Copyright © 2001 by the Genetics Society of America

# The Protein 4.1, Ezrin, Radixin, Moesin (FERM) Domain of Drosophila Coracle, a Cytoplasmic Component of the Septate Junction, Provides Functions Essential for Embryonic Development and Imaginal Cell Proliferation

**Robert E. Ward, IV,[1] Liang Schweizer,[2] Rebecca S. Lamb[3] and Richard G. Fehon**

*Developmental, Cell and Molecular Biology Group, Department of Biology, Duke University, Durham, North Carolina 27708-1000*

Manuscript received March 29, 2001
Accepted for publication June 25, 2001

ABSTRACT

Coracle is a member of the Protein 4.1 superfamily of proteins, whose members include Protein 4.1, the Neurofibromatosis 2 tumor suppressor Merlin, Expanded, the ERM proteins, protein tyrosine phosphatases, and unconventional myosins. Recent evidence suggests that members of this family participate in cell signaling events, including those that regulate cell proliferation and the cytoskeleton. Previously, we demonstrated that Coracle protein is localized to the septate junction in epithelial cells and is required for septate junction integrity. Loss of *coracle* function leads to defects in embryonic development, including failure in dorsal closure, and to proliferation defects. In addition, we determined that the N-terminal 383 amino acids define an essential functional domain possessing membrane-organizing properties. Here we investigate the full range of functions provided by this highly conserved domain and find that it is sufficient to rescue all embryonic defects associated with loss of *coracle* function. In addition, this domain is sufficient to rescue the reduced cell proliferation defect in imaginal discs, although it is incapable of rescuing null mutants to the adult stage. This result suggests the presence of a second functional domain within Coracle, a notion supported by molecular characterization of a series of *coracle* alleles.

THE Protein 4.1 superfamily is composed of a large group of diverse proteins that share an evolutionary conserved protein domain of ~300 amino acids. Members of this family include Protein 4.1, the ezrin, radixin, moesin (ERM) proteins, Talin, the NF2 tumor suppressor Merlin, several protein tyrosine phosphatases, several unconventional myosins, Drosophila Expanded, and Drosophila Coracle (reviewed in Bretscher 1999; Tsukita and Yonemura 1999; Hoover and Bryant 2000). Interest in this superfamily has increased dramatically in recent years with the discovery that the *Neurofibromatosis 2* (*NF2*) tumor suppressor gene encodes a member of the family (Rouleau *et al.* 1993; Trofatter *et al.* 1993) and that other family members also regulate proliferation (Boedigheimer and Laughon 1993; McCartney *et al.* 2000; Morrison *et al.* 2001).

Studies of Erythrocyte Protein 4.1 and the ERM proteins have suggested that family members have two functional domains, one in the N-terminal region and a second near the C terminus. The conserved N-terminal domain, also known as the Protein 4.1, ezrin, radixin, moesin (FERM) domain (Chishti *et al.* 1998), binds to the cytoplasmic tail of several transmembrane proteins. For example, Protein 4.1 binds the transmembrane proteins glycophorin C and the band 3 anion exchanger (Anderson and Lovrien 1984; Pasternack *et al.* 1985), whereas ERM proteins bind CD44, CD43, and ICAM-2 (Tsukita *et al.* 1994; Yonemura *et al.* 1998). Erythrocyte Protein 4.1 contains a second functional domain near the C-terminal region of the protein that interacts with the spectrin/actin cytoskeleton (Correas *et al.* 1986). Similarly, ERM proteins contain a second functional domain near the C terminus that can bind actin directly (Turunen *et al.* 1994). Studies of these proteins demonstrated their ability to crosslink the plasma membrane to the underlying cytoskeleton, leading to the prevailing view that they provide both structural and regulatory functions for the cortical actin cytoskeleton (Marchesi 1985; Algrain *et al.* 1993). However, the subsequent identification of other family members that do not have apparent cytoskeletal binding functions, such as the protein tyrosine phosphatases, suggests that this is not a universal feature within the family.

Coracle, the only Drosophila Protein 4.1 ortholog, shares >60% identity with Protein 4.1 in the FERM domain and >35% identity in the C-terminal 100 amino acids (Fehon *et al.* 1994). Interestingly, Coracle lacks

*Corresponding author:* Richard G. Fehon, B333 LSRC Research Dr., Developmental, Cell and Molecular Biology Group/Department of Biology, Duke University, Durham, NC 27708-1000.
E-mail: rfehon@duke.edu

[1] *Present address:* Howard Hughes Medical Institute, Department of Human Genetics, University of Utah, 15 North, 2030 East, Salt Lake City, UT 84112-5331.

[2] *Present address:* Department of Cell Biology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

[3] *Present address:* Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520.

sequences related to the spectrin-actin binding domain of Protein 4.1, suggesting that it does not have membrane-cytoskeletal crosslinking functions. Coracle is expressed in most epithelial tissues and localizes subcellularly to the septate junction, an apicolateral junction that is structurally and functionally analogous to the vertebrate paranodal septate junction (Bellen *et al.* 1998). Mutations in *coracle* cause a wide range of defects, including failure in dorsal closure, failure in tracheal inflation, necrosis of the salivary glands, and faint denticle belts (Lamb *et al.* 1998; Ward *et al.* 1998). *coracle* function is also required for the establishment or maintenance of the septate junction and specific failure in this process results in a breakdown of the barrier function of the septate junction without affecting apicobasal polarity of the epithelium (Lamb *et al.* 1998).

We previously determined that the FERM domain of Coracle possesses a membrane-organizing function through its interaction with the transmembrane protein Neurexin (Ward *et al.* 1998). Here we use genetic approaches to ask if the FERM domain provides all essential functions of Coracle, or if instead other functional domains are required. The results indicate that the FERM domain alone provides all essential embryonic functions and can rescue a previously identified *coracle* proliferation defect in developing adult epithelia. However, the FERM domain alone is not capable of rescuing mutant animals to the adult stage, suggesting that *coracle* encodes at least one other functional domain. Sequence analysis of *coracle* alleles, together with genetic rescue experiments using mutagenized transgenes, indicate that a second essential functional domain localizes to the conserved C-terminal region. Thus Coracle, like the ERM proteins, contains a C-terminal domain that may function to regulate interactions of its FERM domain or mediate interactions with other proteins.

## MATERIALS AND METHODS

**Drosophila stocks:** For these studies, the following transgenic lines were used: two independent X-linked lines of *P{hs-cor$^{1\text{-}1698}$}* (F97 and M84B); a second chromosome insertion (M7B) and a third chromosome insertion (M7A) of *P{hs-cor$^{1\text{-}383}$}*; and two second chromosome insertions (M7A and M10) and one third chromosome insertion (M2B) of *P{hs-cor$^{378\text{-}1698}$}*.

