The CD59 Family Member Leaky/Coiled Is Required for the Establishment of the Blood–Brain Barrier in *Drosophila*

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The blood–brain barrier of *Drosophila* is established by the subperineurial glial cells that encase the CNS and PNS. The subperineurial glial cells are thin, highly interdigitated cells with epithelial character. The establishment of extensive septate junctions between these cells is crucial for the prevention of uncontrolled paracellular leakage of ions and solutes from the hemolymph into the nervous system. In the absence of septate junctions, macromolecules such as fluorescently labeled dextran can easily cross the blood–brain barrier. To identify additional components of the blood–brain barrier, we followed a genetic approach and injected Texas-Red-conjugated dextran into the hemolymph of embryos homozygous for chromosomal deficiencies. In this way, we identified the 153-aa-large protein Coiled, a new member of the Ly6 (leukocyte antigen 6) family, as being crucially required for septate junction formation and blood–brain barrier integrity. In *coiled* mutants, the normal distribution of septate junction markers such as NeurexinIV, Coracle, or Discs large is disturbed. EM analyses demonstrated that Coiled is required for the formation of septate junctions. We further show that Coiled is expressed by the subperineurial glial cells in which it is anchored to the cell membrane via a glycosylphosphatidylinositol anchor and mediates adhesive properties. Clonal rescue studies indicate that the presence of Coiled is required symmetrically on both cells engaged in septate junction formation.

Introduction

The functionality of neuronal networks primarily depends on the precise regulation of ion concentrations in their environment. The nervous system therefore requires its efficient insulation from circulating blood or hemolymph systems. In consequence, in higher organisms, a blood–brain barrier (BBB) actively ensures a constant ionic environment needed for reliable neuronal function (Carlson et al., 2000; Daneman and Barres, 2005; Abbott et al., 2006; Banerjee and Bhat, 2007; Zlokovic, 2008).

In invertebrates, the BBB is brought about by glial cells, and, despite many differences in the organization of the nervous system in invertebrates and vertebrates, many functional properties are evolutionary conserved. *Drosophila* provides a number of ex-

perimental advantages to study the BBB formation and function (Edenfeld et al., 2005; Edwards and Meinertzhagen, 2010). A variety of glial subtypes have been identified in *Drosophila*, and many tools to visualize and manipulate them have been developed (Klämbt and Goodman, 1991; Ito et al., 1995; Younossi-Hartenstein et al., 2003; Peraanu et al., 2005; Schwabe et al., 2005; Silies et al., 2007; Awasaki et al., 2008; Stork et al., 2008). The insect nervous system is surrounded by hemolymph with a high potassium concentration of ~50 mM (Krans et al., 2010). Perineurial and subperineurial glial (SPG) cells, which also express unidirectionally acting xenobiotic transporters, provide the cellular basis of the BBB (Bainton et al., 2005; Schwabe et al., 2005; Stork et al., 2008; Mayer et al., 2009). In particular, the extensively interdigitating subperineurial glial cells seal the nervous system by forming septate junctions, which mediate a tight cellular contact that prevents uncontrolled paracellular leakage of solutes from the hemolymph into the nervous system (Carlson et al., 2000). Although the organization of septate junctions has been well characterized by EM analysis, almost nothing is known how this intricate structure develops and how its final barrier function is set up.

The notion that the molecular principles underlying glial differentiation are conserved between the mammalian and the *Drosophila* nervous system was also supported by recent work on the molecular components of septate junctions in *Drosophila* and septate-like junctions at the paranode of myelinating glia cells in vertebrates (Baumgartner et al., 1996; Bhat et al., 2001; Girault and Peles, 2002; Poliak and Peles, 2003; Faivre-Sarrailh et al., 2004; Banerjee et al., 2006b; Salzer et al., 2008). Mutants lacking septate junctions between the subperineurial glia show paracellular leakage of potassium into the nervous system (Auld et al., 1995;

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Figure 1. Identification of *coiled*. Ten kilodalton Texas-Red-labeled dextran was injected into stage 17 embryos of the genotypes indicated. Homozygous mutant embryos were identified using GFP-labeled balancer chromosomes. **A–E**, Frontal views of ventral nerve cords or trachea (**A'–E'**). **A**, No penetration of fluorescently labeled dextran was observed into the nerve cord of stage 17 wild-type embryos. **B–E**, Fluorescently labeled dextran penetrates into the ventral nerve cord of embryos homozygous for several different chromosomal deficiencies. The trachea in mutant embryos show a convoluted phenotype and are leaky.

