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A *Drosophila* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the *coracle* gene

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SUMMARY

Protein 4.1 functions to link transmembrane proteins with the underlying spectrin/actin cytoskeleton. To permit a genetic analysis of the developmental role and cellular functions of this membrane-skeletal protein, we have identified and characterized its *Drosophila* homologue (termed D4.1). D4.1 is localized to the septate junctions of epithelial cells and is encoded by the *coracle* gene, a new locus whose primary mutant phenotype is a failure in dorsal closure. In addition, *coracle* mutations dominantly suppress *Ellipse*, a hypermorphic allele of the *Drosophila* EGF-receptor homologue. These data indicate that D4.1 is associated with the septate junction, and suggest that it may play a role in cell-cell interactions that are essential for normal development.

Key words: protein 4.1, EGF-receptor, septate junctions, *Drosophila*, *coracle*

INTRODUCTION

Genetic and molecular studies of *Drosophila* development have identified a number of cell surface receptors or associated proteins that function in cell-cell interactions necessary for proper specification of cell fate. In most cases (see below), these proteins are tightly localized to junctional regions, strongly suggesting that these morphologically distinct regions of cell-cell contact are important for interactions between cells. Three types of junctions commonly occur in *Drosophila* epithelial cells (Poodry and Schneiderman, 1970): (1) adherens junctions, the most apical junctional complex where elements of the cytoskeleton are closely associated with the cell membrane; (2) septate junctions, which lie basal to the adherens junction and may function analogously to vertebrate tight junctions by restricting diffusion between the apical and basolateral domains of the cell membrane (Wood, 1990) and (3) gap junctions that allow the passage of soluble cytoplasmic components between adjacent cells. Together, these junctions are believed to maintain the structural integrity of the epithelium and to organize specialized regions of the cell membrane that function in intercellular communication.

Most receptors and associated proteins identified so far seem to be associated with the adherens junctions. One example is the product of the *Drosophila armadillo* gene, which is homologous to vertebrate β-catenin and is involved in reception of the *wingless*-mediated diffusible intercellular signal (Peifer and Wieschaus, 1990; Peifer, 1993). Two *Drosophila* members of the transmembrane receptor tyrosine kinase family are localized to the apical membrane in the region of the adherens junction, the EGF-receptor homologue (*Egfr*) and *sevenless* (Zak and Shilo, 1992; Tomlinson et al., 1987). Similarly, Notch, which mediates cell-cell interactions during a number of cell fate decisions, is tightly localized to the adherens junction in most epithelial cells (Fehon et al., 1991; R. Fehon and S. Artavanis-Tsakonas, unpublished observations).

Only two proteins associated with the septate junction have been described so far. One, the product of the *discs large* gene (*dlg*; Woods and Bryant, 1991), encodes a protein that shares homology with yeast guanylate kinase and whose function is necessary for growth control during imaginal disc development, as shown by the disc overgrowth phenotype of *dlg* mutants. Because growth regulation within the disc epithelium is thought to depend on cell-cell interactions (Simpson, 1981), it is likely that septate junctions also play an important role in mediating interactions between adjacent cells (Woods and Bryant, 1991). The other protein known to be associated with septate junctions is fasciclin III, which is thought to function as an adhesion protein, (Hortsch and Goodman, 1991; Woods and Bryant, 1994). No molecular components of the *Drosophila* gap junction have yet been described.

In this study, we describe a third protein that is associated with the septate junction, the *Drosophila* homologue of human erythrocyte protein 4.1. Human protein 4.1 was originally identified as a major component of the erythrocyte membrane skeleton, and subsequent biochemical studies have suggested that it may serve to link the spectrin-actin cytoskeleton to the membrane, thereby stabilizing the biconcave erythrocyte shape (Marchesi, 1985). This function is thought to be served through protein-protein interactions with the transmembrane proteins

glycophorin C and band 3 (the anion transporter) mediated by the N-terminal region of protein 4.1. Similarly, sequences near the C terminus of 4.1 interact with spectrin and actin of the membrane skeleton, thereby stabilizing their interactions with each other and with the cell membrane (Bennett, 1989). Consistent with such a role, mutations in the human protein 4.1 gene have been shown to cause elliptocytosis resulting in hemolytic anemia (Conboy et al., 1991a, 1993).

Although most studied in erythrocytes, human protein 4.1 is expressed in a variety of tissues, including the brain, and has been shown to encode tissue-specific isoforms through alternative splicing (Tang et al., 1988, 1990; Conboy et al., 1988, 1991b). The function of protein 4.1 in these cells is not known, but the available data indicate that it may be localized in cellular junctions and in the nucleus (Tang et al., 1990; Correas, 1991). These localizations imply that 4.1 may have functions in non-erythroid tissues that are different from those identified in the erythrocyte model. Indeed, recent molecular studies have revealed a complex pattern of alternative splicing that produces identified, non-erythroid isoforms that lack the putative spectrin-actin binding domain of protein 4.1, suggesting that these forms may not interact with the cytoskeleton in the same manner as the erythrocyte isoform.

