

# A Conserved Functional Domain of *Drosophila* Coracle Is Required for Localization at the Septate Junction and Has Membrane-organizing Activity

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**Abstract.** The protein 4.1 superfamily is comprised of a diverse group of cytoplasmic proteins, many of which have been shown to associate with the plasma membrane via binding to specific transmembrane proteins. Coracle, a *Drosophila* protein 4.1 homologue, is required during embryogenesis and is localized to the cytoplasmic face of the septate junction in epithelial cells. Using in vitro mutagenesis, we demonstrate that the amino-terminal 383 amino acids of Coracle define a functional domain that is both necessary and sufficient for proper septate junction localization in transgenic embryos. Genetic mutations within this domain disrupt the subcellular localization of Coracle and severely affect its genetic function, indicating that correct subcellular

localization is essential for Coracle function. Furthermore, the localization of Coracle and the transmembrane protein Neurexin to the septate junction display an interdependent relationship, suggesting that Coracle and Neurexin interact with one another at the cytoplasmic face of the septate junction. Consistent with this notion, immunoprecipitation and in vitro binding studies demonstrate that the amino-terminal 383 amino acids of Coracle and cytoplasmic domain of Neurexin interact directly. Together these results indicate that Coracle provides essential membrane-organizing functions at the septate junction, and that these functions are carried out by an amino-terminal domain that is conserved in all protein 4.1 superfamily members.

**C**ELL-CELL communication within an epithelium plays vital roles in the maintenance of epithelial character and in correct specification of cell fate. Epithelial tissues exhibit an apicobasal polarity with the apical domain separated from the basolateral domain by a junctional complex. In *Drosophila* and other invertebrate epithelia, the apicolateral junctional complex consists of an apical adherens junction and a more basal septate junction (Poodry and Schneiderman, 1970; Tepass and Hartenstein, 1994). The septate junction, characterized by ladder-like septa in electron micrographs, is an invertebrate specific junction which has been proposed, based upon similar molecular components, to be functionally analogous to the vertebrate tight junction (Willott et al., 1993). The molecular characterization of numerous proteins that function within an array of signaling pathways has revealed that the junctional complex is a primary site for cellular interactions. Studies in *Drosophila* demonstrate that the receptor tyrosine kinases Sevenless and the EGF receptor homo-

logue are localized to the apical junctional complex and enriched at the adherens junction (Tomlinson et al., 1987; Zak and Shilo, 1992). In addition, the Notch receptor and its transmembrane ligands, Delta and Serrate, have also been localized to the adherens junction (Fehon et al., 1991; Thomas et al., 1991; Kooh et al., 1993). Furthermore, Armadillo, the *Drosophila* homologue of  $\beta$ -catenin, is a component of the wingless signaling pathway and makes up part of the molecular architecture of the adherens junction through interactions with junctionally localized cadherins (Peifer and Wieschaus, 1990; Peifer et al., 1991).

The protein 4.1 superfamily comprises a large group of cytoplasmic proteins, many of which have been shown to associate with the plasma membrane (for review see Sato et al., 1992; Arpin et al., 1994; Takeuchi et al., 1994; McCartney and Fehon, 1997; Tsukita et al., 1997; Vaheri et al., 1997). Members of this superfamily include protein 4.1, talin, the ezrin/radixin/moesin (ERM)<sup>1</sup> subfamily, the Neu-

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1. *Abbreviations used in this paper:* CNTR, conserved amino-terminal region; DLG, discs large; ERM, ezrin/radixin/moesin; GST, glutathione-S-transferase; MAGUK, membrane-associated guanylate kinase; NRX, neurexin; PDZ, PSD-95, DLG, ZO-1.

rofibromatosis 2 tumor suppressor Merlin, *Drosophila* Expanded, several protein tyrosine phosphatases (Hendriks et al., 1995; Higashitsuji et al., 1995), and at least two non-muscle myosins (Chen et al., 1996; Weil et al., 1996). The unifying structural characteristic of this family is a region of ~300 amino acids, usually situated near the amino terminus of the protein. Studies of several family members have shown that this region binds to the cytoplasmic tail of specific transmembrane proteins, thereby facilitating their localization to the cytoplasmic face of the plasma membrane (Rees et al., 1990). Interest in the protein 4.1 superfamily has increased because of recent evidence, which suggests that members of this family participate in important cell signaling events. For example, Merlin, the product of the *Neurofibromatosis 2* tumor-suppressor gene, is involved in growth regulation (Rouleau et al., 1993; Trofatter et al., 1993). Additionally, members of the ERM subfamily have been implicated in Rho-dependent signaling (Hirao et al., 1996; Mackay et al., 1997) and in a signaling pathway involving hepatocyte growth factor (Crepaldi et al., 1997).

As the prototypic member of this superfamily, protein 4.1 has been extensively studied. These studies have revealed that the erythrocyte isoform of protein 4.1 cross-links the plasma membrane to the underlying cytoskeleton. This function is carried out through protein-protein interactions with glycophorin C at the membrane using sequences within the conserved amino-terminal domain of protein 4.1, and with spectrin and actin using a more carboxyl-terminal domain (Marchesi, 1985). At the erythrocyte membrane, protein 4.1 exists in a ternary complex with glycophorin C and a palmitoylated glycoprotein, p55 (Marfatia et al., 1994, 1995), a member of a growing family of membrane-associated guanylate kinase (MAGUK) proteins. A defining structural motif of MAGUK proteins is the presence of one to several PDZ domains (PSD-95, DLG, ZO-1), which are known to be involved in protein-protein interactions (Kim et al., 1995). Many signaling molecules found at the plasma membrane contain PDZ domains, leading to the speculation that they function in the clustering of signaling complexes at particular plasma membrane domains (Fanning and Anderson, 1996).

Four members of the protein 4.1 superfamily, *coracle* (*cor*), *Merlin*, *Moesin*, and *expanded* have been identified in *Drosophila* (Boedigheimer and Laughon, 1993; Fehon et al., 1994; McCartney and Fehon, 1996), thereby providing powerful genetic tools to aid in the functional analysis of this protein superfamily. We have previously described a *Drosophila* homologue of protein 4.1, Coracle, that is found at the septate junction in ectodermally derived epithelial cells (Fehon et al., 1994). Mutations in *coracle* are embryonic lethal with defects manifested during the process of dorsal closure. In addition, *coracle* mutations dominantly suppress the rough eye phenotype of *Ellipse*, a hypermorphic allele of the EGF-receptor homologue. Two other proteins that localize to the septate junction in epithelial cells bear striking resemblance to vertebrate proteins known to interact with protein 4.1. The first is *Drosophila* Neurexin (NRX), a member of a growing family of proteins initially identified as synapse-specific receptors (Petrenko et al., 1991; Baumgartner et al., 1996). The cytoplasmic domain of NRX shares 68% similarity to the cyto-

plasmic domain of glycophorin C, and Coracle protein is mislocalized in a strong hypomorphic allele of *Nrx*, suggesting the possibility of a direct interaction between Coracle and NRX similar to that of protein 4.1 with glycophorin C (Baumgartner et al., 1996). The other septate junction resident protein that is similar to a known protein 4.1 interactor is Discs Large (DLG), one of the prototypic members of the MAGUK protein family. Mutations in *dlg* lead to partial to complete loss of septate junctions, loss of apicobasal polarity, and neoplastic overgrowth of imaginal tissue, suggesting that DLG is a critical component of the septate junction (Woods and Bryant, 1991; Woods et al., 1996). Furthermore, a human homologue of DLG, hDLG, has been shown to bind to protein 4.1 (Lue et al., 1994), raising the possibility that Coracle and DLG interact.