Baumgartner et al., 1996; Schulte et al., 2003; Stork et al., 2008). Moreover, a reduction of septate junction length, as can be seen in *Drosophila moody* mutants, impairs BBB function (Bainton et al., 2005; Schwabe et al., 2005). To further decipher the molecular components required for a functional BBB in *Drosophila*, we performed a genetic screen and uncovered the *coiled* gene, which encodes a small glycosylphosphatidylinositol (GPI)-linked protein of the Ly6 (leukocyte antigen 6)/CD59 family. Coiled is secreted to the cell surface, where it mediates cell adhesion. Clonal analysis demonstrates that Coiled is required in two neighboring cells to ensure the correct distribution of septate junction proteins.

Materials and Methods

Molecular genetics, fly work, and cell culture. To generate a UAS*coiled* construct, we subcloned the *coiled* cDNA (LD16147) into pUAST and generated several transgenic strains. For cloning, we used the gateway system. Constructs encoding the different tagged Coiled proteins were synthesized by Genescript. The N-terminal HA tag is placed at position 27, just after the signal sequence, and the C-terminal HA tag is placed at amino acid position 123, a single residue before the GPI attachment site (see Fig. 3). All constructs were sequenced for verification. Transgenic *Drosophila* strains were generated using phiC31-based transformation according to standard procedures (Bischof et al., 2007). Tissue culture experiments were performed as described (Stork et al., 2009). Phosphatidylinositol-specific phospholipase C (PI-PLC) experiments were performed as described (Koh et al., 2008). The insertion of a GFP exon in the *coiled* gene is determined by the Flyprot consortium (<http://www.flyprot.org>). The *coiled in situ* expression data were determined by Berkeley *Drosophila* Genome Project and are available at <http://www.fruitfly.org/cgi-bin/ex/insitu.pl>.

Immunohistochemistry and electron microscopic analysis. Fixation and treatment of tissues for immunohistochemistry was performed as described (Stork et al., 2008). Fluorescently labeled specimens were analyzed using a Carl Zeiss 710 LSM, and orthogonal sections were taken using the Zen software of Carl Zeiss. For electron microscopic analysis, embryos were fixed in glutaraldehyde saturated *n*-heptane, and the

vitelline membrane was removed by hand. After a simultaneous fixation in glutaraldehyde and OsO₄, tissues were embedded in Epon as described (Stork et al., 2008). Ultrathin sections were viewed with a Carl Zeiss 900 TEM.

Dextran uptake. Dextran solutions were injected into stage 17 embryos as described previously (Stork et al., 2008). The kinetics were determined on a Carl Zeiss LSM 710. Z-stacks through the entire ventral nerve cord were recorded. The mean intensity in neuropil region of six neuromers was determined in at least four animals per genotype. The genotype of the embryos was determined using GFP-labeled balancer chromosomes. The laser settings were identical throughout the experiments.

Results

Identification of *coiled*

To identify additional proteins that are required for the establishment of the blood–brain barrier, we injected fluorescently labeled dextran into the body cavity of stage 17 embryos homozygous for chromosomal deficiencies (Fig. 1). The diffusion of fluorescent dye into the CNS was monitored using confocal microscopy. Although wild-type embryos are able to exclude the dye from the CNS, animals with an impaired BBB show dye

penetration into the brain (Bainton et al., 2005; Schwabe et al., 2005; Stork et al., 2008). The injected embryos were then screened for those with a defective blood–brain barrier. From this screen, we identified the deficiency *Df(2L)ED49*, which in homozygosis resulted in a leaky blood–brain barrier (Fig. 1A, E). In addition, we noted dye uptake into trachea, which showed a convoluted phenotype, as it is often found in mutants affecting septate junctions (Fig. 1A'–E') (Wu and Beitel, 2004; Wu et al., 2004, 2007; Bachmann et al., 2008; Laprise et al., 2009, 2010).