Despite the extensive previous biochemical studies of erythrocyte protein 4.1, our understanding of the function of this protein, especially in non-erythroid tissues, is incomplete. We have therefore begun genetic, molecular and cellular analyses of a protein 4.1 homologue which is expressed in a variety of *Drosophila* epithelia during embryonic and imaginal development. In particular, the genetic approach should provide unique assays that will allow us to define cellular and developmental functions of this protein. We describe here the molecular cloning and the initial functional characterization of the *Drosophila* homologue of protein 4.1. We show that this gene encodes several protein isoforms that are closely associated with a part of the lateral cell membrane, the septate junction. The tissue specificity and timing of expression of the *Drosophila* 4.1 homologue closely parallels that of the appearance of septate junctions, suggesting that this protein may form an integral part of this enigmatic cellular junction. Furthermore, using genetic rescue experiments, we identify the *Drosophila* gene that encodes the protein 4.1 homologue, a newly identified gene named *coracle* (*cor*), whose recessive embryonic phenotype is a failure in dorsal closure. The expression and genetic phenotypes of the *coracle*/D4.1 gene suggest that it may play a role in cell-cell interactions that are necessary for proper development.

MATERIALS AND METHODS

Degenerate PCR

An alignment between the human and *Xenopus* protein 4.1 sequences (Fig. 1) was used to design degenerate pcr primers with the following sequences:

primer 1: 5′-AA(T/C) GTI AA(A/G) TT(T/C) TA(T/C) CCI CCI GA(T/C) CC-3′
primer 2: 5′-AC(T/C) TTI GGC CAI GG(A/G) AAI C(G/T)(A/G) TT(A/G/T) AT-3′
primer 3: 5′-GT(A/G) TG(A/G) TG(T/C) TCI AC(A/G) CAI AC(T/C) TTC CA-3′.

Fig. 1. The amino acid sequence of *Drosophila* protein 4.1 and its alignment with the *Xenopus* and human protein 4.1 coding sequences. Sequence identities are shown with gray shading, and a consensus sequence (at least 2 of the 3 match) is shown below each line. Sequences chosen for the design of degenerate oligonucleotide primers used for PCR amplification of the *Drosophila* gene are indicated by arrows above the sequence. Alternatively spliced exonic sequences in the human sequence, termed motifs (Tang et al., 1990), are indicated by bars above the sequence. The positions of the two sequenced *coracle* alleles are shown above the amino acid residues that they mutate to nonsense codons. The DNA sequences of cDNA1 and the other cDNAs have been deposited in the GenBank database.

Inosines were used at fully degenerate positions. Amplification reactions (30 cycles) were performed using *Drosophila* genomic DNA as template under the following conditions: anneal 58°C, 60 seconds; extend 72°C, 60 seconds; melt 94°C, 60 seconds. Standard nucleotide and buffer concentrations were used (GeneAmp kit, Perkin-Elmer-Cetus), and primers were used at 10-50 ρm/rxn. Initial amplification reactions were performed using primers 1 and 2, although subsequent reactions using primers 1 and 3 and cDNA template were also performed. PCR products were cloned into *Sma*I cut Bluescript plasmid (Stratagene) for sequencing.