***coracle* transgenes:** *P{UAS myc cor$^{1\text{-}383}$}* was constructed by the blunt-end ligation of a PCR-amplified fragment of *coracle*, consisting of base pairs 370–1516, into Bluescript vector that had been modified to include an N-terminal myc epitope (LaJeunesse *et al.* 1998). This plasmid was subsequently digested with *Xho*I and *Xba*I to yield a fragment of 1.2 kb, which was ligated into a *Xho*I/*Xba*I-digested pUAST vector (Brand and Perrimon 1993). The sequence of the PCR-amplified region was confirmed by sequence analysis using standard methods. *P{UAS myc cor$^{1\text{-}1698}$}* was constructed by cloning an *Eco*RI, partial *Xba*I fragment (base pairs 1202–5645) from a *Ubiquitin* promoter *coracle* cDNA1 construct (Fehon *et al.* 1994) into an *Eco*RI/*Xba*I-digested pUAST vector. This construct was subsequently digested with *Eco*RI to create a vector into which an *Eco*RI fragment from the Bluescript myc cor$^{1\text{-}383}$ plasmid (described above) was inserted. Transformation of these constructs was performed as described previously (Rebay *et al.* 1993) and three independent transformant lines were established for each construct. For these studies, the following transgenic lines were used: a third chromosome insertion (M19A) of *P{UAS myc cor$^{1\text{-}383}$}* and a third chromosome insertion (M2-1) of *P{UAS myc cor$^{1\text{-}1698}$}*.

**Sequence analysis:** Genomic DNA was obtained using standard methods from wild-type flies and flies homozygous for either *cor$^{8}$*, *cor$^{10}$*, or *cor$^{14}$* that had been rescued to adulthood by ubiquitous expression of a *coracle* cDNA transgene (Fehon *et al.* 1994). Intronic sequence was obtained from PCR products using primers based on exonic sequence that spanned introns. The sequences obtained were subsequently confirmed by comparison with the Drosophila genome sequence (Adams *et al.* 2000). To sequence *coracle* alleles, *coracle* genomic DNA was amplified using intron-specific *coracle* primers. The resulting PCR products were sequenced using the AmpliCycle sequence kit (Perkin-Elmer, Branchburg, NJ).

**Genetic rescue experiments:** For the embryonic rescue experiments, embryos were collected on apple juice plates for 3 hr from parental stocks of *cor$^{5}$/CyO* carrying *P{hs-cor$^{1\text{-}1698}$}*, *P{hs-cor$^{1\text{-}383}$}*, or *P{hs-cor$^{378\text{-}1698}$}*. The embryos were subsequently aged for 4 hr during which time they were divided into two pools, one designated for heat shock and the other for no heat shock, and were then placed on fresh apple juice plates. Roughly 160 embryos were allocated to each pool per experiment. The heat-shock pools were then incubated at 38° for 1 hr. For each pool, all embryos that failed to hatch after 48 hr were mounted in Hoyer's solution and examined for embryonic phenotypes. Four independent collections were conducted for each line. For each collection, the percentages of each phenotype were determined [(number of embryos displaying phenotype/total number of embryos) $\times$ 100], and then the mean of the four percentages plus standard errors were calculated.

The UAS/GAL4 system of Brand and Perrimon (1993) was employed to assess the ability of the FERM domain to provide full genetic rescue to a series of *coracle* alleles. The following *coracle* alleles were recombined onto the ubiquitously expressing GAL4 line *P{GawB}T80 (T80:Gal4)*: *cor$^{4}$*, *cor$^{5}$*, *cor$^{6}$*, *cor$^{8}$*, *cor$^{10}$*, and *cor$^{14}$*. Subsequently, a third chromosome insertion of *P{UAS myc cor$^{1\text{-}383}$}* or *P{UAS myc cor$^{1\text{-}1698}$}* was introduced into these stocks and the following cross was set up:

*w$^{1118}$; P{GawB}T80, cor*/CyO; P{UAS myc cor$^{1\text{-}383(or1698)}$}/+*

$\times$

*w$^{1118}$/Y; P{GawB}T80, cor*/CyO; P{UAS myc cor$^{1\text{-}383(or1698)}$}/+*

Eclosing flies were scored either as balancer class or rescued *coracle* mutant flies. Percentage rescue was determined as the number of rescued *coracle* mutant flies divided by the expected number of transgene-bearing *coracle* homozygous flies (based upon the number of balancer class flies that eclosed) multiplied by 100.

**Histology and dye permeability experiments:** Cuticle preparations, electron microscopy, and junctional permeability experiments were performed as described previously (Lamb *et al.* 1998).

**Clonal analysis:** To generate somatic clones lacking *cor$^{+}$* and instead expressing *cor$^{1\text{-}383}$*, the following cross was performed:

*w$^{1118}$; P{ry$^{+t7.2}$ = neoFRT}43D cor$^{4}$/CyO; P{hs-myc cor$^{1\text{-}383}$}/TM3, P{w$^{+mC}$ = ActGFP}JMR2, Ser$^{1}$*

$\times$

*w$^{1118}$; P{ry$^{+t7.2}$ = neoFRT}43D P{w$^{+mC}$ = piM}46F P{w$^{+mC}$ = piM}47F;*

*MKRS, P{ry$^{+t7.2}$ = hsFLP}/TM6b, Tb*

Eggs were collected for 8 hr and larvae were heat shocked for 1 hr at 37° to induce FLP expression and mitotic recombination. Following clone induction, expression of the *hs-myc* $cor^{1\text{-}383}$ transgene was induced by a 45-min heat shock at 37° and then recovery at 25° for 45 min followed by another 45-min heat shock at 37°. This heat-shock/recovery/heat-shock regimen was repeated every 24 hr. After a final round of heat shock and a 60-min recovery, non-tubby wandering third instar larvae were selected and then further subdivided by green fluorescent protein (GFP) expression. GFP-expressing larvae served as controls, while those that lacked the GFP-marked balancer carried the *P{hs-myc* $cor^{1\text{-}383}$*}* transgene. Wing imaginal discs were then dissected, fixed, and stained using anti-Myc (mAb 9E10) at a concentration of 1:50 as previously described (Fehon *et al.* 1991).

## RESULTS

**Intron/exon boundaries of the *coracle* gene:** Previous studies of the human *Protein 4.1* gene and Drosophila *coracle* gene have shown that both genes encode multiple transcripts due to alternative splicing. Three *coracle* transcripts have been precisely mapped, and Northern blot analysis indicates the presence of several others (Fehon *et al.* 1994). *coracle cDNA 1* (isoform 1) encodes a protein that is predicted to be 1698 amino acids (aa) in length, while *cDNA*s *2* and *3* (isoforms 2 and 3) encode much shorter products of 889 and 703 aa, respectively. As shown in Figure 1, isoforms 2 and 3 differ primarily from isoform 1 in that they lack coding region E, a large (2427 bp), alternatively spliced exonic region.

To determine the number of introns and the precise intron/exon boundaries of the *coracle* gene, we PCR amplified and sequenced genomic DNA from the *coracle* locus and compared it to the known cDNA isoforms. On the basis of these comparisons, *coracle* is composed of 17 exons and 16 introns (Figure 1). In addition, this analysis revealed a complex pattern of splicing that is due to the use of alternative splice acceptor sequences that in some cases are not separated by intervening intronic sequences (Figure 1). Three introns are flanked by alternate splice acceptor sites that result in mRNAs with different coding sequences. The first of these regions, at intron 8 (Figure 1), results in alternative splicing involving regions B and C. Region B contains an alternative splice acceptor that inserts a 12-bp coding region and is used in isoform 3. Region C, which is contiguous with region B in the genomic DNA, is present in isoforms 2 and 3, while in isoform 1, splicing spans from the 5′ end of intron 8, across regions B and C to the 3′ end of the 711-bp intron 9, thereby excising these coding regions. A similar behavior is observed around intron 11, which falls in between regions D and E in the coding sequence. Surprisingly, region E, the largest of the alternatively spliced regions in *coracle* (2427 bp), is not immediately bounded by introns on both ends. Rather, the 3′ end of region E is defined by another alternative splice acceptor site within the large exon that encodes regions E and F (Figure 1). Thus, splicing in this area can span just intron 11 (353 bp; isoform 1), intron 11 plus region E (2780 bp; isoform 2), or intron 11, regions E and F, plus intron 12 (3717 bp; isoform 3). Similar behavior is observed around exon H, although in this case there does not appear to be any alternative splice acceptor within the exon, thereby simplifying the splicing pattern.