To explore the role of Coracle in organizing proteins at the septate junction, we have initiated molecular-genetic and biochemical studies to identify regions that are functionally significant. We show that the amino-terminal 383 amino acids of Coracle define a functional domain that is necessary and sufficient for septate junction localization. In addition, we demonstrate that Coracle binds to the cytoplasmic domain of NRX *in vitro* and that *in vivo* these two proteins display an interdependent relationship that is essential for maintenance of their proper localization at the septate junction.

## Materials and Methods

### *Drosophila* Stocks

All fly stocks were maintained on standard corn meal, yeast, molasses, agar medium. Canton S was used as the wild-type stock. *w*<sup>1118</sup> flies were used for the transformation of *coracle* transgenes using the *mini-w*<sup>+</sup> vector pCaSpeR-hs (Thummel et al., 1988). The *coracle* alleles used in this study are described elsewhere. *Nrx* mutant fly stocks (*Nrx*<sup>46</sup>, *Nrx*<sup>4025</sup>, *Nrx*<sup>4304</sup>, and *Nrx*<sup>14</sup>) were described previously (Baumgartner et al., 1996). *Df*(3L)*vin*<sup>7</sup> was obtained from the Bloomington *Drosophila* stock center at Indiana University (Bloomington, IN).

### Sequencing of Mutant *coracle* Alleles

Genomic DNA was obtained using standard methods from flies homozygous for either *cor*<sup>4</sup> or *cor*<sup>6</sup>, which had been rescued to adulthood by ubiquitous expression of a *coracle* cDNA transgene (Fehon et al., 1994). *coracle* genomic DNA was amplified using intron-specific *coracle* primers. The resulting PCR products were sequenced using the AmpliCycle sequence kit (Perkin Elmer Corp., Branchburg, NJ). The sequence was verified on both strands.

### *coracle* Transgenes

The transformation vector pCaSpeR-hs (Thummel et al., 1988) was modified to include an amino-terminal myc epitope by annealing the two primers consmyc S (sense) 5'-AAT TCA CCA TGG AGC AAA AGC TCA TTT CTG AAG AGG ACT TGA GGC CTA A and consmyc A (anti-sense) 5'-GAT CTT AGG CCT CAA GTC CTC TTC AGA AAT GAG CTT TTG CTC CAT GGT G, which produce a duplex with overhanging EcoRI and BglII ends. This fragment was subsequently cloned into an EcoRI/BamHI cut pCaSpeR-hs vector (all restriction enzymes were obtained from New England Biolabs Inc., Beverly, MA). Digestion of the modified vector with StuI allowed PCR generated *coracle* transgenes to be cloned in-frame immediately downstream of the myc epitope.

The following *coracle* transgenes were generated by PCR amplification from a full length *coracle* cDNA clone (Fehon et al., 1994): *cor* 1-383 (bp 370-1,516), *cor* 1-323 (bp 370-1,337), *cor* 1-167 (bp 370-868), *cor* 106-167 (bp 685-868), *cor* 106-383 (bp 685-1,516), *cor* 207-383 (bp 988-1,516), *cor* 378-1698 (bp 1,501-5,544), *cor* 378-1591 (bp 1,501-5,141), *cor* 1585-1698

(bp 5,122–5,544). The sequence of all PCR amplified regions was confirmed by sequencing using standard methods (Fehon et al., 1991).

To specifically immunoprecipitate *cor<sup>4</sup>* mutant protein from a large collection of embryos, a transformation construct providing genetic rescue of *coracle* mutations that lacked the epitope recognized by the guinea pig anti-Coracle antibody was constructed by fusing the *Drosophila* ubiquitin promoter (Lee et al., 1988) to the cDNA2 isoform of *coracle* (Fehon et al., 1994) and placing this expression cassette within pCaSperR-4 (Thummel et al., 1988). For this construct, a 1.3-kb EcoRI/PvuII fragment representing a portion of the 3' end of cDNA2 was ligated into an EcoRI/PvuII (partial) cut pRmHa-3 construct containing the 3' end of *coracle*. The resulting plasmid was digested with EcoRI and XbaI to excise a 2.8-kb fragment which contained the 3' half of cDNA2 and the ADH polyadenylation signal. This fragment was ligated along with an 885-bp SacI/EcoRI fragment containing the 5' half of cDNA2 into a SacI/XbaI digested RHXpHSS7-pUp2 vector. A 6.1-kb NotI fragment containing the ubiquitin promoter, the *coracle* cDNA2 coding sequence and the ADH polyadenylation signal was then excised and subcloned into a NotI digested pCaSpeR-4 vector. Transformation of all these constructs was performed as described (Rebay et al., 1993). For each construct from 4–10 independent transformant lines were established.

### Transient Transfection of Schneider 2 Cells

The maintenance, transfection, induction, and immunofluorescent analysis of Schneider 2 (S2) cells were performed as described (Fehon et al., 1990). The heat shock regimen consisted of a 30-min heat shock at 38°C followed by 2 h recovery at 25°C.

### Antibody Preparation, Immunolocalization, and Cuticle Preparations

A DLG fusion protein was generated by cloning a PCR-generated fragment of nucleotides 2,653–3,360 comprising amino acids 757–993 (Woods and Bryant, 1991) into SmaI cut pGEX 2 (Smith and Johnson, 1988; Pharmacia Diagnostics AB, Uppsala, Sweden). The fusion protein was purified as described (Frorath et al., 1991). Polyclonal antiserum was raised against the DLG fusion protein in mice. Mouse monoclonal antiserum against the myc epitope was obtained as monoclonal supernatant from cell line 1-9E10.2 (Evan et al., 1985) using standard procedures (Harlow and Lane, 1988). Guinea pig polyclonal antiserum against Coracle has been previously described (Fehon et al., 1994). Rabbit polyclonal antiserum against NRX was provided by H. Bellen (Baylor College of Medicine, Baylor, TX) and has been previously described (Baumgartner et al., 1996). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Mutant embryos were unambiguously identified either using marked balancers that express  $\beta$ -gal driven by the *ftz* promoter or by counterstaining the embryos with antibodies against Coracle or NRX. Immunostaining of embryos was performed as described (Fehon et al., 1991). For the genetic rescue of NRX localization, wild-type embryos or embryos of the genotypes *cor<sup>2</sup>*; *hscor 1–383* or *cor<sup>2</sup>*; *hscor 378–1698* were collected for 2 h at 25°C, aged for 4 h at 25°C, heat shocked for 1 h at 38°C, and then allowed to recover for 7 h at 25°C. The embryos were subsequently fixed and stained as described above. A Zeiss LSM410 laser scanning confocal microscope with a krypton/argon laser (Carl Zeiss Inc., Thornwood, NY) was used for the confocal microscopy. Antibodies were used at the following dilutions: anti-Coracle 1:10,000; anti-DLG 1:250; anti-myc 1:100; and anti-NRX 1:5,000. Cuticle preparations were mounted in Hoyer's medium (Ashburner, 1989).