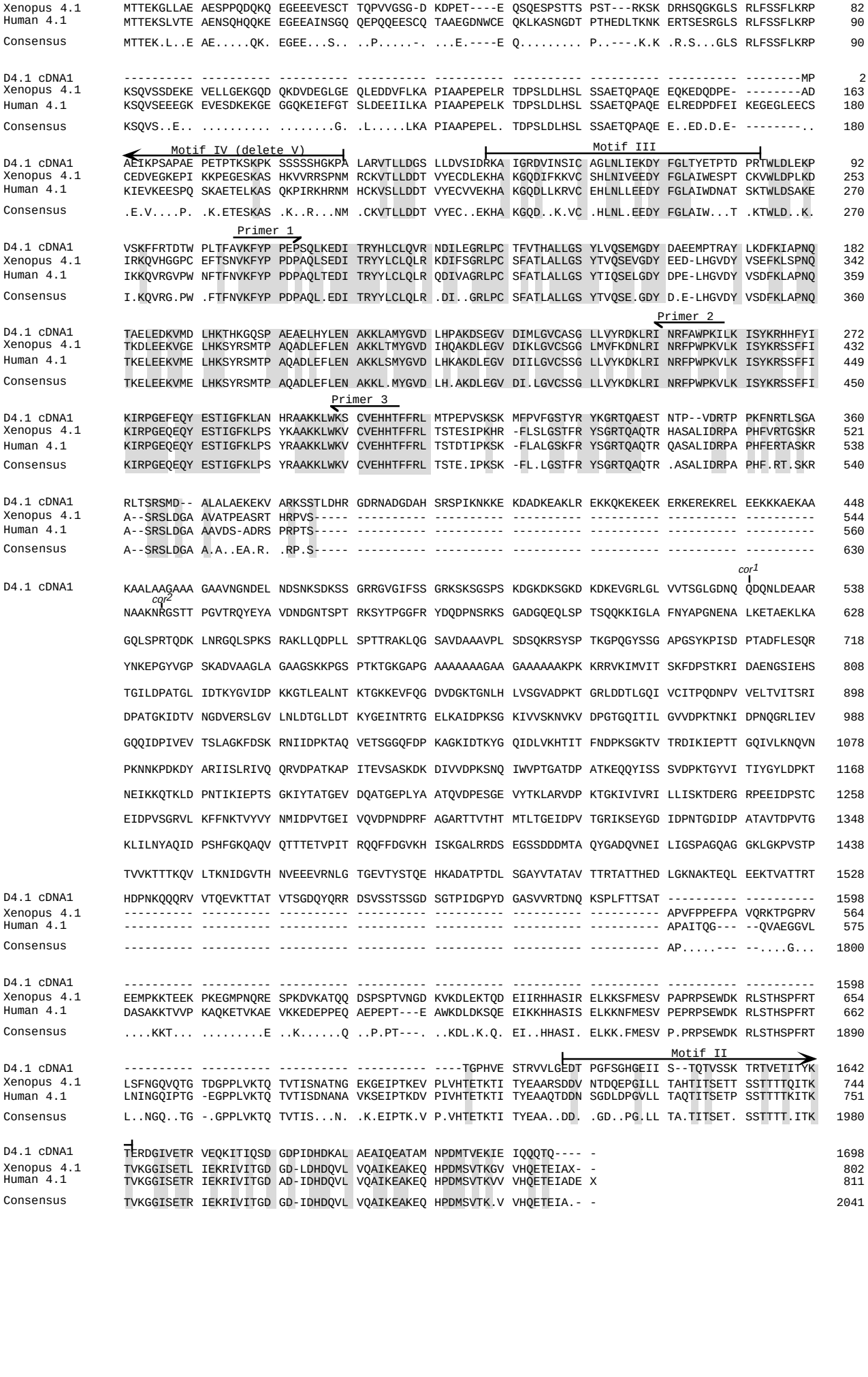
Isolation and sequencing of cDNA clones

We screened two libraries for D4.1 cDNAs, the 12-24 hour λgt10 library of Poole et al. (1985) and a λzap 2-14 hour embryonic library (C. Delidakis and R. Fehon, unpublished data; Stratagene). The λgt10 library was screened using the subcloned degenerate pcr product as probe, while the λzap library was screened with a probe made from the 3′ end of region E, which is unique to cDNA1, in order to isolate clones containing the 3′ end of cDNA1. The λgt10 clones were digested with *Eco*RI and subcloned into Bluescript for sequencing. The λzap clones were excised to form Bluescript clones according to manufacturer's instructions.

Sequencing was performed on single- or double-stranded templates from the Bluescript clones using the dideoxynucleotide chain-termination method (the Sequenase kit, US Biochemicals). Sequence reactions were done according to manufacturer's instructions except that for double-stranded sequencing the following modifications were employed: precipitated, alkaline-denatured template DNA (15-20 μg) was resuspended in reaction buffer with 0.1 μg T4 gene 32 protein (Boehringer), label mix was diluted 3-fold, and Sequenase enzyme was diluted 5-fold. Both strands were sequenced for all clones and dITP was used when required to resolve sequence compressions. Sequences were read by hand and assembled using the GeneWorks analysis program (Intelligenetics). Codon preference analysis was performed using a *Drosophila* codon preference table provided by the MacVector sequence analysis program (IBI). The BLAST (Altschul et al., 1990) server on the NCBI GenBank database was used for sequence homology searches.

Antibody preparation

Two D4.1 fusion proteins were used as immunogens in mice for the experiments described here. The first, from the N-terminal region of the protein (Fig. 2A), was made from PCR-amplified sequences (bp 586 to 1560) blunt-end ligated (Rebay et al., 1991) into the *Sma*I site of pGEX3 fusion protein vector (Smith and Johnson, 1988; Pharmacia). The second fusion protein was similarly amplified from a more C-terminal region unique to cDNA1 (bp 2193 to 4225) and also cloned into pGEX3. Purified fusion protein was prepared as described by Smith and Johnson, (1988) or by Frorath et al., (1991), depending on solubility of the protein. Polyclonal antisera and hybridoma cell lines were made as previously described (Fehon et al., 1990).