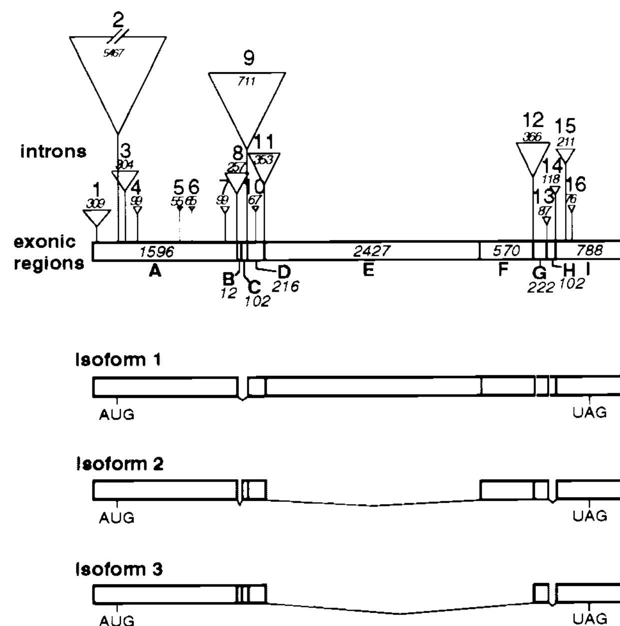


Figure 1.—Molecular organization of the *coracle* gene and its major products. Above is a map indicating the sizes (in italics) and locations of exonic and intronic sequences in the *coracle* gene. Boxes (A–I) show all identified exonic regions from sequencing three distinct cDNA isoforms (isoforms 1–3). The triangles (numbered 1–16) indicate the positions of introns based on comparisons between cDNA and genomic sequences. Most, but not all, junctions between exonic sequences coincide with intron/exon boundaries. However, note that at least two such junctions (B/C and E/F) represent alternative splice acceptor sites rather than intron/exon boundaries.

**Structural analysis of the *coracle* locus reveals a region dispensable for viability:** The observed complex pattern of alternative splicing raises the possibility that different isoforms may have different functions during development. This notion is supported by our previous observation that the $cor^1$ and $cor^2$ alleles are associated with nonsense mutations that should affect only isoform 1 (Fehon *et al.* 1994). The observation that these mutations display a fully penetrant, embryonic lethal phenotype implies that isoform 1 encodes functions that are not present in the other isoforms and, therefore, that region E is likely to contain an essential functional domain. Alternatively, it is possible that while functionally equivalent, the expression level of the other isoforms is too low to provide sufficient *coracle* function for viability, consistent with previous observations that the alternative mRNA splice forms are expressed at lower levels than isoform 1 (Fehon *et al.* 1994).

**TABLE 1**

**Specific *coracle* isoforms can replace endogenous *coracle* function**

| $cor^+$ transgene | Null *coracle* allele | % viability |
|---|---|---|
| Isoform 1 | $cor^4$ | 98 (981) |
| Isoform 2 | $cor^4$ | 77 (1524) |
| Isoform 3 | $cor^4$ | 79 (1345) |
| Isoform 1 | $cor^5$ | 76 (426) |
| Isoform 2 | $cor^5$ | 67 (1072) |
| Isoform 3 | $cor^5$ | 106 (552) |

Viability as determined by number of rescued flies/number of expected rescued flies (based upon eclosed balancer class flies) × 100. Numbers in parentheses indicate number of balancer class flies eclosed. $cor^+$ transgene expression is under the control of the ubiquitin promoter. In the absence of any transgene, $cor^4$ and $cor^5$ animals are completely inviable.

To distinguish between qualitative and quantitative functional differences between the three Coracle isoforms, we constructed *P*-element-based transgenes encoding isoforms 1, 2, and 3 under the control of the Drosophila *Ubiquitin* promoter. Use of the *Ubiquitin* promoter ensures that all three isoforms are expressed at roughly equivalent levels and that they are expressed in all tissues throughout embryonic development. To test the ability of these isoforms to rescue *coracle* lethal mutations, independent insertion lines of each were crossed into the appropriate *coracle* mutant background, and viability of the homozygous mutant class was scored (Table 1). All three of the tested isoforms provided sufficient *coracle* function to rescue >67% of the expected *coracle* mutant offspring bearing null alleles ($cor^4$ and $cor^5$; Table 1), indicating that all encode the essential *coracle* functions when expressed ubiquitously and at sufficient levels. In all cases both males and females were fertile, allowing us to maintain stocks of rescued homozygous mutant animals. In comparison to isoform 1, isoform 2 lacks coding sequences within regions E (2427 bp) and H (102 bp), and isoform 3 lacks, in addition, region F (570 bp). In comparison to isoform 3, isoform 1 lacks coding sequences within regions B (12 bp) and C (102 bp). Thus, the observation that isoforms lacking these regions can rescue severe loss-of-function *coracle* mutations indicates that, although in combination regions B, C, E, F, and H encode 1071 amino acid residues, they do not contain any essential functional domains. Likewise, the observation that each of these isoforms rescues $cor^5$, a null *coracle* allele (Lamb *et al.* 1998), indicates that each encodes all essential *coracle* functions and thus that these functions are restricted to coding regions A, D, G, and I of Figure 1.

**The FERM domain of Coracle provides all essential embryonic functions:** Our prior structure/function analysis revealed that the N-terminal 383 amino acids ($COR^{1\text{-}383}$; the FERM domain) constitute the smallest N-terminal fragment capable of proper subcellular localization (Ward *et al.* 1998). In addition, this region was shown to constitute an important functional domain that has membrane-organizing activity. We therefore tested the idea that the Coracle FERM domain possesses all essential genetic function by determining if ubiquitous expression of this domain could rescue null mutant animals to the adult stage. Heat-shock-driven expression of $COR^{1\text{-}383}$ failed to produce any rescued adult offspring in a $cor^5$ null mutant background ($n$ = 83 balancer class flies eclosed). However, similar expression of a *cDNA1* transgene ($cor^{1\text{-}1698}$) rescued 57% of the expected $cor^5$ mutant flies ($n$ = 66 balancer class flies eclosed).