Thus, we concluded that *Df(2L)ED49* uncovered a gene required for epithelial barrier formation. This notion is supported by the finding that additional deficiencies overlapping the genomic region deleted in *Df(2L)ED49* lead to identical dye penetration phenotypes [*Df(2L)Exel8003*; *Df(2L)BCS16*; *Df(2L)ED80*] (Fig. 1B–D). According to the genome annotation (Dowell et al., 2001; Tweedie et al., 2009), the deficiency *Df(2L)ED49* removes only seven transcription units, and the function of five of these transcription units can be tested by additional alleles (Fig. 1E) (see Fig. 3A–E) (Hahn and Jackle, 1996; Royet et al., 1998). Of the five mutants tested, only a transposon insertion into the second coding exon of *CG2813* PBac[f05607] (abbreviated as *coiled^P*) displayed a dye penetration phenotype in homozygosis and in trans to the deficiency *Df(2L)ED49* (Figs. 2D–F, 3D). The lethality and the dye penetration phenotype associated with the transposon insertion can be reverted by precise excision (data not shown). Based on its mutant phenotype, we originally termed the gene *leaky*. Recently, the same gene has been named *coiled* (*cold*) (Nilton et al., 2010).

To characterize the blood–brain barrier defects observed in *coiled* mutants in more detail, we compared the dye penetration characteristics of *coiled* mutant animals with other mutants known to affect the blood–brain barrier (Fig. 2). *neurexinIV* mutant animals do not form septate junctions and have a permeable

Figure 2. *coiled* mutants show similar BBB defects as *neurexinIV* mutants. Differently sized fluorescently labeled dextran molecules were injected into stage 17 embryos of the following genotypes: *w¹¹¹⁸*, *coiled⁰⁵⁶⁰⁷*, *nrxIV⁴³⁰⁴*, and *moody^{Δ17}*. Images shown were taken 2 min after injection. The different sized dextrans are indicated; all images were taken with a Carl Zeiss LSM 710 microscope. Homozygous embryos were recognized using GFP-labeled balancer chromosomes. **A–C**, A *w¹¹¹⁸* embryo is used as wild-type control. **D–F**, In *coiled* mutants, smaller dyes readily penetrated into the nerve cord. Larger dyes penetrate into the nerve cord but not as easily as the 10 kDa dextran. **G–I**, In *neurexinIV* (*nrxIV*) mutant embryos, all sizes of dextran molecules penetrated comparable with *coiled* mutants. **J–L**, Dye penetration in *moody* mutants. **M**, Quantification of 10 kDa dextran uptake over 60 min in the genotypes indicated.

blood–brain barrier (Baumgartner et al., 1996; Stork et al., 2008). In contrast, homozygous *moody* mutants show reduced septate junctions and have a mildly affected blood–brain barrier (Bainton et al., 2005; Schwabe et al., 2005). The barrier defects can be assessed by the kinetics of dye penetration and by determining the penetration of differently sized dextrans (Stork et al., 2008).

In these assays, *coiled* animals are characterized by very similar, although somewhat less pronounced, permeability to high-molecular-weight dyes compared with *neurexinIV* mutants (Fig. 2). In *neurexinIV* mutants, 10, 70, and 500 kDa labeled dextran molecules can easily penetrate into the CNS. Similar penetration defects are observed in *coiled* mutant animals (Fig. 2*D–I*). In contrast, *moody* mutants only show a clear penetration defect for 10 kDa labeled dextran, whereas relatively little penetration is noted for larger dextran molecules (Fig. 2*J–L*). The similarity of the *coiled* and the *neurexinIV* mutant phenotypes became even more evident when we compared the kinetics of dye uptake by determining the increase of fluorescence intensity in the CNS over 1 h (Fig. 2*M*). Together, these data point to a strong impairment of the blood–brain barrier in *coiled* mutants.

The coiled gene

The *coiled* gene generates a small transcript from three exons and encodes a 153-aa-long protein with a predicted molecular weight of ~15 kDa. The presence of a signal sequence, which is encoded in the first exon, suggests that the protein is transported to the secretory pathway. Additional structural motives suggest that Coiled is tethered to the membrane by a GPI anchor (Fig. 3*F*) (see below). Position-specific iterated BLAST searches identified Coiled as a member of the Ly6/CD59 protein family (Nilton et al., 2010). Within the *Drosophila* genome, a large number of Ly6/CD59-like proteins have been described (Hijazi et al., 2009). The most closely related protein is Boudin, which is required for the formation of septate junctions in the developing tracheal system (Hijazi et al., 2009). To address where *coiled* is expressed, we performed *in situ* hybridization experiments and generated several antibodies directed against different peptides derived from the Coiled protein. Unfortunately, none of the Coiled antibodies allows detection of the endogenous Coiled protein in fixed specimens. The mRNA is expressed in tissues that form septate junctions (embryonic hindgut, trachea, epidermis, and the dorsal pouch). Faint expression can also be detected in the nervous system (Berkeley Drosophila Genome Project) (Tomancak et al., 2007) (data not shown).

