin colocalization in cultured human cells (Berditchevski et al., 1997), primarily at cell contacts. In addition, integrins are linked to actin via adaptor proteins, such as talin, that bind to specific sequences in the intracellular tail of integrins (reviewed by Arnaout et al., 2002). Basigin family proteins have no known binding motifs inside the cell for actin-binding proteins and there is little conservation between D-basigin and mouse basigin in the intracellular tail. However, direct interactions between D-basigin and actin-binding proteins cannot be ruled out.

Although D-basigin colocalizes with actin at cell contacts in culture, its effects on internal cell structure may result from alterations in either microfilaments or, more indirectly, microtubules. Although organelle anchoring has not been studied in Drosophila, in many systems, nuclei are anchored in their final positions by attachment to actin (Apel et al., 2000). However, in fly photoreceptors, both nuclei (Fan and Ready, 1997; Patterson et al., 2003) and mitochondria (Stowers et al., 2002) require microtubules for their proper migration (Fan and Ready, 1997; Patterson et al., 2003). Our mitochondrial placement defect is also similar to that seen in the motor axons of kinesin mutants (Hurd and Saxton, 1996). There are direct physical links between microfilaments and microtubules, as well as interactions between the two (Rodriguez et al., 2003; Cao et al., 2004), so that an affect on one of these cytoskeletal elements may also affect changes in the other.

MMP-independent functions of D-basigin

There are several reasons to conclude that many functions of D-basigin in the fly do not depend on its putative role as an MMP inducer. When we made mosaics in which MMP function is missing in the eye, there was no effect on photoreceptor cell structure and no effect on the placement of glial cell nuclei. Likewise, when we misexpressed Drosophila TIMP (tissue specific inhibitor of MMPs) (Page-McCaw, 2003) in photoreceptors we also saw no effect on cell structure.

There is an even stronger reason to believe that D-basigin has MMP-independent functions in the fly. Bsg265 mutants are embryonic lethal. If D-basigin acted only through MMPs, then MMP mutants would also be embryonic lethal; this is not the case. MMP-1-null mutants survive through the second larval instar, MMP-2-null mutants survive into the pupal stage, and double mutants also survive well into the larval stages (Page-McCaw et al., 2003).

Mouse basigin and D-basigin

Mouse basigin can replace the function of D-basigin in the fly visual system. The homology between fly and mouse basigin lies in the external and transmembrane domains, and in the six cytoplasmic residues closest to the membrane, that form a short positive stretch. Comparing basigin and gp55 from several species we see the following consensus: Y E K R/K R/K R/K R/K/N. Embigin, the most divergent family member, shows a similar sequence, Y T H K K K (mouse). Beyond this, there is little or no homology in the intracellular portion of the molecules. This pattern of sequence conservation between basigin from many species is consistent with the observations that the extracellular portion of basigin has biological activity (Guo et al., 1997) and that basigin interacts with proteins in the plane of the membrane.

There is also congruence of function between basigin in flies and mammals during development. For example, rod cells in the Bsg knockout mouse retina exhibit gross morphological differences, having smaller outer segments (Ochrietor et al., 2001). Mouse basigin is expressed in both retinal neurons and Müller cell glia (Ochrietor et al., 2003). Anti-basigin can block neuronal-glial adhesion in disassociated cultures from avian retina (Fadool and Linser, 1993). Drosophila basigin also affects neuron-glia interactions (our unpublished data). Flies or mice mutant for the basigin gene both have defects in olfaction. Thus, Bsg knockout mice are unable to respond to noxious odors (Igakura et al., 1996); and, in flies, a P-element mutation in an upstream non-coding exon from the bsg class 1 and 2 transcripts leads to a loss of sensitivity to noxious odors (Anholt et al., 2002). In addition, Bsg knockout mice are also male sterile (Igakura et al., 1998; Saxena et al., 2002). Intriguingly, a screen for male sterile mutants in Drosophila identified a fly line containing a P-element insertion in bsg about 500 bp upstream from the P-element previously described (Castrillon et al., 1993). It was based on this insertion that the locus was originally called gelded (gel). However, our bsg265 deletion allele compliments the original gel allele for male sterility. This makes it unclear, at present, if the male sterility phenotype is really due to a mutation in bsg. Lastly, in mice, a knockout that eliminates basigin remains viable, whereas in flies bsg265 mutants are lethal. The most likely explanation for this difference is that mammals contain three basigin family members that may be required for different aspects of development whereas flies contain only one.

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