Motif IV (delete V)
Motif III
Primer 1
Primer 2
Primer 3
cor1
cor2
Motif II

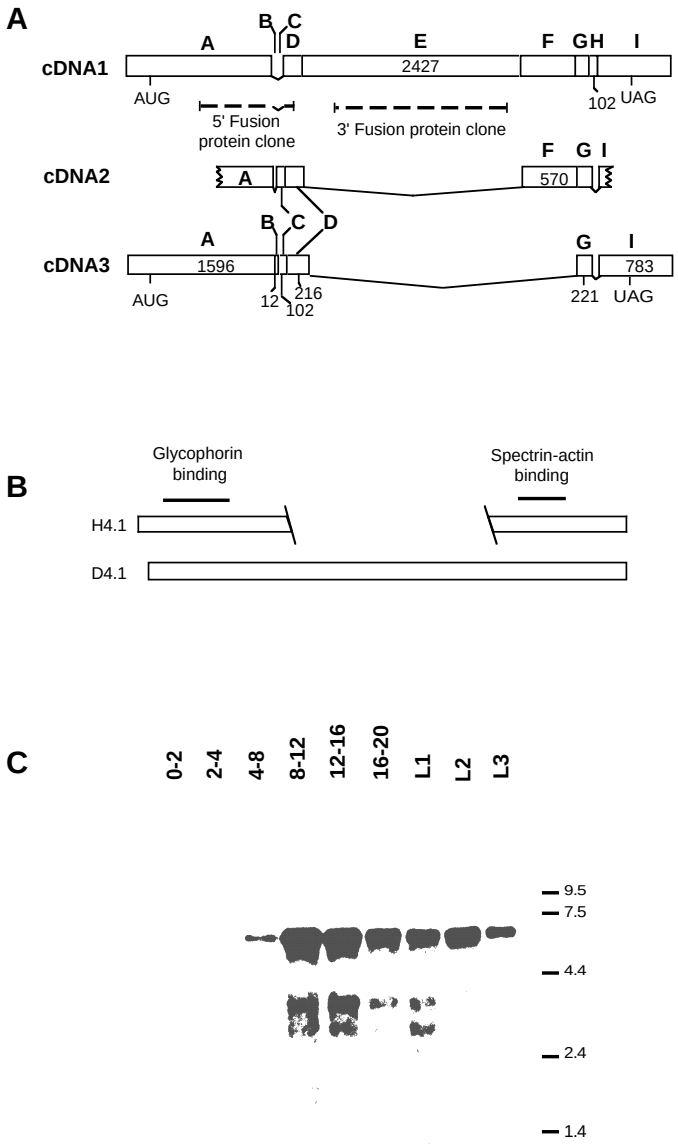


Fig. 2. D4.1 structure and alternative splice forms. (A) Three characterized embryonic cDNA clones are shown diagrammatically. Exonic regions identified by comparison of the three differently spliced cDNAs are indicated by letters (A-I) at the top. Numbers indicate the size, in bp, of each exonic region. cDNA2 is truncated at either end, as indicated by the jagged lines. AUG and UAG indicate start and stop sites respectively. The two fusion protein clones used to raise antibodies are indicated by dashed lines below cDNA1. (B) Cartoons of the human erythrocyte protein 4.1 isoform (H4.1) and the *Drosophila* cDNA1 isoform (D4.1) are shown aligned with each other and the cDNA maps in A). Shaded areas indicate regions of sequence conservation. Gap in the middle of the human sequence denotes the size difference between it and the *Drosophila* cDNA1 coding sequence. Bars above the human sequence indicate the approximate locations of the glycophorin and spectrin/actin binding domains of erythrocyte protein 4.1 (Conboy et al., 1991b). (C) Northern blot of poly(A)$^+$ mRNA prepared from staged *Drosophila* embryos (0-20 hours after egg laying) and larvae (first, second, and third larval instars - L1, L2, L3). The blot was probed with radiolabeled DNA from the 5′ region that is shared by all of the alternatively spliced cDNAs. In addition to a predominant form at 6.3 kb, there are several more weakly expressed species of lower molecular weight. Positions of size markers are shown at right.

Cuticle preparations and SEM

Cuticle preparations were mounted in Hoyer's medium according to standard protocols (Ashburner, 1989). SEM was performed as previously described (Rebay et al., 1993).

Histology and immunoblotting

Embryos and imaginal discs were prepared for immunofluorescence and immunocytochemistry as previously described (Fehon et al., 1991), with the exception that we found that the PLP fixative (Van Vactor et al., 1991) gave better preservation of D4.1 on imaginal tissues than the standard paraformaldehyde fixative. D4.1/rhodamine phalloidin double labeling experiments were done using rhodamine phalloidin (Molecular Probes) according to manufacturers instructions. A BioRad MRC-600 laser scanning confocal microscope with a krypton/argon laser was used for the confocal microscopy. For immunoelectron microscopy of imaginal disc tissues, we dissected the tissues as usual but followed the techniques of Van Vactor et al. (1991) for fixation and staining, with the exception that 0.1% saponin (Sigma) was used to permeabilize tissue.