### Immunoprecipitation and Western Blot Analysis

Wild-type and *cor<sup>4</sup>* mutant embryos ubiquitously expressing the cDNA2 isoform of Coracle (Fehon et al., 1994) were collected for 3 h at 25°C, and then aged for ~12–14 h at room temperature. Transgenic embryos expressing Cor 1–383 or Cor 378–1698 were collected for 3 h at 25°C, aged for ~10–12 h at room temperature, and were then heat shocked for 1 h at 38°C followed by a 2 h recovery at 25°C. Embryos were then dechorionated in 50% commercial bleach (12% sodium hypochlorite) for 3 min and rinsed several times in ice-cold lysis buffer (20 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM DTT, 1% Triton X-100). Embryos were homogenized in a 10 $\times$  vol of ice-cold lysis buffer that included PMSF and leupeptin at a 1:1,000 dilution in a tight-fitting dounce homogenizer. The homogenate was drawn through a 27 gauge tuberculin needle

5–10 times, and then centrifuged at 16,000 *g* for 15 min at 4°C. After removing the upper lipid layer, the soluble lysate was collected and incubated for 30–60 min with primary antibody. Primary antibodies used were guinea pig anti-Coracle at 1:200 and anti-myc 1-9E10.2 at 1:1. Immunoreactive complexes were precipitated by centrifugation (8,000 *g* for 1 min at 4°C) following incubation with zysorbin (Zymed Labs Inc., South San Francisco, CA) for 30–60 min at 4°C. The complexes were washed 8–10 times in lysis buffer, and then boiled in 1 $\times$  SDS sample buffer (Laemmli, 1970). Protein samples separated on 3–15% SDS-PAGE were transferred to nitrocellulose for 1 h at 100 V at 4°C. Blots were blocked in 5% nonfat milk in TBS plus 0.1% Tween-20 for 1 h at room temperature, and then incubated overnight at 4°C in primary antibody (anti-NRX at 1:10,000, anti-DLG at 1:5,000). After washing in TBS Tween-20 and incubation in blocking reagent with horseradish peroxidase-coupled secondary antibodies for 3–4 h at room temperature, the immunoreactive proteins were visualized using chemiluminescent autoradiography (Pierce, Rockford, IL).

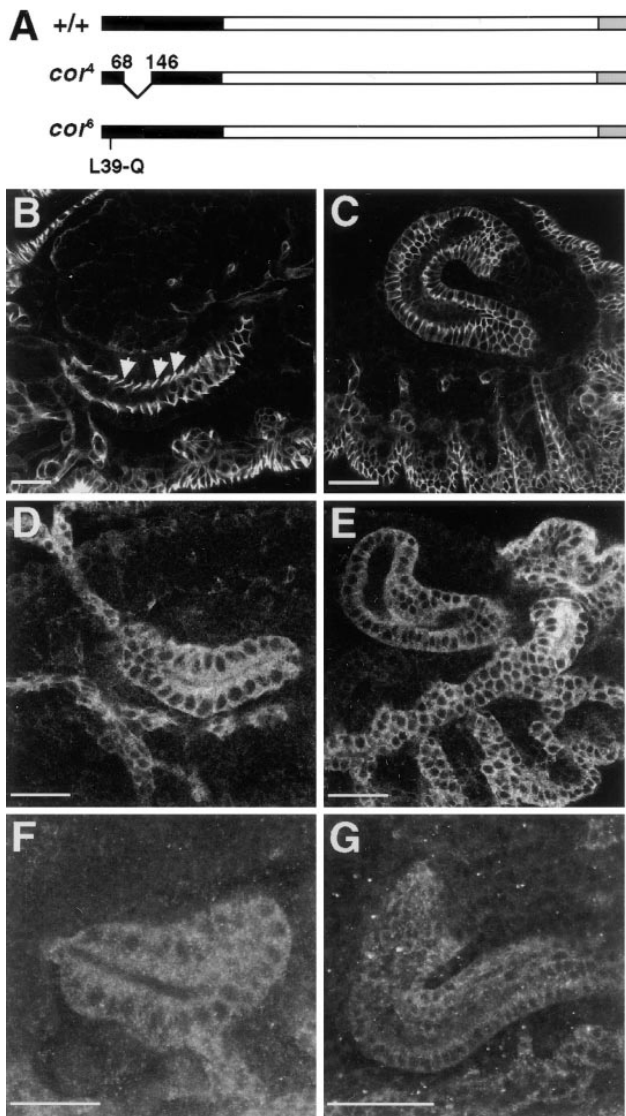
### In Vitro Binding Studies

A Coracle fusion protein was constructed by cloning the entire *coracle* cDNA3 sequence (Fehon et al., 1994) into the SmaI site of pGEX 2TK (Pharmacia Diagnostics AB). Purified Coracle fusion protein was prepared as described (Frangioni and Neel, 1993) with the exception that 1% Triton X-100 was used in place of sarkosyl. The glutathione-S-transferase (GST) moiety was removed by thrombin cleavage followed by adsorption to glutathione agarose (Sigma Chemical Co., St. Louis, MO). Purified Coracle was labeled with <sup>32</sup>P using  $\gamma$ -<sup>32</sup>P dATP and the catalytic subunit of protein kinase (from bovine heart, Sigma Chemical Co.) according to manufacturers instructions. Labeled protein was purified from unincorporated nucleotides through a G25 column (Pharmacia Diagnostics AB) and fractions containing the highest concentration of TCA precipitable counts were used for the binding reactions. A Coracle fusion protein of amino acids 608–1,285 has been previously described (Fehon et al., 1994), and was purified as just described. A NRX fusion protein was constructed by cloning the cytoplasmic domain of NRX (bp 3,993–4,127) into the SmaI site of pGEX 3. NRX fusion protein was purified as just described. GST-NRX was covalently linked to CNBr-activated Sepharose (Pharmacia Diagnostics AB) at a concentration of ~1 mg protein per ml beads and blocked in binding buffer (20 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 5 mM DTT) plus 1% BSA for at least 2 h at 4°C. As a control, purified GST was covalently linked to CNBr-activated Sepharose (Pharmacia Diagnostics AB) at a concentration of ~1 mg protein per ml beads. 100  $\mu$ l of a 50% slurry of NRX or GST-linked beads (~50  $\mu$ g protein) was incubated with 5–10  $\mu$ g of <sup>32</sup>P-labeled Coracle in 250–400  $\mu$ l of binding buffer  $\pm$  a 10-fold molar excess of unlabeled proteins for 30 min at room temperature with gentle shaking. After washing three times with binding buffer, the beads were added to 3 ml of scintillation fluid (Safety-Solve, Research Products International Corp., Mount Prospect, IL) and counted in a scintillation counter (LS6000SC; Beckman, Fullerton, CA). Three replicates were performed for each treatment and means plus standard errors were determined.

## Results

### Coracle Protein Produced in *cor<sup>4</sup>* and *cor<sup>6</sup>* Mutant Embryos Is Mislocalized to the Cytoplasm

Coracle protein is normally localized to the septate junction in ectodermally derived epithelial cells (Fehon et al., 1994). Using optical microscopy, this localization is most obvious in the polarized epithelia of the salivary glands and hindgut (Fig. 1, B and C, respectively). To identify regions of Coracle responsible for this localization, we examined its subcellular localization in a collection of *coracle* point mutations with moderate to severe phenotypes. Two severe alleles, *cor<sup>4</sup>* and *cor<sup>6</sup>*, produce Coracle protein which is mislocalized to the cytoplasm (Fig. 1, D–G). The Coracle protein in *cor<sup>4</sup>* mutant embryos is expressed at nearly wild-type levels (Fig. 1), but with a molecular mass roughly 10 kD smaller than the wild-type protein (data not shown). Sequence analysis revealed that the molecular le-



**Figure 1.** Coracle protein is mislocalized in embryos homozygous for the *cor<sup>4</sup>* and *cor<sup>6</sup>* mutations. (A) Diagrammatic representation of Coracle protein showing the molecular lesions responsible for the *cor<sup>4</sup>* and *cor<sup>6</sup>* alleles. The CNTR is depicted in black. (B–G) Confocal optical sections of salivary glands (B, D, and F) and hindguts (C, E, and G) from wild-type (B and C), *cor<sup>4</sup>* (D and E) and *cor<sup>6</sup>* (F and G) mutant embryos stained with guinea pig polyclonal antibodies against Coracle. In this and all subsequent figures, anterior is to the left. In wild-type epithelia, Coracle is localized to the apicolateral membrane in the region of the septate junction (arrows in B), whereas in *cor<sup>4</sup>* and *cor<sup>6</sup>* tissues, Coracle is found throughout the cytoplasm. Bars, 25  $\mu$ m.