Although the FERM domain was found to be incapable of providing full genetic rescue, we sought to investigate the range of functions provided by this domain. To accomplish this, we induced the expression of $COR^{1\text{-}383}$ in a null genetic background and looked for rescue of the *coracle* embryonic defects. Loss of *coracle* function during embryogenesis results in a failure of dorsal closure, necrosis of the salivary glands, thinning and delamination of the ectodermal cuticle, and tracheal inflation defects (Fehon *et al.* 1994; Lamb *et al.* 1998; Ward *et al.* 1998). Heat-shock induction of $COR^{1\text{-}383}$ rescued every defect

**TABLE 2**

**Percentage of unhatched embryos displaying dorsal open phenotype**

| Parental stock | no HS | HS |
|---|---|---|
| $cor^5/CyO$ | 45.9 ± 2.8 (282) | 39.4 ± 1.9 (247) |
| $P\{hs\text{-}cor^{1\text{-}1698}\}$ (F97); $cor^5/CyO$ | 37.8 ± 3.0 (180) | 9.8 ± 1.2 (218) |
| $P\{hs\text{-}cor^{1\text{-}1698}\}$ (M84B); $cor^5/CyO$ | 39.9 ± 3.8 (173) | 6.9 ± 1.7 (153) |
| $cor^5$ $P\{hs\text{-}cor^{1\text{-}383}\}$ (M7B)/$CyO$ | 28.2 ± 3.6 (214) | 3.9 ± 1.5 (193) |
| $cor^5/CyO$; $P\{hs\text{-}cor^{1\text{-}383}\}$ (M7A) | 34.8 ± 1.4 (260) | 1.6 ± 0.9 (244) |
| $cor^5$ $P\{hs\text{-}cor^{378\text{-}1698}\}$ (M7A)/$CyO$ | 31.9 ± 1.6 (313) | 32.9 ± 4.4 (340) |
| $cor^5$ $P\{hs\text{-}cor^{378\text{-}1698}\}$ (M10)/$CyO$ | 19.4 ± 3.5 (274) | 20.3 ± 0.7 (300) |
| $cor^5/CyO$; $P\{hs\text{-}cor^{378\text{-}1698}\}$ (M2B) | 31.3 ± 5.0 (168) | 27.9 ± 3.7 (168) |

Values are mean ± standard error. Sample sizes are given in parentheses and are the number of cuticles scored. We find that the CyO/CyO embryos display a completely penetrant embryonic lethality with no discernible cuticular defect. Therefore, a completely penetrant dorsal open phenotype associated with the *coracle* allele would be expected to produce only 50% dorsal open cuticles. HS, heat shock.

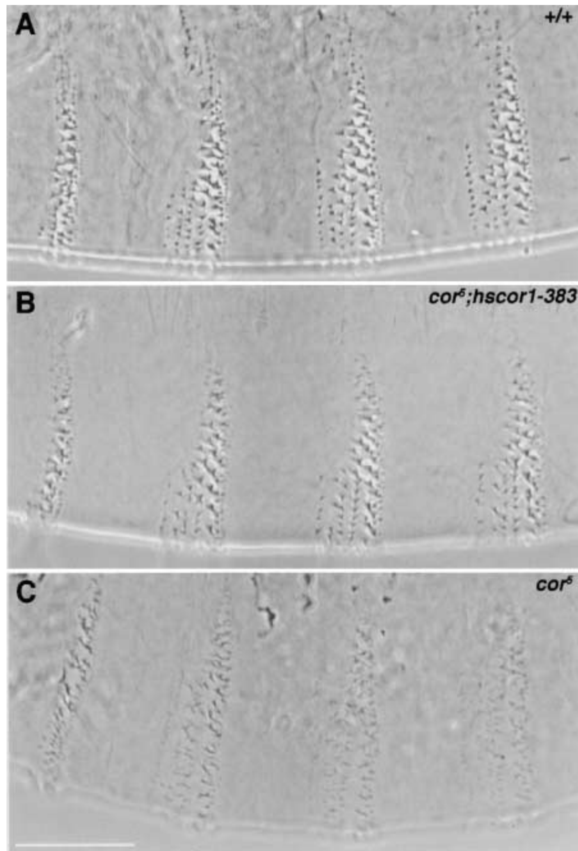


Figure 2.—The FERM domain of Coracle is sufficient to rescue the weak cuticle phenotype of *cor*$^{5}$ mutant embryos. Cuticle preparations showing a ventrolateral view of the denticle belts from the first four abdominal segments of wild-type (A), *cor*$^{5}$; *P{hs-cor*$^{1\text{-}383}$*}* (B), and *cor*$^{5}$ (C) embryos. The embryo in (B) has been heat shocked to induce the expression of COR$^{1\text{-}383}$. The denticle belts in the "rescued" embryo (B) are nearly wild type in appearance. Note that although the denticles are fainter in the *cor*$^{5}$ mutant embryo, the overall pattern is normal. Bar, 50 μm.

associated with loss of *coracle* function in the embryo (Table 2; Figure 2). For example, the expression of COR$^{1\text{-}383}$ in a *cor*$^{5}$ background resulted in a significant reduction in the number of embryos displaying a dorsal open phenotype (Table 2). This observation held true for two independent transgenic lines of *P{hs cor*$^{1\text{-}383}$*}*, and the magnitude of the rescue was equal to the rescue of the dorsal closure defect of *cor*$^{5}$ by heat-shock induction of two independent transgenic lines of *P{hs cor*$^{1\text{-}1698}$*}* (Table 2). In contrast, rescue of the dorsal closure defect of *cor*$^{5}$ was never produced by the induction of COR$^{378\text{-}1698}$, a derivative of the full-length protein lacking the FERM domain (Table 2). The failure of COR$^{378\text{-}1698}$ to rescue the dorsal closure defect was not due to the inadequate expression of this protein. Immunoblot analysis of embryonic lysates from the three transgenic lines expressing COR$^{378\text{-}1698}$ indicates that the recombinant protein is being efficiently expressed (data not shown).

In addition to rescuing the dorsal closure defects associated with loss of *coracle* function, expression of COR$^{1\text{-}383}$ rescued all the other embryonic defects described above. Expression of COR$^{1\text{-}383}$ in these embryos greatly reduced the cuticular thinning (Figure 2), as well as the necrosis of the salivary glands and the tracheal inflation defect (data not shown). Furthermore, a small percentage of *cor*$^{5}$ mutant embryos expressing COR$^{1\text{-}383}$ hatched as larvae, indicating that expression of the FERM domain alone is sufficient for complete embryonic development, although these animals died as larvae (data not shown).

Previous work indicated that Coracle serves an essential structural role in the pleated septate junctions of embryonic epithelia (Lamb *et al.* 1998). To test the idea that the FERM domain provides all functions necessary for septate junction structure and function, we induced the expression of COR$^{1\text{-}383}$ in *cor*$^{5}$ mutant embryos and examined the septate junctions. Whereas loss of *coracle* function results in embryonic epithelia that lack the individual septae that characterize the septate junction (Figure 3D), expression of COR$^{1\text{-}383}$ restored the normal appearance of the septate junctions (Figure 3G). To assess the function of the septate junctions in similarly rescued embryos, we investigated the permeability of the salivary gland epithelium to a rhodamine-labeled dextran. Injection of this marker into the hemocoel of stage 16 mutant embryos results in the rapid accumulation of the dye into the lumen of the salivary gland (Figure 3F and Lamb *et al.* 1998). In contrast, the salivary gland epithelium of *cor*$^{5}$ mutant embryos expressing COR$^{1\text{-}383}$ was found to restrict the diffusion of the labeled dextran (Figure 3I). Taken together, these results indicate that the FERM domain provides all functions that are essential for the characteristic structure of the septate junction and for the formation of a transepithelial barrier.