Immunoblots were performed as previously described (Fehon et al., 1990; Rebay et al., 1993).

Isolation of coracle alleles

b pr c px sp males were mutagenized with EMS and mated to Elp^{B1} females according to standard procedures (Grigliatti, 1986). The resulting F_1 progeny were screened for males in which the Elp^{B1}/+ dominant rough eyed phenotype was suppressed. Such individuals were crossed to Elp^{B1}/CyO females and dominant suppressors which bred true and segregated with the second chromosome were retained for further analysis. Two independent alleles of a locus, which we called *coracle*, were recovered from approximately 10,000 mutagenized chromosomes in this screen.

Genetic rescue experiments

A transformation construct for testing genetic rescue of the *coracle* mutations was prepared by fusing the *Drosophila* ubiquitin promoter (Lee et al., 1988) to D4.1 cDNA1, and placing this within the pCaSpeR-4 P-element transformation vector. For this construct, a 3294 bp *Eco*RI/*Kpn*I fragment (bp 1202 to 4495) and a 1150 bp *Kpn*I/*Xho*I fragment (bp 4496 to 5645) from the 3′ end of cDNA1 were ligated into Bluescript plasmid that had been digested with *Eco*RI/*Xho*I. The resulting construct was *Eco*RI/*Xho*I digested to excise the 3′ end of cDNA1, and ligated together with an 886 bp *Sac*I/*Eco*RI fragment (bp 316 to 1201) from the 5′ end of cDNA1 into *Sac*I/*Sal*I digested pRmHa-3, a metallothionein-promoter expression plasmid (Bunch et al., 1988). To place cDNA1 behind the ubiquitin promoter, a 2.0 kb *Sal*I/*Xba*I fragment from pUp-2 (R. DuBreuil, U. Chicago) containing the promoter was filled and ligated into the *Hpa*I site of RXHpHSS7 (constructed by ligating a linker containing *Hpa*I, *Eco*RI and *Xba*I sites into *Eco*RI/*Xba*I digested pHSS7; I. Rebay, unpublished data). The metallothionein-cDNA1 construct was then partially digested with *Eco*RI and *Xba*I to excise the entire cDNA1 coding sequence together with the Adh polyadenylation signal from pRmHa-3 and subcloned into the ubiquitin-pHSS7 construct. The resulting ubiquitin-promoter driven cDNA1 construct was then excised using flanking *Not*I sites and subcloned into the pCaSpeR-4 transformation vector. Injections were performed as previously described (Rebay et al., 1993).

RESULTS

Cloning and sequence analysis of the *Drosophila* 4.1 (D4.1) homologue

The human and *Xenopus* protein 4.1 genes are approximately 70% identical over a stretch of 560 amino acids at the N termini of the two proteins (Fig. 1). We therefore used alignments between the human and *Xenopus* sequences to design degenerate oligonucleotide primers for PCR reactions to amplify homologous *Drosophila* sequences (Fig. 1; primer sequences and details of methods are described in Materials and Methods). Reactions using these primers and *Drosophila* genomic DNA as template produced a 550 bp amplification product. This product was subcloned, sequenced and found to be capable of encoding a protein that is approx. 70% identical to human 4.1. cDNA clones from two *Drosophila* libraries were then isolated for sequence analysis (see Materials and Methods). The assembled sequence from two overlapping cDNAs forms a message of 5915 bp with a single open reading frame that encodes a protein of 1698 amino acids. The sequence of this protein is presented in Fig. 1, together with its alignment with the human and *Xenopus* proteins. The *Drosophila*, *Xenopus* and human sequences are 54% identical over a stretch of 350 aa in the N-terminal region. Within this region there is significant but lower identity with other members of the protein 4.1 gene family, including ezrin (31% over 229 aa), talin (22% over 194 aa), moesin A and B (30% over 229 aa), merlin (27% over 229 aa) and the two human tyrosine phosphatase genes (37% over 287 aa). In addition, D4.1 shares sequence similarity with the human and *Xenopus* 4.1 genes in a smaller region at the C terminus that is not shared with any other members of the 4.1 gene family (indicated by shading in Figs 1, 2B). The intervening 1200 aa of D4.1 sequence shows no similarity to any sequences in the GenBank database.