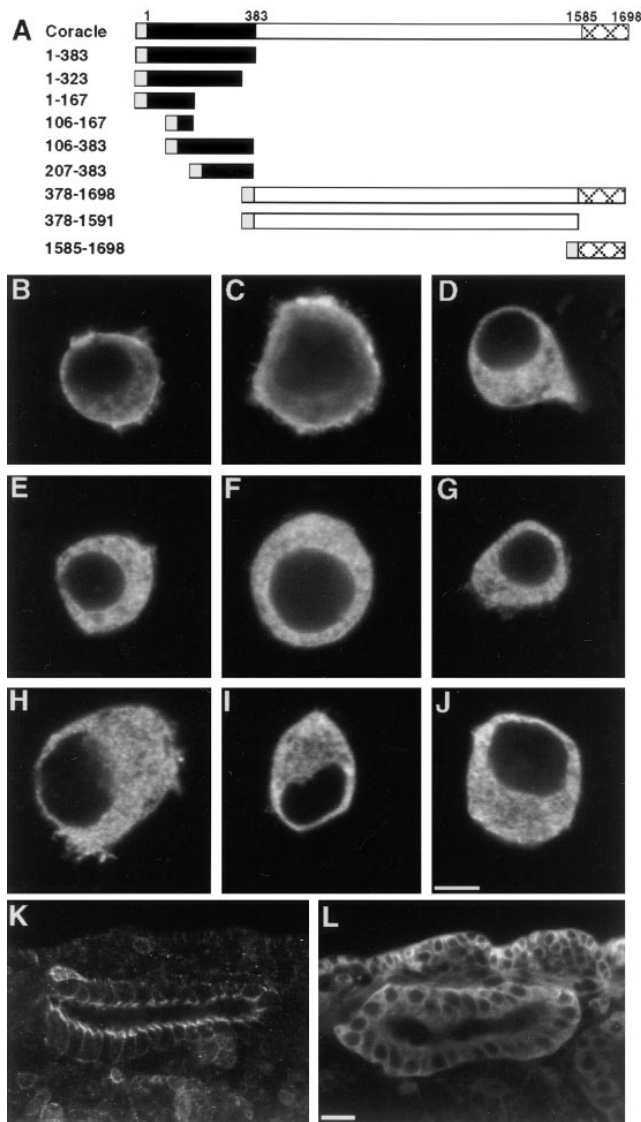
sion responsible for this allele is an in-frame deletion of 78 amino acids (amino acids 68–146) in the conserved amino-terminal region (CNTR; Fig. 1 A). Analysis of the *cor<sup>6</sup>* mutation showed that it is a missense mutation of Leu<sup>39</sup> to Gln in the CNTR (Fig. 1 A). Coracle protein in embryos homozygous for *cor<sup>6</sup>* is expressed at much lower levels based upon immunofluorescence (Fig. 1) and by immunoblot (data not shown). These results, together with the observation that both *cor<sup>4</sup>* and *cor<sup>6</sup>* behave genetically as strong hypomorphs (Lamb, R.S., R.E. Ward, and R.G. Fe-

hon, manuscript in preparation), suggest that correct subcellular localization is essential for Coracle function.

### *The Amino-terminal 383 Amino Acids of Coracle Are Necessary and Sufficient for Localization to the Septate Junction*

To address more definitively the role of the CNTR in subcellular localization, we initiated a structure/function analysis of Coracle by generating the *coracle* deletion mutations shown in Fig. 2. These constructs were transfected into *S2 Drosophila* cultured cells, heat shocked to induce expression, and analyzed by indirect immunofluorescence to determine the subcellular localization of the expressed proteins. Although *S2* cells do not form an epithelium or display junctions, transiently transfected full-length Coracle is found predominately associated with the plasma membrane (Fig. 2 B), consistent with its membrane association in embryonic epithelia and in imaginal discs. Similarly, Cor 1–383, encoding the entire CNTR (Fehon et al., 1994), is also predominately associated with the plasma membrane, indicating that this region is sufficient for membrane localization in *S2* cells (Fig. 2 C). To delimit the region responsible for this subcellular localization, we generated several smaller fragments of the amino-terminal region and expressed them in *S2* cells. In every case, the resulting recombinant protein was found in the cytoplasm and showed no appreciable association with the plasma membrane (Fig. 2, D–G). Even Cor 1–323, which only removes 60 amino acids from the carboxyl terminus of the CNTR, was cytoplasmically localized (Fig. 2 D), indicating that an intact CNTR is necessary for correct subcellular localization. Consistent with this notion, Cor 378–1698, which contains all of Coracle except the CNTR, is also found throughout the cytoplasm as are all smaller fragments derived from this region (Fig. 2, H–J).

To assess the *in vivo* function of these recombinant proteins, we generated transgenic flies using P-element-mediated germline transformation and determined the subcellular localization of the induced proteins during embryogenesis by indirect immunofluorescence. In all cases, the subcellular localization of these recombinant proteins was consistent with our observations in the *S2* cell assay. Specifically, the CNTR fragment, Cor 1–383, is associated with the membrane in the region of the apical junctional complex in a pattern that is very similar to the localization of Coracle expressed in wild-type embryos (compare Figs. 2 K to 1 B). Cor 1–383 displays the same pattern of localization in embryos homozygous for *cor<sup>5</sup>* (data not shown), a null genetic background that has no detectable Coracle protein (Lamb, R.S., manuscript in preparation), indicating that sequences within the CNTR alone are responsible for this localization. Also consistent with the *S2* cell assay, Cor 378–1698 in transgenic embryos is found throughout the cytoplasm and is excluded from the nucleus (Fig. 2 L). The expression levels of the smaller fragments derived from the CNTR were usually substantially reduced, although in all cases where the recombinant proteins were detected, they were found throughout the cytoplasm and not associated with the membrane (data not shown). Taken together, these experiments demonstrate that the amino-terminal 383 amino acids are both necessary and sufficient



**Figure 2.** The CNTR of Coracle is necessary and sufficient for membrane localization in S2 cultured cells and septate junction localization in transgenic embryos. (A) Diagrammatic representation of Coracle deletion mutants. The CNTR is depicted in black, the myc epitope tag in grey, and the carboxy-terminal-conserved region in broad crosshatch. (B–J) Confocal optical sections of S2 cells transfected with full length *coracle* (B) or *coracle* deletion constructs (C–J) stained with antibodies against Coracle (B) or the myc epitope (C–J). (B) full length Coracle. (C) Cor 1–383. (D) Cor 1–323. (E) Cor 1–167. (F) Cor 106–167. (G) Cor 106–383. (H) Cor 378–1698. (I) Cor 378–1591. (J) Cor 1585–1698. Wild-type Coracle and Cor 1–383 show distinct membrane localization whereas the other deletion constructs are primarily cytoplasmic. (K and L) Confocal optical sections of salivary glands in transgenic embryos expressing Cor 1–383 (K) or Cor 378–1698 (L) stained with antibodies against the myc epitope. Note that the apicolateral localization of Cor 1–383 in transgenic embryos is similar to that of Coracle in wild-type embryos (Fig. 1 B), whereas Cor 378–1698 is distributed throughout the cytoplasm. Bars, (B–J) 5  $\mu\text{m}$ ; (K and L) 10  $\mu\text{m}$ .

for correct subcellular localization in cultured S2 and embryonic epithelial cells.