To more fully investigate the role of the FERM domain throughout development we wished to assess the ability of COR$^{1\text{-}383}$ to rescue defects unique to postembryonic stages. Since complete loss of *coracle* function is embryonic lethal, the use of clonal analysis was necessary to determine the function of *coracle* in imaginal tissues. Clones of null or strongly hypomorphic *coracle* mutant cells are at a growth disadvantage in heterozygous imaginal tissues (R. S. Lamb and R. G. Fehon, unpublished observations). For example, clones of *cor*$^{4}$ mutant cells fail to persist except as very small cell clusters, much smaller than the corresponding twin spot (Figure 4A). However, expression of COR$^{1\text{-}383}$ throughout the imaginal tissue by daily heat-shock induction fully rescues this growth disadvantage (Figure 4, B–F). This result indicates that the function of the FERM domain is required for normal proliferation in imaginal tissues.

**A second functional domain is required after embryogenesis:** The observation that the FERM domain is sufficient for embryonic development and for normal proliferation in imaginal cells, and yet is incapable of rescuing animals carrying a null allele to the adult stage,

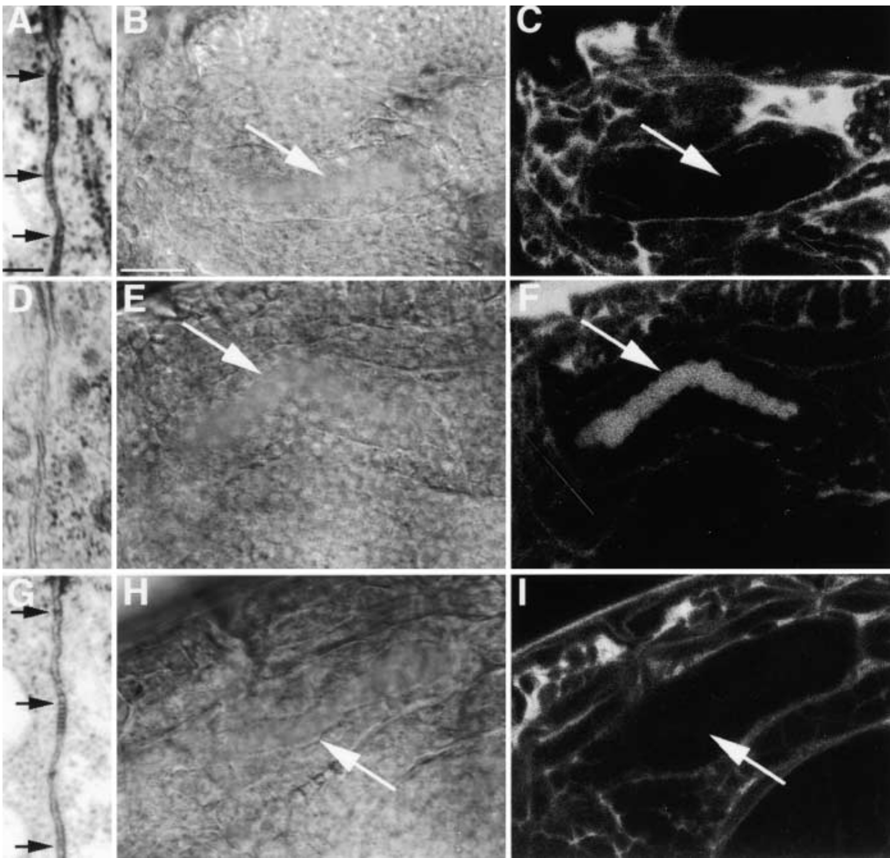


FIGURE 3.—The FERM domain is sufficient to promote normal septate junction structure and function. (A, D, and G) Transmission electron micrographs of epidermis from late stage 17 wild-type (A), *cor⁵* (D), and *cor⁵*; *P{hs-cor¹⁻³⁸³}* (G) embryos induced to express the *coracle* transgene. The individual septae that characterize the pleated septate junction in wild-type tissue (solid arrows in A) are missing in *cor⁵* tissue. Expression of $COR^{1\text{-}383}$ in *cor⁵* mutant embryos restores the normal appearance of the septate junction (solid arrows in G). (B, C, E, F, H, and I). The transepithelial barrier function of the septate junction is disrupted in *cor⁵* mutant embryos and can be restored by the expression of $COR^{1\text{-}383}$. Confocal optical sections of stage 16 wild-type (B and C), *cor⁵* (E and F), and *cor⁵*; *P{hs-cor¹⁻³⁸³}* embryos (H and I) showing the diffusion of a 10-kD rhodamine-dextran injected into the hemocoel (dextran shown in C, F, and I, corresponding differential interference contrast images in B, E, and H). Note that in the wild-type embryo and the mutant embryo expressing the FERM domain the dextran fails to cross the salivary gland epithelium (open arrow), whereas in the *cor⁵* mutant embryo the dextran freely crosses the salivary gland epithelium. Bars: A, D, G, 100 nm; B, C, E, F, H, I, 10 μm.

suggests the presence of another essential functional domain that maps outside the FERM domain and functions at later stages of development. As a means to identify this putative functional domain, we sequenced the coding regions of the *coracle* locus in several *coracle* alleles (Table 3; FEHON *et al.* 1994; LAMB *et al.* 1998). We previously reported that mutations in $cor^4$ and $cor^6$ specifically affect the FERM domain, result in mislocalization of the mutant Coracle protein, and display fully penetrant embryonic lethality (LAMB *et al.* 1998; WARD *et al.* 1998). $cor^8$ and $cor^{10}$ are both weakly hypomorphic alleles that result from missense mutations within or immediately C-terminal to the FERM domain, but do not affect subcellular localization (Table 3; data not shown). Consistent with the notion that the FERM domain is affected in $cor^8$ and $cor^{10}$ animals, both alleles display predominately embryonic lethality (LAMB *et al.* 1998). In contrast, lethal phase analysis of $cor^{14}$ indicates that it is one of the weakest alleles, showing nearly penetrant larval lethality (LAMB *et al.* 1998). Sequence and immunoblot analyses (data not shown) reveal that $cor^{14}$ results from a nonsense mutation at $Arg^{1607}$, truncating the protein at the start of a C-terminal region that is well conserved between Coracle and its vertebrate homologs, but not found in any other family member (FEHON *et al.* 1994). The subcellular localization of $COR^{14}$ is normal (data not shown). Taken together, these results suggest a molecular organization in which the FERM domain is necessary for embryonic development and cell proliferation, whereas a second region near the C terminus contains a functional domain that seems dispensable in embryos but necessary for some aspect of post-embryonic development.