To further determine the Coiled expression pattern, we used the GFP–exon trap insertion *CPTT⁰⁰¹²⁷⁷* located in the first intron (Fig. 3*A*). The GFP–exon is spliced in frame and generates an N-terminally tagged Coiled protein. This protein variant, however, appears nonfunctional, and homozygous *CPTT⁰⁰¹²⁷⁷* flies die during embryonic development. During embryonic development, this Coiled fusion protein is expressed by all septate-junction-forming tissues. In the nervous system, Coiled is expressed by all subperineurial glial cells that form the blood–brain barrier. Likewise, Coiled is specifically expressed by the subperineurial glia, elevated levels can be detected at the cell–cell junction (Fig. 4*C*, arrows).

Coiled is required for septate junction formation in the subperineurial glia

The *coiled* mutant phenotype is characterized by a defective blood–brain barrier. The overall organization of the nervous system as well as the distribution of all glial cells, however, appeared normal (Fig. 4*F–K*). To further determine whether abnormal septate junctions between the subperineurial glial cells could account for the disrupted barrier function in *coiled* mutants, we determined the ultrastructural phenotype using transmission electron microscopic analysis. In wild-type stage 17 embryos, subperineurial glial cells are characterized by extensive septate

Figure 3. Genomic region of *coiled*. **A**, Schematic view of the genomic region encompassing the *coiled* gene. The location and orientation of eight genes is depicted by arrows. The extent of three deficiencies is indicated. The *Df(2L)ED49* affects seven transcription units (*Nle*, *CG2807*, *lpk2*, *CG2813*, *Ptth*, *Pph13*, and *gsc*) in the region 21E2. The function of five of these genes can be addressed using specific mutants (Flybase). The exon–intron structure of *coiled* and the integration sites of the transposon insertions used in this study are indicated. **B–E**, Ventral views of ventral nerve cords of the genotypes indicated. Only homozygous *CG2813^{f05607}* embryos show a disrupted blood–brain barrier, and fluorescently labeled dextran penetrates into the nervous system (**D**). **F**, Schematic organization of the four different Coiled constructs used in this study.

junctions (Fig. 5A,B) (Tepass and Hartenstein, 1994). Homozygous mutant stage 17 embryos, which were selected with the help of GFP-expressing balancer chromosomes, lack septae between subperineurial glial cells, explaining the disrupted blood–brain barrier (Fig. 5C,D). To better understand the disruption of septate junctions observed in *coiled* mutants, we analyzed the distribution of well-characterized septate junction components: NeurexinIV, Coracle, Discs large, and Fasciclin III. In epithelial tissues such as the epidermis and the hindgut, these proteins are associated with septate junctions and prominently localize to a region in the apical part of the basolateral membrane (Woods and Bryant, 1991; Baumgartner et al., 1996; Lamb et al., 1998; Wu and Beitel, 2004) (Figs. 6, 7). In contrast, *coiled* mutants show a mislocalization of these septate-junction-associated proteins along the entire basolateral membrane domain (Fig. 6). In conclusion, these data show that *coiled* is required for the establishment of septate junctions.

Coiled acts cell autonomously to control blood–brain barrier integrity

To further verify that the Coiled protein is required for the blood–brain barrier formation, we performed rescue experiments using the Gal4/UAS system. We generated UAS constructs that allow the expression of wild-type as well as N- and C-terminally tagged Coiled protein variants (Fig. 3F). After integration of these constructs in the same chromosomal landing site (86Fb) using phiC31-mediated in-

tegration, we confirmed similar expression strength by Western blot analyses (Bischof et al., 2007) (see Fig. 9).