### Similarities between *coracle* and *Neurexin* Embryonic Phenotypes

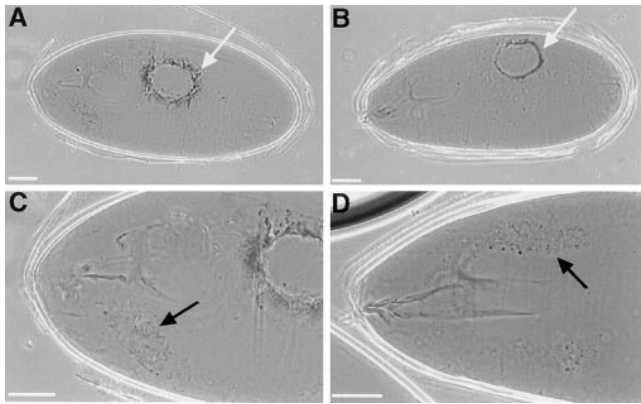
Given the evidence that protein 4.1 localizes to the erythrocyte membrane through an interaction with the cytoplasmic tail of glycophorin C (Marchesi, 1985), it seems likely that Coracle localizes to the plasma membrane via an interaction with a septate junction resident transmembrane protein. Two recent results implicate NRX in this possible interaction with Coracle at the septate junction. First, Coracle localization is altered in *Nrx* mutant embryos (Baumgartner et al., 1996), and second, 8 of the 12 amino acid residues shown to be required for the binding of protein 4.1 to glycophorin C are conserved in NRX (Littleton et al., 1997). We therefore sought to examine further the interaction between Coracle and NRX through comparison of their genetic phenotypes, by more extensive investigation of the localization requirements for each protein, and through biochemical assays for direct physical interactions.

A previous study (Baumgartner et al., 1996) has reported defects in dorsal closure in severe *Nrx* alleles similar to those reported for *coracle* alleles (Fehon et al., 1994). To understand better the relationship between these genes, we compared the embryonic phenotypes of *coracle* and *Nrx* mutant embryos. Cuticle preparations of *coracle* and *Nrx* mutant embryos display similar dorsal closure defects (Fig. 3, A and B), although the penetrance of this phenotype is greater in *coracle* than in *Nrx* mutant embryos. The most penetrant phenotype shared between *coracle* and *Nrx* is a “weakening” of the cuticle in which the ventral denticle belts are faint, with finer, less opaque, and less abundant denticles. Additionally, both *coracle* and *Nrx* mutants display a very penetrant necrosis of the salivary glands (Fig. 3, C and D). Two other phenotypes reported for *Nrx* mutants (Baumgartner et al., 1996) are apparent in strong *coracle* alleles: the tracheae fail to inflate, and muscular contractions are severely diminished late in embryogenesis (data not shown).

### Interdependence of *Coracle* and *NRX* for Proper Localization

Baumgartner and colleagues (1996) have recently reported that in embryos mutant for a strong *Nrx* allele, Coracle protein was no longer tightly localized to the septate junction. To confirm and extend this observation, we have examined the subcellular localization of Coracle in embryos homozygous for null (*Df[3L]vin<sup>7</sup>*) and strongly hypomorphic (*Nrx<sup>46</sup>*, *Nrx<sup>4025</sup>*, and *Nrx<sup>4304</sup>*) *Nrx* alleles by indirect immunofluorescence. In all these cases, Coracle protein is mislocalized in the mutant embryos (Fig. 4). Rather than being tightly associated with the septate junction, the majority of the Coracle protein, although still membrane associated, has a more basolateral distribution (Fig. 4, compare D with G). In addition, some of the Coracle protein is distributed throughout the cytoplasm. Furthermore, the overall expression level of Coracle in the internal epithelia of the salivary gland and hindgut is considerably reduced relative to the expression in the epidermis. This mislocal-





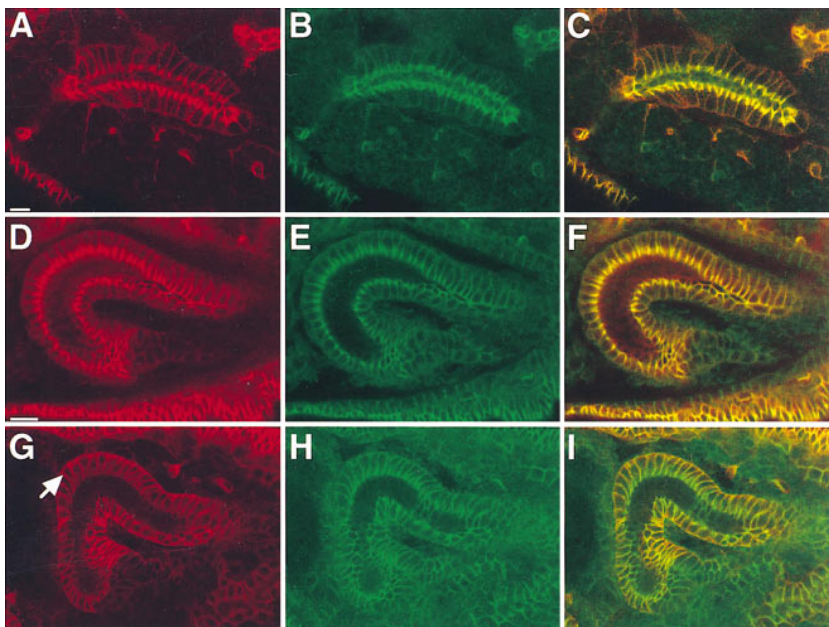
**Figure 3.** *coracle* and *Nrx* mutant embryos display similar defects in dorsal closure and in the salivary glands. Cuticle preparations of *cor*<sup>5</sup> (A and C), *Nrx*<sup>46</sup> (B) and *Nrx*<sup>4304</sup> (D) mutant embryos. The dorsal holes in *coracle* and *Nrx* mutant embryos are very similar (white arrows in A and B), as is the necrosis of the salivary glands (black arrows in C and D). Bars, 50  $\mu$ m.

ization of Coracle is specific and does not reflect a gross alteration in the integrity of the septate junction, as judged by the nearly wild-type appearance of DLG in these embryos (compare Fig. 4 E with 4 H). This result suggests that the targeting of Coracle to the septate junction requires either a direct interaction with NRX or a NRX-dependent complex.