If this model is correct, expression of the FERM domain may rescue *coracle* alleles bearing defects in the N-terminal functional domain but not those bearing defects in the putative C-terminal domain. To test this idea we used the GAL4/UAS system (BRAND and PERRIMON 1993) to drive expression of a *P{UAS myc cor¹⁻³⁸³}* transgene using endogenous enhancer elements, thereby eliminating the stress induced by repeated heat shocks. The *T80* enhancer trap line was used to express GAL4 ubiquitously in the embryo, beginning approximately at germ-band retraction, and in the imaginal tissues throughout development (R. WARD and D. LAJEUNESSE, unpublished observations). Expression of $COR^{1\text{-}383}$ by *T80:GAL4* was incapable of providing rescue to the adult stage for the strongest alleles whose lesions affect the FERM domain, $cor^4$ and $cor^6$, although it was sufficient to provide moderate rescue to the weaker alleles $cor^8$ and $cor^{10}$ (Table 3). Many of the rescued adults displayed mild phenotypes reminiscent of hypomorphic *coracle* escapers (LAMB *et al.* 1998), such as slightly roughened eyes (data not shown), suggesting that although the

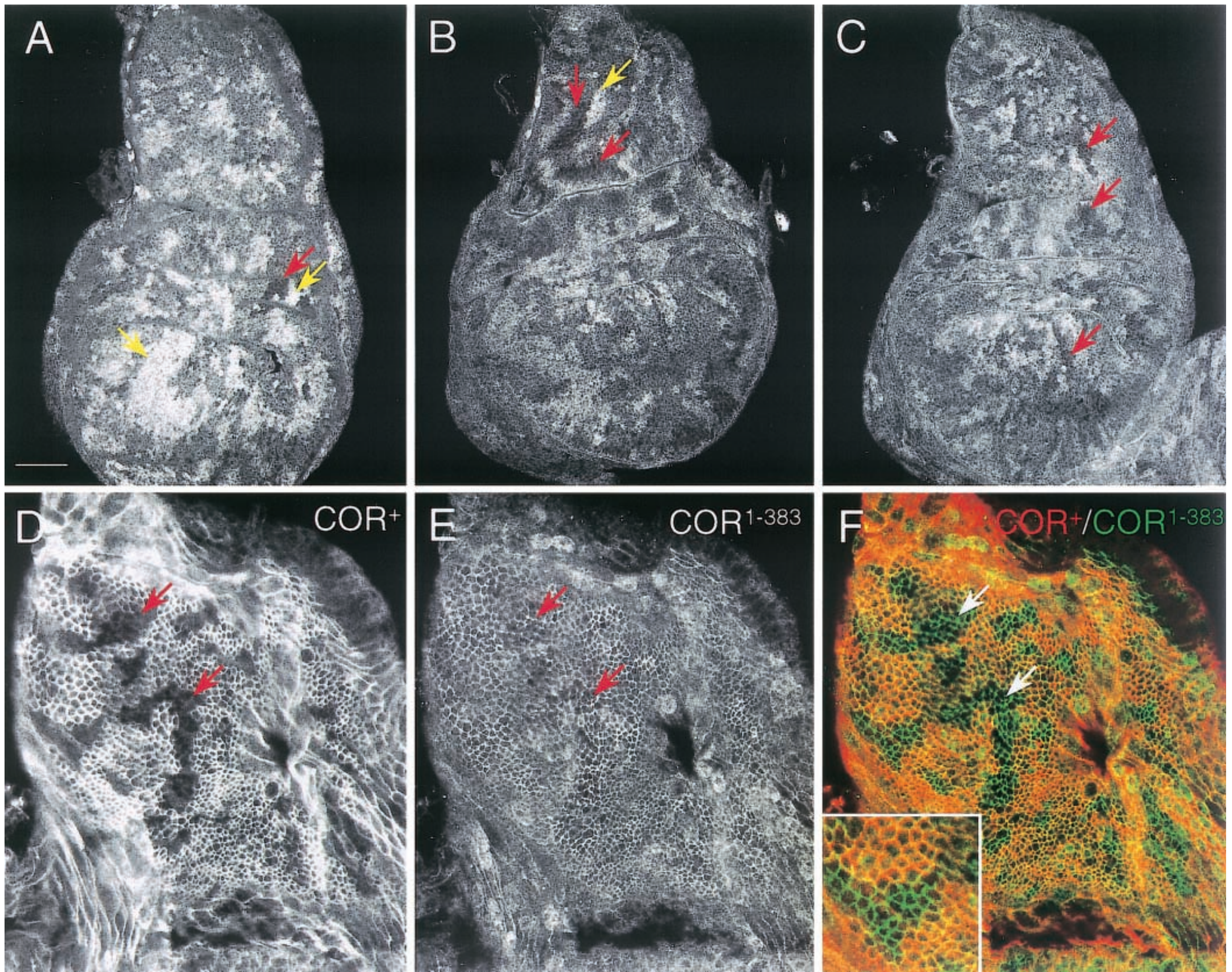


Figure 4.—The FERM domain is sufficient to rescue the *coracle* cell viability phenotype. Somatic mosaic clones of homozygous $cor^4$ mutant cells were induced at 48 hr in a heterozygous background using the FLP/FRT recombination system. Homozygous mutant clones are marked by the absence of the PiMyc marker (red arrows), while homozygous wild-type sister clones induced at the same time are indicated by higher levels of Myc staining (yellow arrows). (A) Homozygous $cor^4$ mutant cells in clones (red arrows) survive poorly in the wing imaginal epithelium, as indicated by their relatively small size and low frequency relative to the simultaneously induced sister wild-type clone (yellow arrows). (B and C) Homozygous $cor^4$ mutant clones induced in sibling larvae that also express $COR^{1\text{-}383}$ under the control of the heat-shock promoter. The two wing imaginal discs shown in B and C have a higher frequency of $cor^4$ mutant clones relative to A, and these clones are larger, indicating that the FERM domain provides essential *coracle* functions for viability in clones. (D–F) Tangential optical section through the apical ends of epithelial cells in the notal region of imaginal disc shown in C, stained for wild-type Coracle (D), Myc-tagged $COR^{1\text{-}383}$ expressed under the heat-shock promoter (E), and the two proteins combined (F). Large clones of $cor^4$ mutant cells (red arrow in D) that express $COR^{1\text{-}383}$ (red arrows in E) are readily visible. Merged image (F) demonstrates that $COR^+$ and $COR^{1\text{-}383}$ are coexpressed at the plasma membrane in the apical region. Inset at lower left in F shows higher magnification view of membrane localization of $COR^{1\text{-}383}$ in the mutant clone (green cells) and its colocalization with $COR^+$ in surrounding wild-type cells (orange cells). Bars: A–C, 50 μm; D and E, 25 μm.

rescue was substantial, it was not complete. However, the rescued $cor^8$ and $cor^{10}$ animals were found to be fertile even though this was rarely, if ever, detected in *coracle* escapers (Lamb *et al.* 1998).

In contrast to the observed rescue of the $cor^8$ and $cor^{10}$ alleles, expression of $COR^{1\text{-}383}$ by *T80:GAL4* had no effect on the viability of $cor^{14}$ animals (Table 3). Control experiments, in which $COR^{1\text{-}1698}$ was driven by the *T80:GAL4* driver, produced moderate rescue of $cor^{14}$ (Table 3), which, taken with the failure of $COR^{1\text{-}383}$ to provide genetic rescue to $cor^{14}$, strongly supports the idea of a second functional domain outside the FERM domain. However, no rescue of $cor^5$, a null allele, was observed in these control experiments, indicating that the *T80:GAL4* driver did not fully recapitulate the expression pattern of endogenous *coracle* (Table 3).

**TABLE 3**

**Genetic rescue of *coracle* alleles by *T80:GAL4; P{UAS cor}***

| Allele | Class[b] | Molecular lesion | % viability ($n$)[a] | |
|---|---|---|---|---|
| | | | *P{UAS cor$^{1\text{-}383}$}* M19A | *P{UAS cor$^{1\text{-}1698}$}* M2-1 |
| *cor$^5$* | Strong | ND | 0 (725) | 0 (1071) |
| *cor$^4$* | Strong | Δ $I^{68}$-$T^{146}$ | 0 (241) | ND |
| *cor$^6$* | Strong | $L^{39}$ to Q | 0 (692) | ND |
| *cor$^8$* | Weak | $D^{368}$ to A | 35.1 (502) | 64 (354) |
| *cor$^{10}$* | Weak | $A^{393}$ to S | 18.9 (649) | ND |
| *cor$^{14}$* | Weak | $R^{1607}$ to stop | 0 (929) | 32 (1982) |

[a] Viability as determined by number of rescued flies/number of expected rescued flies (based upon eclosed balancer class flies) × 100, with $n$ = balancer class flies eclosed.
[b] Based upon embryonic phenotype (see Lamb *et al.* 1998).
ND, not done.