During *daughterless*Gal4-driven ubiquitous expression of UAS*coiled* in *coiled* mutants, lethality was rescued and fluorescently labeled dextran was not able to cross the blood–brain barrier (data not shown). Because the closely related Ly6 member Boudin acts in a non-autonomous manner (Hijazi et al., 2009), we next examined the autonomy of *coiled* function. When we expressed untagged or C-terminally tagged Coiled protein in the *engrailed* pattern, we noted a rescue of septate junction assembly in all *engrailed*-expressing cells (Fig. 7) (data not shown). In a wild-type hindgut, NeurexinIV or Coracle is strictly localized to a short apical stretch of the lateral cell membrane (Figs. 6, 7A). In *coiled* mutants, expression spreads uniformly around the basolateral membrane domain. During *engrailed* directed expression of a C-terminally tagged Coiled protein in a mutant *coiled* background, NeurexinIV and Coracle recruitment to a short stretch of the lateral cell membrane is restored only within the *engrailed* domain (Fig. 7B,D).

In contrast, expression of an N-terminally tagged Coiled protein is not able to rescue the lethality associated with *coiled* mutants and does not change the NeurexinIV or Coracle expression defects, although both proteins are expressed from the same chromosomal location. Likewise, the GFP-tagged Coiled protein variant generated in the

exon trap line *CPTI⁰⁰¹²⁷⁷* appears nonfunctional, and homozygous *CPTI⁰⁰¹²⁷⁷* flies as well as *CPTI⁰⁰¹²⁷⁷/Df(2L)ED49* are lethal.

In a next step, we expressed *coiled* in a pan-glial manner (using *repo*Gal4) to ask whether *coiled* acts cell autonomously within the blood–brain barrier. This expression paradigm does not rescue the lethal phenotype associated with *coiled* but completely restores blood–brain barrier integrity of *coiled* mutants (Fig. 7G). Expression in neurons driven by *elav*Gal4 rescues neither the blood–brain barrier integrity nor the lethality (data not shown). Thus, Coiled appears to act cell autonomously to control the formation of septate junctions.

Septate junction formation requires symmetric Coiled expression

Septate junctions connect two neighboring cells. Both cells involved express a large number of proteins, which need to be correctly assembled in septate junctions. We wondered whether Coiled is required on both neighboring cells or whether expression in one cell would be sufficient to trigger septate junction formation. To test this, we expressed Coiled together with cytoplasmic GFP in a striped pattern in the epidermis using the *engrailed*-Gal4 driver (Fig. 7, *en* >> *coiled*). We determined the expression of Coracle and NeurexinIV along membranes that are flanked by two GFP- and thus Coiled-expressing cells (Fig. 8A,

Figure 5. Electron microscopic analysis of septate junction formation. Electron micrographs focusing on the CNS blood–brain barrier of late-stage 17 embryos. **A, B**, Wild-type embryo. The boxed area in **A** is shown in **B**. The subperineurial glial cells (spg) establish prominent electron-dense ladder-like septate junctions (sj, arrowhead). **C, D**, In *coiled* mutant embryos, no septate junctions can be detected.

Figure 6. Septate junction proteins are mislocalized in *coiled* mutants. The figure shows embryonic hindgut from stage 16 wild-type and *coiled* mutant embryos as indicated. **A**, In the wild type, NeurexinIV (Nrx IV) is localized to a small stretch of the basolateral membrane. **B**, In *coiled* mutant embryos, the NeurexinIV expression domain expands and is found around the entire cell. A similar expansion of the expression domain was observed for Coracle expression (**C, D**, Cora), Fasciclin III expression (**E, F**, Fas III), and Discs large expression (**G, H**, Dlg).

nally tagged Coiled protein can rescue the blood–brain barrier defect. When we expressed the protein in the blood–brain-barrier-forming subperineurial glial cells together with UAS-CD8-GFP using SPG-Gal4, we noted a very weak expression colocalizing with the general membrane marker (Fig. 9F–I). During expression of the N-terminally tagged Coiled protein, which is not able to rescue the mutant phenotype, we noted a much stronger labeling, but most of the protein appeared outside the subperineurial glial cells within the nervous system (Fig. 9D, E). Therefore, the N-terminal-tagged protein appears not to be properly attached to the membrane and is thus secreted. Importantly, both the C-terminal and the N-terminal pro-

tein are expressed at very similar levels in the blood–brain barrier as shown by Western blot (Fig. 9J). We thus conclude that most of the functional C-terminally tagged Coiled protein is buried in the septate junctions in which it escapes detection by antibodies.