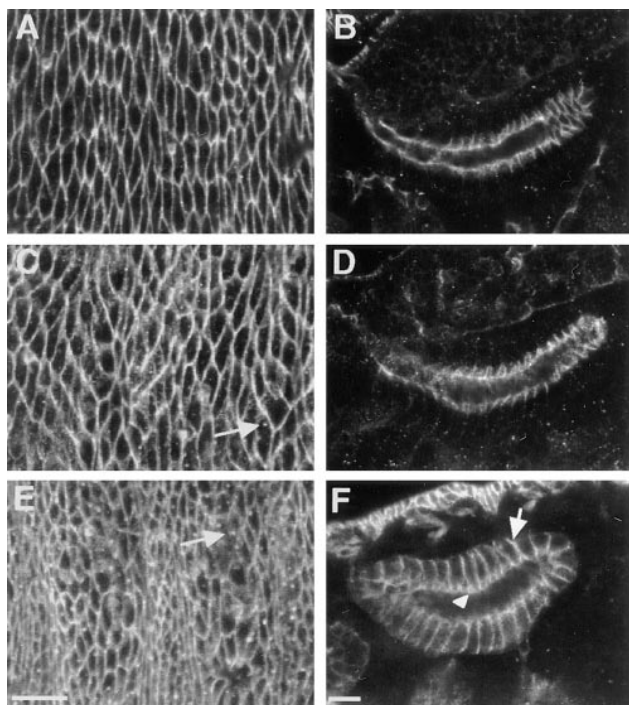
A previous study (Baumgartner et al., 1996) has indicated that in contrast to the dependence of Coracle localization on NRX expression, NRX does not depend on Coracle expression for its localization. However, the *coracle* allele used for this study, *cor*<sup>2</sup>, expresses a protein with an intact CNTR that localizes to the cell membrane (Fehon et al., 1994) and retains partial function (Lamb, R.S., manuscript in preparation). It is, therefore, still possible that

NRX is dependent on Coracle for proper subcellular localization. To address this question, we took advantage of recently isolated *coracle* alleles (Lamb, R.S., manuscript in preparation). In weaker *coracle* alleles, NRX protein targets correctly to the septate junction in embryonic salivary glands (Fig. 5 D). However, in null and strongly hypomorphic *coracle* alleles (*cor*<sup>5</sup> and *cor*<sup>6</sup>, respectively), NRX extends basolaterally so that it is expressed along the entire lateral membrane (Figs. 5 F and 6 B). In addition, we have observed punctate cytoplasmic NRX staining in the salivary glands and in the epidermis of both weakly and strongly hypomorphic *coracle* embryos (Fig. 5, C and E). This effect on the localization of NRX is specific, as we find no detectable difference in the localization of DLG in *coracle* mutant embryos (data not shown).

Given the observations that Coracle function is required for maintenance of NRX localization at the septate junction and that the CNTR of Coracle is sufficient to correctly target Coracle to the septate junction, it is possible that sequences within the CNTR are sufficient for the maintenance of NRX localization. To test this idea, we induced expression of the CNTR in the *cor*<sup>5</sup> null genetic background and examined the subcellular localization of NRX by indirect immunofluorescence. NRX protein is mislocalized along the entire basolateral membrane in *cor*<sup>5</sup> mutant embryos (Fig. 6 B). In contrast, *cor*<sup>5</sup> embryos that have been induced to express Cor 1–383 display NRX localization identical to that of wild-type embryos (Fig. 6, compare C with A). However, *cor*<sup>5</sup> mutant embryos that express the remainder of Coracle (Cor 378–1698) fail to “rescue” the NRX localization defect and display a NRX distribution identical to *cor*<sup>5</sup> mutant embryos (Fig. 6, compare D with B). Taken together, these results indicate that there is an interdependence of Coracle and NRX for proper localization to the septate junction, and that sequences within the CNTR of Coracle are essential for this function.



**Figure 4.** Coracle protein is mislocalized in *Nrx* mutant embryos. Confocal optical sections of salivary glands (A–C) and hindguts (D–I) from wild-type (A–F) and *Nrx*<sup>46</sup> mutant embryos (G–I) doubly stained with antibodies against Coracle (red) and either NRX (green in B and C) or DLG (green in E, F, H, and I). Coracle and NRX colocalize along the apicolateral membrane in the region of the septate junction in wild-type embryos (A–C). Similarly, DLG colocalizes with Coracle at the septate junction in wildtype embryos (D–F). However, in *Nrx*<sup>46</sup> mutant embryos Coracle protein spreads along the entire lateral membrane (arrow in G) and is found in the cytoplasm whereas DLG protein remains concentrated in the region of the septate junction. Bars, 10  $\mu$ m.

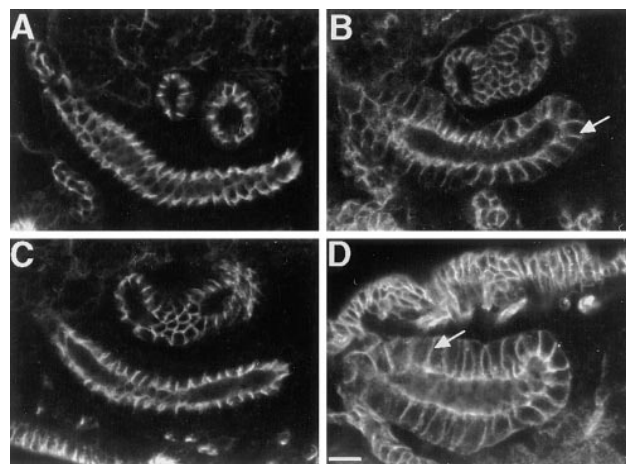


**Figure 5.** NRX protein is mislocalized in *coracle* mutant embryos. Confocal optical sections of epidermis (A, C, and E) and salivary glands (B, D, and F) in wild-type (A and B), *cor<sup>10</sup>* (C and D), and *cor<sup>6</sup>* (E and F) mutant embryos stained with antibodies against NRX. In *cor<sup>6</sup>* mutant embryos NRX spreads basally along the lateral membrane (arrow in F), although some enhanced NRX staining is still apparent in the region of the septate junction (arrowhead in F). Note also the punctate cytoplasmic staining in the epidermis of both *cor<sup>10</sup>* and *cor<sup>6</sup>* mutant embryos (arrows in C and E). Bars, 10  $\mu$ m.

### Interactions between Coracle and Neurexin

The observed codependence of Coracle and NRX for normal localization to the septate junction suggests that these proteins interact at the cytoplasmic face of the septate junction. We have addressed this idea in two ways, by asking if Coracle and NRX coprecipitate in extracts of embryonic tissues, and by examining the binding of Coracle and NRX in vitro. Consistent with the notion that they do interact, immunoprecipitation of Coracle from wild-type embryonic lysates results in a coprecipitation of NRX (Fig. 7 A). This coprecipitation is specific to Coracle and NRX, because DLG, another septate junction associated protein, is not detected in anti-Coracle immunoprecipitates (Fig. 7 B).