Previous studies of protein 4.1, ezrin and talin have suggested that the C-terminal halves of these proteins have a primarily α-helical structure that interacts with elements of the cytoskeleton (Rees et al., 1990). Chou-Fasman and Garnier computer-based structural analyses both predict a region of α-helical structure that extends for approx. 400 aa immediately C terminal to the highly conserved domain of D4.1 (the first 370 aa). However, the additional 800 aa of sequence in D4.1 that extends beyond this putative α-helical domain is predicted by these structural analyses to be composed primarily of β-sheet structures. No such extended β-sheet domain has been proposed for any of the other protein 4.1 family members. We also note that the N-terminal conserved region in D4.1 appears to have several hydrophobic domains that could interact with the cell membrane.

In addition to the 5.9 kb cDNA (Fig. 1; cDNA1 in Fig. 2A), two other cDNA clones were analyzed using a combination of sequencing and PCR. As Fig. 2A shows, these other cDNAs are identical for 1596 bp at the 5′ end (with the exception of cDNA 2, which appears to have been truncated during cloning), but diverge by alternative splicing 3′ to this point. cDNA 2 contains a 102 bp insert (region C, Fig. 2A), and cDNA 3 contains the same insert, plus a second one of 12 bp just 5′ to the first (region B). In both cases, a continuous open reading frame is maintained through these inserts to 3′ sequences that are shared with cDNA1.

The existence of different D4.1 cDNAs clearly suggests that D4.1 gene transcripts are alternatively spliced, as has been described for the human gene (Tang et al., 1990; Conboy et al., 1991b). However, there does not seem to be a general correlation between the protein motifs that are alternatively spliced in the human gene and those that are spliced in the *Drosophila* gene. The alternative N terminus in human 4.1 that is formed by the insertion of a 17 bp sequence (termed motif IV) and the deletion of an 80 bp sequence (Motif V; Tang et al., 1990) is not apparent in the *Drosophila* cDNAs we have isolated. Nor is there any evidence of a potential coding region 5′ to the identified start site as would be expected if alternative splicing similar to that identified for human 4.1 occurs in D4.1. However, sequences similar to splice motifs II and III in the human gene are present in the *Drosophila* and *Xenopus* genes (Fig. 1). We also note that the region corresponding to the spectrin/actin binding domain in the erythroid 4.1 protein is not conserved in any of the D4.1 splice forms that we have so far characterized (Fig. 2B), although this region is highly conserved between the human and *Xenopus* genes. While it is possible that other, uncharacterized splice forms exist that do share similarity in the spectrin/actin binding domain, this result implies that the major *Drosophila* 4.1 protein isoforms do not interact with the cytoskeleton in the manner hypothesized in the erythrocyte model.

D4.1 expression

To gain insight into possible functions of D4.1, we have examined its embryonic and imaginal expression at the mRNA and protein levels. Northern blots were performed using a probe from the 5′ region shared by all of the cDNAs (region A, Fig. 2A). The results of this experiment are shown in Fig. 2C. A predominant mRNA of approx. 6.3 kb is first detected at 4-8 hours of development, and becomes much more abundantly expressed in the 8-12 hour interval. Expression remains prevalent throughout later embryonic and larval stages. In addition, this probe, which contains sequences common to all three of the cDNAs (Fig. 2A), hybridizes to at least five other less abundantly expressed mRNAs, ranging in size from 1.2 to 3.6 kb (Fig. 2C). The sizes of the three cDNAs (5.9, 3.5 and 2.9 kb) determined by sequence analysis correlate well with the measured sizes of the mRNAs in northern blots (6.3, 3.6 and 2.9 kb respectively). Thus it is likely that the cDNAs represent authentic alternatively spliced mRNAs from the D4.1 gene. Consistent with the notion that cDNA1 corresponds to the predominant 6.3 kb mRNA, a probe prepared from region E (Fig. 2A), which is specific to cDNA1, hybridizes only to this mRNA species (data not shown).

To examine protein expression, polyclonal and monoclonal antibodies were prepared using fusion protein antigens from two regions of the D4.1 coding sequence (indicated in Fig. 2A). The first antigen included sequences from the 5′ end of the coding sequence that is shared by all three cDNAs, and therefore should recognize all protein isoforms expressed by the alternatively spliced cDNAs. The second contained sequences from region E, which is specific to the product of cDNA1. These antisera were used to determine the expression pattern of D4.1 during *Drosophila* development. Consistent with northern (Fig. 2C) and immunoblot (data not shown) analyses, protein expression was not detectable on whole-mount embryos before embryonic stage 12 (Campos-Ortega