## DISCUSSION

**Structural aspects of the *coracle* locus:** Genetic rescue experiments and molecular characterization of several *coracle* alleles suggest that Coracle is a modular protein having at least two separate functional domains. The finding that each of the three identified *coracle* isoforms was capable of rescuing *coracle* mutant animals to the adult stage (Table 1) indicates that regions A, D, G, and I harbor all essential functional domains. Interestingly, regions A (the N-terminal ~400 aa) and I (the C-terminal ~100 aa) encode the Coracle sequences that are most highly conserved with human Protein 4.1 (>60% and >35% identity, respectively). Consistent with the notion that these are essential domains, many of the mutations thus far identified in *coracle* alleles fall within these two regions. *cor$^4$*, *cor$^6$*, *cor$^8$*, and *cor$^{10}$* all result from either in-frame deletion or missense mutation within region A. *cor$^{14}$* results from a nonsense mutation at $Arg^{1607}$, truncating the protein within region I. Taken together, these data suggest the existence of two important functional domains in Coracle, one corresponding to the conserved FERM domain and the other corresponding to the C-terminal 100 amino acids.

Interestingly, all *coracle* mutations that are predicted to affect the N-terminal functional domain are embryonic lethal, whereas *cor$^{14}$*, which truncates the C-terminal domain, shows no embryonic lethality. This observation raises the possibility that the N-terminal functional domain is required to complete embryonic development, whereas the C-terminal domain is required at a later stage. Ectopic expression experiments using just the FERM domain strongly support this supposition. Embryos completely lacking *coracle* function display a range of defects including failure in dorsal closure, thinning of the cuticle, necrosis of the salivary glands, and an inability to inflate the trachea at the end of embryogenesis. Expression of the N-terminal 383 amino acids fully rescues all of these defects (Table 2 and Figure 2). Additionally, the *cor$^8$* and *cor$^{10}$* mutations, which affect the FERM domain and cause embryonic lethality, can be rescued to viable, fertile adults by the ubiquitous expression of just the FERM domain (Table 3). In contrast, *cor$^4$* and *cor$^6$* mutant animals, which also have molecular lesions within the FERM domain, are not rescued by ubiquitous expression of $COR^{1\text{-}383}$ (Table 3). Interestingly, the *cor$^4$* and *cor$^6$* mutant proteins display abnormal subcellular localizations (Ward *et al.* 1998), raising the possibility that correct subcellular localization is as crucial for the function of the C-terminal domain as it is for the function of the FERM domain. Also, *cor$^1$* and *cor$^2$*, which have an intact FERM domain display embryonic defects, but like most nonsense mutations these alleles show reduced protein expression (Fehon *et al.* 1994).

Our experiments strongly suggest the existence of an essential functional domain within the C-terminal region of Coracle. Even though *cor$^{14}$* is one of the weakest alleles tested in these experiments, ectopic expression of the FERM domain is incapable of rescuing *cor$^{14}$* animals. In contrast, *cor$^{14}$* is rescued by expression of a full-length *coracle* transgene (Table 3), indicating that a region outside the FERM domain is necessary for viability. *cor$^{14}$* results from a nonsense mutation at $Arg^{1607}$, suggesting that the C-terminal functional domain includes sequences within the highly conserved C-terminal 100 amino acids.

The proposed modular organization of the functional domains within Coracle leads to the following prediction: alleles that specifically alter only one functional domain should complement alleles that affect only the other functional domain. We have previously reported on such an experiment (Lamb *et al.* 1998). If alleles that dramatically reduce the expression level of the mutant protein or alter their subcellular localization are excluded, examination of these data bears out this hypothesis. Specifically, combinations involving *cor$^8$* and *cor$^{10}$* (specifically affecting the N-terminal domain) and *cor$^{14}$* (specifically affecting the C-terminal domain) support this hypothesis. Although all three of these alleles are recessive lethal (either homozygous or over a deficiency),

*cor*[10] is >75% viable when heterozygous with *cor*[14], and *cor*[8] weakly complements *cor*[14] (Lamb *et al.* 1998). This result is in agreement with the results obtained here using molecular genetic approaches and strongly supports our conclusions on the modular nature of the functional domains within Coracle.

**Cellular functions of the Coracle domains:** Our previous studies have suggested that most, if not all, of the embryonic defects associated with loss of *coracle* function are due to an inability to maintain a physiologically "tight" epithelium (Lamb *et al.* 1998). In studies described here, we demonstrate that ectopic expression of the FERM domain in *coracle* mutant embryos is sufficient to rescue all of the described embryonic defects, raising the possibility that this rescue was accomplished by restoring the integrity of the septate junction. Ultrastructural and physiological analyses confirmed this hypothesis (Figure 3), demonstrating that this domain provides an essential structural function at the septate junction in embryonic epithelia.

Although our results indicate that the FERM domain provides an essential structural function during embryonic development, it is less clear what role this domain plays in post-embryonic development. Loss of *coracle* function in imaginal epithelia results in a proliferative disadvantage that is ameliorated by expression of just the FERM domain (Figure 4), but does not alter overall epithelial integrity or polarity (Lamb *et al.* 1998). A number of cell signaling pathways have been implicated in controlling cell proliferation and growth in imaginal epithelia. Included among these are the epidermal growth factor, Wingless, Notch, and the Dpp pathways (Diaz-Benjumea and Garcia-Bellido 1990; Burke and Basler 1996; Johnston and Edgar 1998). Mutations that perturb the proper transmission of these signals produce imaginal defects similar to those we report here for *coracle*. Additionally, *coracle* was originally identified as a dominant suppressor of *Egfr*[Ellipse], a hypermorphic allele of the epidermal growth factor receptor homolog (Fehon *et al.* 1994). It is possible that the FERM domain facilitates the transduction of one or more of these signaling cascades by binding to and thereby localizing an important intracellular factor or factors. Our continuing efforts to identify additional genes that interact with *coracle* will undoubtedly help resolve the function of this domain in regulating proliferation.

What, then, is the role of the C-terminal domain? By analogy with Protein 4.1 and the ERM proteins, we suspect that this region in Coracle contains a protein-binding domain. In the ERM proteins, the C-terminal domain is thought to regulate the function of the FERM domain via an intramolecular interaction (Gary and Bretscher 1995; Reczek and Bretscher 1998). It is not known yet if similar interactions may occur in Coracle or Protein 4.1. In addition, recent experiments have identified several potential protein-protein interactions mediated by this domain. For example, the immunophilin FKBP13 interacts with the C-terminal region of Protein 4.1G, one of several Protein 4.1 paralogues in mammals (Walensky *et al.* 1998). If such interactions occur via the Coracle C-terminal domain, they could function to anchor additional proteins to the region of the septate junction. In contrast, two recent studies have suggested interactions between this same domain of Protein 4.1 and proteins known to function in the nucleus. These proteins are NuMA, the nuclear mitotic apparatus protein (Mattagajasingh *et al.* 1999; Ye *et al.* 1999), and PIKE, a putative regulator of $PI_3$ kinase activity in the nucleus (Ye *et al.* 2000). At the moment the functional significance of these interactions is not known, nor do we know if Coracle has similar interactions. Further experiments, particularly using genetic approaches, will be required to determine their functional significance. However, both our data and data regarding putative protein-protein interactions are consistent with a function for the C-terminal domain that is distinct from that of the FERM domain and may differ significantly from current ideas about Protein 4.1/Coracle functions.