Based upon the observation that the CNTR of Coracle is sufficient for the maintenance of NRX localization, it seemed likely that the coprecipitation of NRX with Coracle was mediated through the CNTR. To examine this possibility, we prepared lysates from embryos homozygous for *cor<sup>4</sup>* (a deletion of 78 amino acids within the CNTR) carrying a *cor<sup>+</sup>* transgene that lacks the epitope recognized by the guinea pig antibody, but provides full genetic function (see Materials and Methods; Schweizer, L.Z., and R.G. Fehon, unpublished observations). Immunoprecipitation of Coracle from this lysate precipitates only the *cor<sup>4</sup>*-derived Coracle protein. NRX protein does not co-



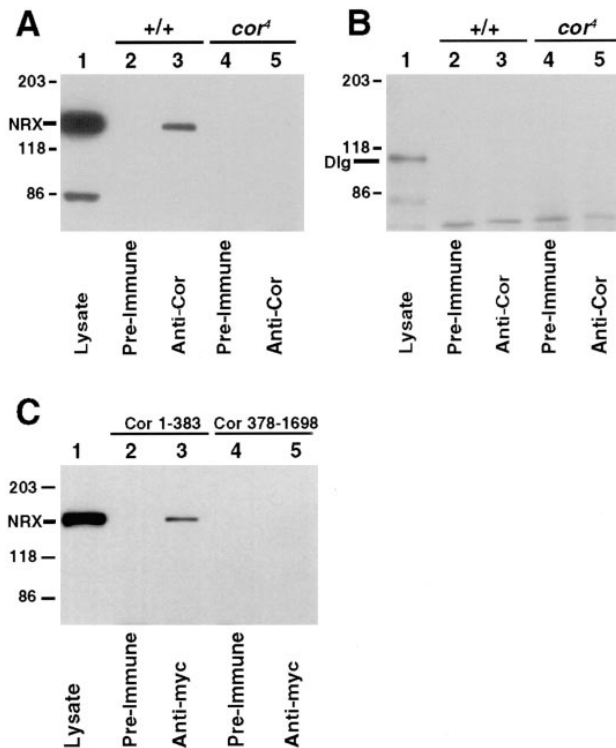
**Figure 6.** The CNTR of Coracle can rescue the mislocalization of NRX in *coracle* mutant embryos. NRX staining in salivary glands from wild-type embryos (A), *cor<sup>5</sup>* mutant embryos carrying the *hscor 1–383* transgene uninduced (B), *cor<sup>5</sup>; hscor 1–383* induced to express the recombinant protein (C), or *cor<sup>5</sup>; hscor 378–1698* induced (D). NRX localizes along the entire lateral membrane in *cor<sup>5</sup>* embryos that do not express the CNTR (arrows in B and D) whereas *cor<sup>5</sup>* mutant embryos induced to express the CNTR display properly localized NRX (compare C with A). Bar, 10  $\mu$ m.

precipitate with the *cor<sup>4</sup>* protein (Fig. 7 A), indicating that the CNTR of Coracle is necessary for the observed interaction with NRX. Furthermore, immunoprecipitates of just the CNTR (Cor 1–383) from transgenic embryos did coprecipitate NRX, whereas immunoprecipitates from embryos expressing the rest of Coracle (Cor 378–1698) did not (Fig. 7 C), indicating that the CNTR is not only necessary but also sufficient for interaction with NRX. Thus the essential sequences for binding NRX or a NRX-containing complex are contained within the amino-terminal 383 amino acids of Coracle.

To determine whether the coprecipitation of Coracle and NRX in embryonic lysates was mediated through a direct interaction between these two proteins, we examined binding in vitro between bacterially expressed recombinant proteins. A Coracle fusion protein representing the cDNA3 isoform of *coracle* (Fehon et al., 1994) specifically bound to the cytoplasmic domain of NRX immobilized onto a column. This interaction was efficiently competed with a 10-fold molar excess of unlabeled Coracle (Table I). However, this interaction was not significantly affected with a 10-fold molar excess of unlabeled GST nor with a Coracle fusion protein containing the central divergent region of Coracle (amino acids 608–1,285). Similar binding was observed between the NRX cytoplasmic tail and just the CNTR of Coracle (data not shown). These results demonstrate that Coracle and NRX interact directly via the CNTR of Coracle and the cytoplasmic tail of NRX.

### Discussion

We have dissected the Coracle protein structurally to identify functionally important regions of this member of the protein 4.1 superfamily. Using in vitro mutagenesis, we



**Figure 7.** NRX coimmunoprecipitates with Coracle from embryonic lysates. (A) Embryonic lysates from wild-type embryos (lanes 2 and 3) or *cor*<sup>4</sup> embryos (lanes 4 and 5, see Materials and Methods) were immunoprecipitated with guinea pig polyclonal anti-Coracle antibody (lanes 3 and 5) or preimmune serum (lanes 2 and 4). After 3–15% SDS-PAGE and transfer to nitrocellulose, they were immunoblotted with anti-NRX antibodies. Lane 1 is a whole cell lysate. Molecular weight standards in kD are indicated on the left. Sample loading was adjusted to ensure equal quantities of wild-type or *cor*<sup>4</sup>-derived Coracle protein in the immunoprecipitates. Note the presence of NRX in anti-Coracle immunoprecipitates from wild-type but not *cor*<sup>4</sup> mutant embryonic lysates. (B) The same blot as in A was stripped (Kaufmann et al., 1987) and immunoblotted with anti-DLG antibodies. DLG does not coprecipitate with Coracle in wild-type embryonic lysates. (C) Embryonic lysates from wild-type embryos expressing hsCor 1–383 (lanes 2 and 3) or hsCor 378–1698 (lanes 4 and 5) were immunoprecipitated with mouse monoclonal anti-myc antibodies to specifically immunoprecipitate the induced recombinant protein (lanes 3 and 5) or with preimmune serum (lanes 2 and 4). The immunoprecipitates were subjected to the same treatment as in A. Induction and immunoprecipitation of recombinant proteins was verified by immunoblot (data not shown). Note the coprecipitation of NRX with Cor 1–383 but not with Cor 378–1698.

have shown that the amino-terminal 383 amino acids of Coracle are sufficient and necessary for membrane association in S2 *Drosophila* cultured cells, and for correct subcellular localization to the septate junction in transgenic embryos. Genetic mutations that affect this domain disrupt the subcellular localization of Coracle and severely affect its genetic functions. Furthermore, the localization of Coracle and the transmembrane protein Neurexin to the septate junction display an interdependent relationship, suggesting that they interact with one another. Consistent with this notion, immunoprecipitation and in vitro

**Table I.** In Vitro Binding between Coracle and NRX

Bound protein	Labeled protein	Competition	% relative binding*
NRX	<sup>32</sup> P-COR	none	100
GST	<sup>32</sup> P-COR	none	33.9 ± 8.3
NRX	<sup>32</sup> P-COR	10 × COR	36.6 ± 3.4
NRX	<sup>32</sup> P-COR	10 × GST	90.9 ± 3.1
NRX	<sup>32</sup> P-COR	10 × COR <sup>608–1285</sup>	118.0 ± 8.8

\*Relative to COR and NRX, no competition ± standard error

binding assays demonstrate that the amino-terminal 383 amino acids of Coracle and the cytoplasmic domain of Neurexin interact directly.

The finding that the amino-terminal 383 amino acids (the CNTR) of Coracle is responsible for septate junction localization correlates well with similar studies of other protein 4.1 family members. The corresponding region of erythrocyte protein 4.1, the 30-kD domain, has been shown to be required for membrane association through binding to the cytoplasmic domain of glycophorin C and the anion exchanger (Anderson and Lovrien, 1984; Pasternack et al., 1985). Likewise, in ERM proteins, the amino-terminal conserved region has been shown to be responsible for localization to the cytoplasmic face of the cell membrane in cultured cells (Algrain et al., 1993), possibly through an interaction with CD44 (Tsukita et al., 1994). Taken together, these results suggest that sequences conserved in all protein 4.1 superfamily members serve as a subcellular targeting domain.