To further test whether membrane anchorage is required for *coiled* function, we generated a construct encoding a C-terminally tagged Coiled protein lacking the GPI-attachment site (Fig. 3). When expressed in S2 cells, this protein is secreted (data not shown). We then generated transgenic flies and inserted the construct in the chromosomal landing site 86Fb. This construct fails to rescue the *coiled* mutant phenotype. However, the expression of the C-terminally tagged secreted protein was identical to what we found for the C-terminally tagged GPI-anchored Coiled protein (data not shown).

In conclusion, Coiled protein appears integrated into the septate junctions in which it cannot be detected by immunohistochemical methods, although it can easily be detected by Western blots. A secreted Coiled protein can still be incorporated into the cell junctions but is nonfunctional, indicating that Coiled-mediated cell–cell adhesion is relevant for the integrity of the blood–brain barrier.

Discussion

The *Drosophila* blood–brain barrier is formed by the subperineurial glial cells, which are interconnected by extensive bands of septate junctions, which prevent uncontrolled paracellular leakage of solutes into the nervous system. To better understand the function of the blood–brain barrier, we undertook a genetic screen looking for mutants in which the barrier function is compromised. Here we report the identification of a small GPI-linked cell-surface protein, Coiled, as a novel component organizing septate junctions in *Drosophila*.

The BBB is generated during late embryonic stages by the subperineurial glia (Bainton et al., 2005; Schwabe et al., 2005). These cells remain in place until adult stages, and the septate junctions established remain stable over a very long time. Work on the tracheal system and the developing ectoderm has revealed a large number of genes required for the establishment of septate junctions (Fehon et al., 1994; Baumgartner et al., 1996; Behr et al., 2003; Genova and Fehon, 2003; Paul et al., 2003; Schulte et al., 2003; Faivre-Sarrailh et al., 2004; Llimargas et al., 2004; Wu and Beitel, 2004; Wu et al., 2004, 2007; Banerjee et al., 2006a; Furuse and Tsukita, 2006; Hijazi et al., 2009; Nelson et al., 2010; Nilton et al., 2010). To date, ~18 cell-surface proteins have been described that are all structurally associated with septate junctions. Among these, some mediate homophilic adhesion, such as the GPI-linked protein Lachesin or the Ig-domain protein Fasciclin3, whereas other proteins mediate heterophilic adhesion, such as NeurexinIV or Gliotactin (Bieber et al., 1989; Snow et al., 1989; Auld et al., 1995; Genova and Fehon, 2003; Schulte et al., 2003; Llimargas et al., 2004; Strigini et al., 2006). Interestingly, septate junction organization appears identical in the CNS and other ectodermal derivatives, and, for many septate junction components, a disruption of the blood–brain barrier integrity has been demonstrated.

Here we have shown that the Ly6 protein family member Coiled mediates homophilic adhesion and is also required for septate junction formation. In line with the morphological results, we determined dye penetration kinetics similar to mutants lacking the *nrxIV* gene, which is also crucially required for septate junction formation. A GFP–exon trap indicates that Coiled is specifically expressed in the subperineurial glia in which it is enriched in septate junctions. The GFP-tagged protein is not functional, and most of it appears to accumulate in the cytoplasm. In contrast, expression of an N-terminally HA-tagged Coiled variant, which is also nonfunctional, becomes secreted and diffuses in the nervous system. When we expressed a C-terminally tagged Coiled protein, we could not easily detect the protein in tissue but in Western blots, suggesting that the C-terminally tagged Coiled protein is masked when integrated in septate junctions. A C-terminally tagged, truncated Coiled variant lacking the GPI anchor, which cannot rescue the mutant *coiled* phenotype, also appears to be weakly expressed. This indicates that the mutant protein is incorporated into the septate junctions, but as a result of the lack of its GPI anchor, it cannot mediate adhesive functions and is nonfunctional.