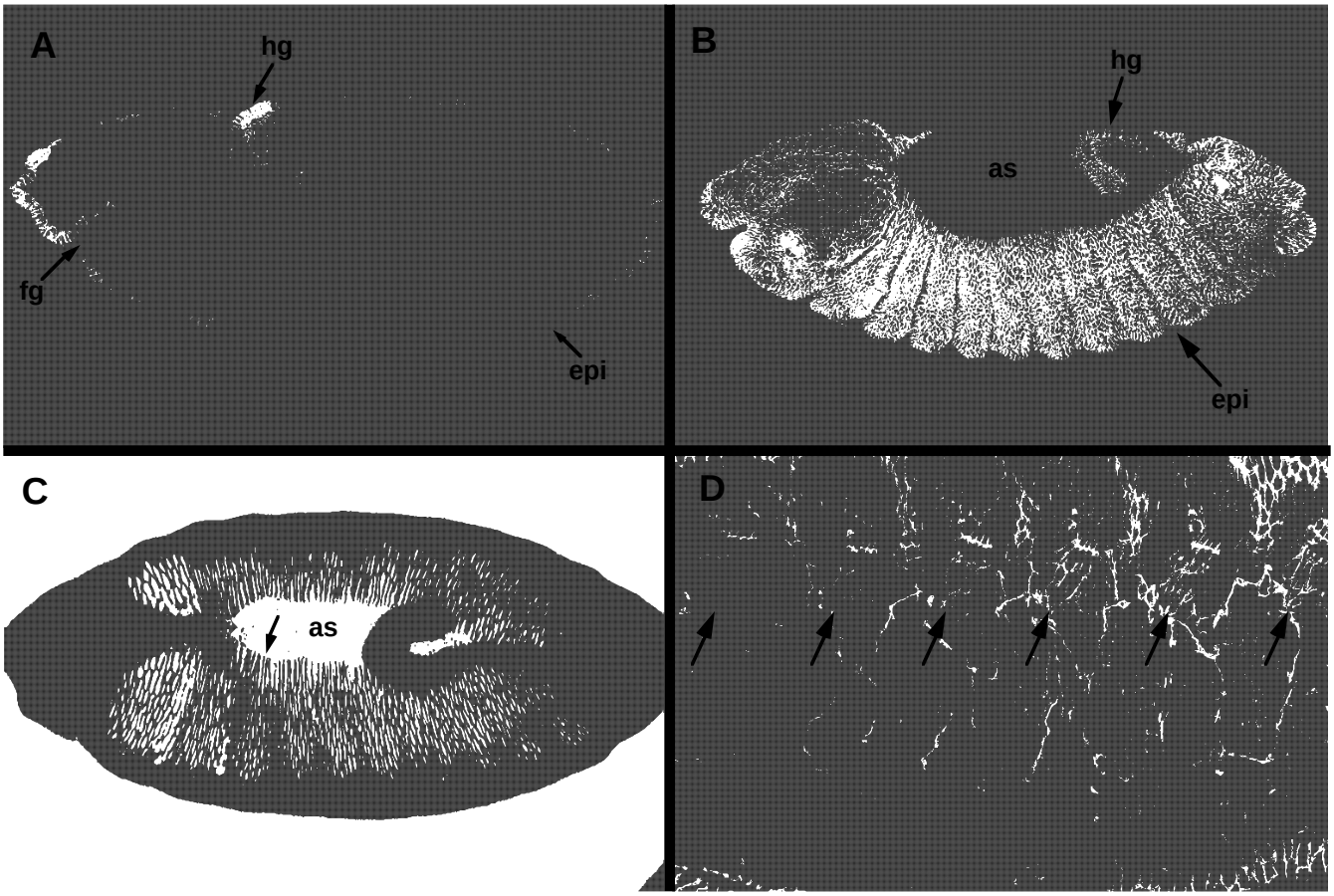


Fig. 3. Expression of D4.1 protein during embryonic development. Embryos were stained with mouse polyclonal antibodies that recognize the cDNA1-unique region E fusion protein. Identical results were obtained with antibodies against the 5′ end of D4.1 (not shown). (A) Confocal optical section of a stage 12 embryo, at the time that D4.1 is first expressed. The antibody stains epidermis (epi), foregut (fg), and hindgut (hg) rudiments. There is no apparent staining in the mesodermal or neuronal derivatives. (B) Projection of confocal sections of a stage 13 embryo, which is undergoing germband shortening and dorsal closure. Note that while D4.1 expression is strong in the epidermis, it is not expressed in the amnioserosa (as). (C) Dorsal view of a late stage 14 embryo, stained with anti-D4.1 primary antibody and an HRP-coupled secondary antibody. Prominent membrane associated staining is apparent in all epidermal cells, but not in the amnioserosa. Also note that D4.1 is not expressed along the leading edge of the dorsal epidermis (arrow), although it is expressed on the other surfaces of these cells. (D) Projection of confocal sections showing the pentascolopidial chordotonal organs (arrows) just beneath the lateral epidermis.