Although the CNTR is well conserved between different members of this superfamily, it is likely that the differences in sequence between them may affect interactions with different transmembrane partners and thereby provide subcellular specificity. For example, in *Drosophila*, Coracle is localized to the septate junction whereas the other family members, Moesin, Merlin, and Expanded, are found primarily in the region of the adherens junction (Boedigheimer and Laughon, 1993; McCartney and Fehon, 1996). Once correctly targeted to a specific subdomain of the membrane, each protein may then serve unique functions. Erythrocyte protein 4.1 and the ERM proteins appear to function as membrane-cytoskeletal crosslinkers, whereas other family members, such as the protein tyrosine phosphatases, have divergent functions that are not directly related to cytoskeletal binding (McCartney and Fehon, 1997).

The identification and characterization of the *cor*<sup>4</sup> mutation, which results from an in-frame deletion of 78 amino acids within the CNTR, provides insight into the functions of Coracle and its localization to the septate junction. Although *cor*<sup>4</sup> protein is expressed at essentially normal levels, it does not localize correctly to the septate junction (Fig. 1, D and E). Similarly, *cor*<sup>6</sup>, which results from an amino acid substitution at Leu<sup>39</sup> within the CNTR (but outside of the region deleted in *cor*<sup>4</sup>), produces a protein that is also mislocalized to the cytoplasm. Attempts to subdivide this region into smaller pieces using in vitro mutagenesis result in proteins that cannot localize to either the cell membrane or to the septate junction (Fig. 2). Taken together, these mutagenesis studies indicate that the CNTR operates as a single functional unit. Further-



more, the observation that both *cor*<sup>4</sup> and *cor*<sup>6</sup> mutant embryos display severe embryonic phenotypes (Lamb, R.S., R.E. Ward, and R.G. Fehon, manuscript in preparation) suggests that an intact CNTR is essential not only for subcellular localization, but also for function in the context of the developing embryo. However, the CNTR does not appear to provide full genetic function as determined by the inability of *hscor 1-383* to rescue a null *coracle* allele by repeated daily heat shocks (data not shown). Further studies are underway to more fully examine the properties of this functional domain.

Previous work has indicated that whereas Coracle is dependent on NRX for its localization, NRX is not dependent on Coracle (Baumgartner et al., 1996). We have confirmed that Coracle localization is dependent on NRX, but find in addition that in strong *coracle* alleles, NRX protein is no longer tightly confined to the septate junction and instead spreads basolaterally (Fig. 5). However, the mislocalization of NRX in *coracle* mutants is qualitatively different from that of Coracle in *Nrx* mutants. Whereas Coracle shows no apparent localization to the septate junction in *Nrx* mutants, in *coracle* mutants there is an enrichment of NRX at the septate junction. One interpretation of this result is that targeting of NRX to the septate junction occurs properly in cells lacking Coracle, but that subsequent maintenance of NRX localization is disrupted. Alternatively, it is possible that there is residual Coracle function in even the strongest alleles, although phenotypic and biochemical evidence indicates that this is not the case (Lamb, R.S., R.E. Ward, and R.G. Fehon, manuscript in preparation).

The evidence presented here indicates that Coracle functions to organize the transmembrane protein NRX to the septate junction. Similarly, previous studies have shown that transfection of ezrin into mouse thymoma cells leads to the redistribution of ICAM-2 in cellular protrusions known as uropods (Helander et al., 1996). In addition, the observed correlation between the rapid redistribution of ERM proteins and dynamic cell surface projections upon growth factor stimulation (Bretscher, 1989) suggests that this membrane-organizing function is a general property of the protein 4.1 superfamily. Our observation that this activity is contained entirely within the CNTR indicates that this conserved domain has similar functions in all family members.

The interdependence between Coracle and NRX for proper localization suggests that at least one other protein in the presumptive septate junction serves as the initial target for both proteins to be properly localized. Based upon the protein 4.1 paradigm of a ternary complex consisting of protein 4.1, glycophorin C, and p55 (Marfatia et al., 1995), we would predict that a PDZ repeat-containing protein is a part of the complex containing Coracle and NRX. The most likely existing candidate for this additional protein is DLG based upon its extensive sequence similarity to p55. DLG is expressed maternally, and initially is uniformly distributed along the lateral membrane and to a lesser extent throughout the cytoplasm. Coincident with the expression of Coracle and NRX, this subcellular localization is refined to the presumptive septate junction (Woods and Bryant, 1991). This expression pattern might be expected of a protein that serves to “prepat-

tern” the septate junction. However, we have failed to detect any interaction between Coracle and DLG by immunoprecipitation (Fig. 7), and we have observed no genetic interaction between *coracle* and *dlg* mutant alleles (data not shown). In addition, the embryonic defects associated with *dlg* mutants (Perrimon, 1988) are different from those of *coracle* and *Nrx*. These results suggest that DLG is not involved in a ternary complex together with Coracle and NRX, despite its structural similarity with p55. The question of whether there is another PDZ repeat-containing protein that functions to stabilize Coracle-NRX interactions remains to be answered, although the structural similarities between the respective *Drosophila* and human proteins strongly suggest that one exists. Additionally, the recent identification of EBP50 as a PDZ repeat-containing protein that associates with ERM proteins (Reczek et al., 1997) suggests that an interaction with a PDZ repeat-containing protein may be a ubiquitous feature of protein 4.1 members. Regardless, the results described here strongly suggest that at least one other component is involved in Coracle/NRX localization and function. Further studies are currently underway to identify this other possible component using a combination of genetic and biochemical approaches.

The structural components and signaling molecules that function together at the septate junction implicate this region in essential signaling mechanisms required for proper cellular communication and epithelial integrity. The phenotype resulting from zygotic loss of *dlg* function suggests a requirement for septate junctions in the control of cell proliferation and apicobasal polarity (Stewart et al., 1972; Woods and Bryant, 1989; Woods et al., 1997). *Nrx* mutant embryos display neuromuscular defects apparently due to a breakdown in the blood-brain barrier, and subtle morphological defects in the ultrastructure of the septate junction, suggesting that the septate junction acts as a barrier to paracellular flow (Baumgartner et al., 1996). In addition, the extracellular domain of NRX has EGF and laminin G repeats (Baumgartner et al., 1996), raising the possibility that it serves as a cell surface receptor. In support of this hypothesis, Neurexins were first identified as synaptic receptors (Petrenko et al., 1991), and have subsequently been proposed to function as receptors in synaptic targeting of neurons (Ullrich et al., 1995). The interdependence between Coracle and NRX for proper localization and their similar embryonic phenotype suggests that they function together in signaling events. The molecular details of these signaling processes are currently unclear, as are their functions. However, the range of observed *coracle* phenotypes, including embryonic dorsal closure and proliferation defects (Lamb, R.S., R.E. Ward, and R.G. Fehon, manuscript in preparation), suggests that either more than one signaling mechanism is involved, or that this signal has multiple functions during development. Given the membrane-organizing activity of the CNTR of Coracle that we describe here, it is likely that Coracle and the other protein 4.1 family members function to target receptors and their intracellular effectors to specific membrane domains. Precedence for this type of signal-mediating molecular scaffold has been found in the multiple PDZ repeat-containing protein InaD, which functions to bring components of the phototransduction pathway together into a “transdu-

cisome” (Tsunoda et al., 1997). Given the increasing evidence that protein 4.1 family members function in concert with PDZ repeat-containing proteins (Marfatia et al., 1994, 1995; Reczek et al., 1997), the hypothesis that Coracle and other protein 4.1 family members link together the molecular components of a signal cascade is particularly intriguing. The identification and characterization of other components of this protein complex at the cytoplasmic face of the septate junction will add to our understanding of the functions of Coracle and the other members of this highly conserved protein superfamily in the context of signal integration during development.

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