Coiled is a member of the Ly6 family characterized by a conserved set of 10 cysteine residues. The 3D structure of the Ly6/CD59 proteins has been resolved and resembles the one shown by certain snake neurotoxins, which target specific nicotinic acetylcholine receptors (Tsetlin, 1999; Huang et al., 2007). The CD59 protein, a small glycosylated protein, is linked to the plasma membrane via a GPI anchor. A prime function of CD59 is during complement activation, when it inhibits the assembly of the terminal membrane attack complex and thereby prevents damage to “self” cells (Meri et al., 1990). Ly6 proteins are involved in early steps of T-cell-receptor-mediated T-cell activation (Kimberley et al., 2007). Recent studies provided evidence that Ly6-related proteins mediate cell adhesion and interact with unknown ligands on the antigen-presenting cells, which leads to T-cell suppression (Bamezai and Rock, 1995; Brakenhoff et al., 1995; Hänninen et al., 1997; Holt et al., 2001; Longhi et al., 2005; Kimberley et al., 2007). Interestingly, the human E48 antigen, another member of the Ly6 family, is required in lymphocyte extravasation through the endothelium (Eshel et al., 2002). Interactions of Ly6 protein family members with their target proteins at the plasma membrane may also occur in *cis*-. This has been observed during the activation of Syk kinase in immune cells or during the modulation of cholinergic receptor function in neurons (Miwa et al., 1999; Halova et al., 2002; Miwa et al., 2006; Choo et al., 2008).

Members of the Ly6 protein family are widely expressed outside the immune system (Blake et al., 1993; Harris et al., 2003; Bamezai, 2004; Papanastasiou et al., 2007). In newts, expression of an Ly6-related molecule called Prod1 has been linked to regeneration as a marker for local identity (da Silva et al., 2002; Brockes

Figure 7. *coiled* acts cell autonomously. **A–D**, *Drosophila* hindgut from stage 16 embryos stained for NeurexinIV and Coracle as indicated. **A**, In wild-type embryos, NeurexinIV is localized to septate junctions. **B**, In *coiled* mutant embryos expressing a C-terminally tagged Coiled protein in the *engrailed* pattern, the localization of NeurexinIV to the septate junctions is restored in the *engrailed*-expressing cells (arrowhead). **C**, No rescue of NeurexinIV expression can be detected in the *engrailed* domain after expression of an N-terminally tagged Coiled protein. **D**, Expression of the C-terminally tagged Coiled protein rescues the expression of Coracle. **E–G**, Expression of Coiled in glial cells rescues the integrity of the blood–brain barrier. Frontal views of ventral nerve cords are shown after injection of fluorescently labeled dextran. **E**, Wild-type stage 17 embryo. No dye penetrates into the nerve cord. **F**, Mutant *coiled* stage 17 embryo injected with 10 kDa labeled dextran. The dye penetrates into the nerve cord. **G**, Mutant *coiled* stage 17 embryo expressing Coiled in the *repo* pattern. Blood–brain barrier function is restored and no dye penetrates into the nerve cord.

Figure 8. Symmetric Coiled expression is required for septate junction formation. **A**, Frontal view of a single confocal section of a stage 16 mutant *coiled* embryo expressing Coiled and GFP in the *engrailed* pattern, stained for Coracle (red) and GFP (green) expression. The plain of view is indicated in the orthogonal section (arrow). The length of septate junctions is rescued to the normal length among cells within the *engrailed* domain (hexagon), whereas between mutant and rescued cells the Coracle expression domain is expanded as in the mutant tissue (oval and rectangle, respectively). The relative Coracle expression domain length is quantified in **B**. $n = 60$ cells. $+/+$ indicates Coracle expression domain length between rescued cells, $+/-$ indicates Coracle expression domain length between rescued and mutant cells, and $-/-$ indicates Coracle expression domain length between two mutant cells.

and Kumar, 2005). Another member of the Ly6 protein family, such as the secreted SLURP-1 protein, which lacks the GPI anchor, has been linked to the skin disorder Mal de Meleda (Fischer et al., 2001). In mammals, recent transcriptome analyses have