We thank the Bloomington Drosophila stock center at Indiana University for fly stocks. We thank D. LaJeunesse (University of North Carolina, Greensboro), D. Kiehart (Duke University, Durham, NC), and J. Genova (Duke University, Durham, NC) for valuable suggestions and discussions. This work was supported by grant no. RPG-97-026-04-DDC from the American Cancer Society to R. G. Fehon. R. E. Ward and R. S. Lamb were supported by National Institutes of Health training grants GM-07754 and GM-07184, respectively.

## LITERATURE CITED

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne *et al.*, 2000 The genome sequence of Drosophila melanogaster. Science **287:** 2185–2195.

Algrain, M., O. Turunen, A. Vaheri, D. Louvard and M. Arpin, 1993 Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. J. Cell Biol. **120:** 129–139.

Anderson, R. A., and R. E. Lovrien, 1984 Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. Nature **307:** 655–658.

Bellen, H. J., Y. Lu, R. Beckstead and M. A. Bhat, 1998 Neurexin IV, caspr and paranodin—novel members of the neurexin family: encounters of axons and glia. Trends Neurosci. **21:** 444–449.

Boedigheimer, M., and A. Laughon, 1993 *expanded*: a gene involved in the control of cell proliferation in imaginal discs. Development **118:** 1291–1301.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

Bretscher, A., 1999 Regulation of cortical structure by the ezrin-radixin-moesin protein family. Curr. Opin. Cell Biol. **11:** 109–116.

Burke, R., and K. Basler, 1996 Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. Development **122:** 2261–2269.

Chishti, A. H., A. C. Kim, S. M. Marfatia, M. Lutchman, M. Hanspal *et al.*, 1998 The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. [letter] Trends Biochem. Sci. **23:** 281–282.

Correas, I., T. L. Leto, D. W. Speicher and V. T. Marchesi, 1986 Identification of the functional site of erythrocyte protein 4.1 involved in Spectrin-actin associations. J. Biol. Chem. **261:** 3310–3315.

Diaz-Benjumea, F. J., and A. Garcia-Bellido, 1990 Genetic analysis

of the wing vein pattern of Drosophila. Roux's Arch. Dev. Biol. **198:** 336–354.

Fehon, R. G., K. Johansen, I. Rebay and S. Artavanis-Tsakonas, 1991 Complex cellular and subcellular regulation of Notch expression during embryonic and imaginal development of *Drosophila*: implications for Notch function. J. Cell Biol. **113:** 657–669.

Fehon, R. G., I. A. Dawson and S. Artavanis-Tsakonas, 1994 A *Drosophila* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the *coracle* gene. Development **120:** 545–557.

Gary, R., and A. Bretscher, 1995 Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. Mol. Biol. Cell **6:** 1061–1075.

Hoover, K. B., and P. J. Bryant, 2000 The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. Curr. Opin. Cell Biol. **12:** 229–234.

Johnston, L. A., and B. A. Edgar, 1998 Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. Nature **394:** 82–84.

LaJeunesse, D. R., B. M. McCartney and R. G. Fehon, 1998 Structural analysis of Drosophila merlin reveals functional domains important for growth control and subcellular localization. J. Cell Biol. **141:** 1589–1599.

Lamb, R. S., R. E. Ward, L. Schweizer and R. G. Fehon, 1998 *Drosophila coracle*, a member of the protein 4.1 superfamily, has essential structural functions in the septate junctions and developmental functions in embryonic and adult epithelial cells. Mol. Biol. Cell **9:** 3505–3519.

Marchesi, V. T., 1985 Stabilizing infrastructure of cell membranes. Annu. Rev. Cell Biol. **1:** 531–561.

Mattagajasingh, S. N., S. C. Huang, J. S. Hartenstein, M. Snyder, V. T. Marchesi *et al.*, 1999 A nonerythroid isoform of protein 4.1R interacts with the nuclear mitotic apparatus (NuMA) protein. J. Cell Biol. **145:** 29–43.

McCartney, B. M., R. M. Kulikauskas, D. R. LaJeunesse and R. G. Fehon, 2000 The neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation. Development **127:** 1315–1324.

Morrison, H., L. S. Sherman, J. Legg, F. Banine, C. Isacke *et al.*, 2001 The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev. **15:** 968–980.

Pasternack, G. R., R. A. Anderson, T. L. Leto and V. T. Marchesi, 1985 Interactions between protein 4.1 and band 3. J. Biol. Chem. **260:** 3676–3683.

Rebay, I., R. G. Fehon and S. Artavanis-Tsakonas, 1993 Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell **74:** 319–329.

Reczek, D., and A. Bretscher, 1998 The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. J. Biol. Chem. **273:** 18452–18458.

Rouleau, G. A., P. Merel, M. Lutchman, M. Sanson, J. Zucman *et al.*, 1993 Alteration in a new gene encoding a putative membrane-organizing protein causes neurofibromatosis type 2. Nature **363:** 515–521.

Trofatter, J. A., M. M. MacCollin, J. L. Rutter, R. Eldridge, N. Kley *et al.*, 1993 A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell **72:** 791–800.

Tsukita, S., and S. Yonemura, 1999 Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. J. Biol. Chem. **274:** 34507–34510.

Tsukita, S., K. Oishi, N. Sato, J. Sagara, A. Kawai *et al.*, 1994 ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J. Cell Biol. **126:** 391–401.

Turunen, O., T. Wahlström and A. Vaheri, 1994 Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. J. Cell Biol. **126:** 1445–1453.

Walensky, L. D., P. Gascard, M. E. Fields, S. Blackshaw, J. G. Conboy *et al.*, 1998 The 13-kD FK506 binding protein, FKBP13, interacts with a novel homologue of the erythrocyte membrane cytoskeletal Protein 4.1. J. Cell Biol. **141:** 143–153.

Ward, R. E., R. S. Lamb and R. G. Fehon, 1998 A conserved functional domain of Drosophila coracle is required for localization at the septate junction and has membrane-organizing activity. J. Cell Biol. **140:** 1463–1473.

Ye, K., D. A. Compton, M. M. Lai, L. D. Walensky and S. H. Snyder, 1999 Protein 4.1N binding to nuclear mitotic apparatus protein in PC12 cells mediates the antiproliferative actions of nerve growth factor. J. Neurosci. **19:** 10747–10756.

Ye, K., K. J. Hurt, F. Y. Wu, M. Fang, H. R. Luo *et al.*, 2000 Pike. A nuclear gtpase that enhances PI3kinase activity and is regulated by protein 4.1N. Cell **103:** 919–930.

Yonemura, S., M. Hirao, Y. Doi, N. Takahashi, T. Kondo *et al.*, 1998 Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. J. Cell Biol. **140:** 885–895.

Communicating editor: K. V. Anderson