Figure 9. Coiled mediates adhesive functions. S2 cell aggregation assay. **A**, After transfection with C-terminally tagged Coiled, cell aggregates were detected after anti-HA staining (green). **B**, After transfection with N-terminally tagged Coiled, no cell aggregates are formed. **C**, Western blot. S2 cells were transfected with a C-terminally tagged Coiled protein. Cell extract (C) and cell culture supernatant (M) were probed with anti-HA antibody. In the presence of PI-PLC, HA-tagged Coiled protein is released into the supernatant. In the absence of PI-PLC, no Coiled protein can be detected in the supernatant. **D–I**, Confocal sections showing *SPG*Gal4-driven expression of C- or N-terminally tagged Coiled protein and CD8GFP in the ventral nerve cord of third-instar larvae. **D**, Orthogonal section through the nerve cord. The position is indicated by a dashed line in **E**. In animals with the genotype *SPG*Gal4 *UAS*CD8GFP *UAS*Coiled^{N-terminal HA}, Coiled expression (green) is found outside the *moody*-positive subperineurial glial cells (asterisk) within the entire nerve cord. The arrow indicates N-terminally tagged Coiled accumulation in areas flanking the neuropil. **F**, **G**, In animals with the genotype *SPG*Gal4 *UAS*CD8GFP *UAS*Coiled^{C-terminal HA} from the same chromosomal landing sites, only weak Coiled expression (green) is found in the *moody*-positive subperineurial glial cells (red, asterisk). **G**, The dashed line indicates the plane of section in the orthogonal section shown in **F**. **H**, Expression of CD8GFP in the *moody* domain. **I**, Expression of Coiled^{C-terminal HA} in the *moody* domain. The arrows indicate Coiled expression at the borders of the subperineurial cells. **J**, Western blot of embryos expressing N-terminal or C-terminal HA-tagged Coiled protein in all glial cells using the *repo*Gal4 driver strain.

shown that Ly6-like proteins are also expressed by the blood–brain barrier forming endothelial cells (Daneman et al., 2010).

Surprisingly, the *Drosophila* genome encodes a similar large number of Ly6 family proteins as the human genome (41 vs 45 family members) (Galat, 2008; Hijazi et al., 2009). The different proteins are thought to exert a large range of molecular functions. The cell-surface protein Sleepless is involved in the control of sleeping behavior, the Retroactive protein is involved in the assembly of tracheal luminal chitin, and the secreted protein Boudin is involved in septate junction formation in the tracheal system (Moussian et al., 2006; Koh et al., 2008; Hijazi et al., 2009; Wu et al., 2010). As a common denominator, all different Ly6 family members are implicated in the recognition of extracellular cues, ranging from cell adhesion and signal transduction to cell activation processes.

GPI-linked proteins can in principle be localized to internal vesicles or to the plasma membrane. Unfortunately, we were unable to generate specific antibodies to allow the detection of endogenous Coiled expression in tissues. However, the fact

that S2 cells transfected with *UAS*Coiled were able to form aggregates and the fact that PI-PLC treatment resulted in the shedding of Coiled into the cell supernatant suggest that Coiled is indeed a cell-surface protein, as has been demonstrated for many other members of the Ly6 family (see above). The closely related Boudin protein shows a broad cellular distribution and can act non-autonomously on septate junction formation (Hijazi et al., 2009).

Coiled is crucially required for the formation of septate junctions among subperineurial glial cells. A core component of the *Drosophila* septate junction is NeurexinIV whose expression requires differential splicing (Baumgartner et al., 1996; Stork et al., 2009). Correct membrane localization of NeurexinIV is brought about by the GPI-linked protein Contactin. Surprisingly, loss of Contactin is relatively well tolerated, and the BBB is only moderately affected (Faivre-Sarrailh et al., 2004). Recently, it was suggested that Coiled is also required for the correct trafficking of NeurexinIV, and Coiled was reported to exhibit a vesicular expression within cells (Nilton et al., 2010). This vesicular distribution may reflect recycling Coiled protein because membrane expression appears rather low. Based on the PI-PLC treatment and the cell aggregation experiments, we propose that Coiled acts primarily at the cell surface. In line with this notion, a secreted, full-length N-terminally tagged Coiled protein cannot rescue the blood–brain barrier phenotype. In addition, it is interesting to note that the secreted Coiled protein does not appear to distribute in a random manner throughout the nervous system but rather appears to associate with other glial cells. Thus, either cortex and neuropil

glial cells specifically phagocytose the Coiled protein or express a yet unknown Coiled receptor, possibly another member of the Ly6 family (Hijazi et al., 2009). The function of Coiled could therefore provide adhesiveness to prime the formation of septate junctions and to allow the subsequent integration of the NeurexinIV-based adhesive system. How Coiled and possibly other Ly6 domain proteins stabilize NeurexinIV to structure septate junctions must be addressed in future experiments.

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