and Hartenstein, 1985), just at the onset of germ band retraction (Fig. 3A). D4.1 protein appears simultaneously in the epidermis, hindgut and foregut. As the germ band retracts, expression increases in these tissues (Fig. 3B,C) and also appears in the tracheal branches and salivary glands as each begins to develop. D4.1 is not expressed at detectable levels within the CNS or any of its derivatives, although it is expressed within some sensory neural cells, such as the scolopidia of the pentascolopidial chordotonal organ (Fig. 3D; Campos-Ortega and Hartenstein, 1985). Once established, this pattern of expression is maintained throughout embryonic development. Comparisons of staining patterns between the N-terminal antibody that recognizes all cDNA isoforms and a more C-terminal antibody that recognizes only the protein encoded by cDNA1 (Fig. 2A), showed no obvious differences. This result suggests that all of the cDNAs are expressed in the same tissues in embryos, although it is possible that the smaller forms are expressed in a subset of tissues or in quantities too small to be readily detected in embryo whole mounts. During postembryonic development, D4.1 is abundantly expressed in the wing and eye imaginal discs but not in the central nervous system. Other imaginal tissues have not yet been examined for expression.

Subcellular distribution of D4.1

During embryonic and imaginal development, D4.1 protein is closely associated with the cell membrane in all expressing cells, as is readily apparent in Fig. 3B,C. Furthermore, in tall columnar epithelia, such as in the hindgut, there is an obvious polarity of D4.1 expression toward the apicolateral epithelial surface (Fig. 4A). To examine this distribution in more detail, we prepared wing imaginal epithelia for confocal and immunoelectron microscopy and stained them using a monoclonal anti-D4.1 antibody. In the confocal microscope, imaginal disc cells showed intense staining in regions of cell contact just below the apical epithelial surface, just as we observed in embryonic epithelia (Fig. 5). In the electron microscope, D4.1 protein is similarly localized to lateral contacts below the most

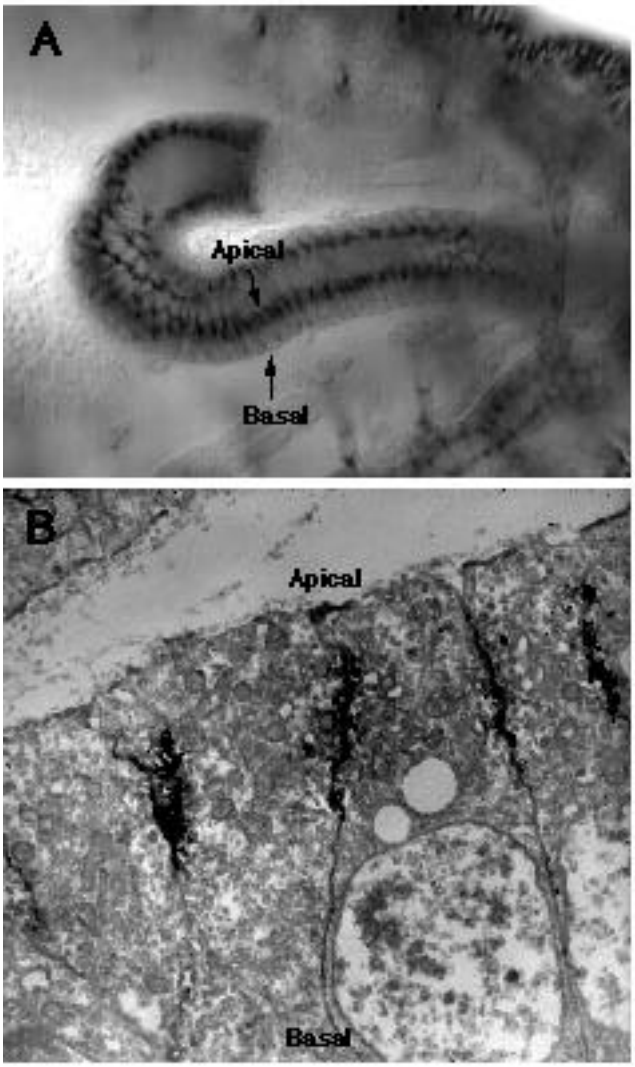


Fig. 4. D4.1 protein is localized to the septate junction in epithelia. (A) Optical section of the embryonic hindgut stained with polyclonal anti-D4.1 and an HRP-coupled secondary antibody. Arrows indicate the apical and basal surfaces of the single-layered hindgut epithelium. Staining is associated with the lateral cell membrane, in the apical domain. (B) Electron micrograph of the wing imaginal disc epithelium stained with monoclonal antibody C566.9C, an HRP-coupled secondary, and silver intensified (see Materials and Methods for details). Staining is visible as a dark precipitate associated with the cell membrane just below the apical cell surface. This region corresponds to the septate junction.

apical junctional region (Fig. 4B). Based on its position, this region of the cell membrane appears to coincide with the septate junction (Poodry and Schneiderman, 1970), although the antibody staining and the fixation procedure used in these preparations obscure the characteristic 'ladder-like' appearance of this junctional complex. Our sequence analysis has revealed no signal sequence or transmembrane domain as expected for a secreted or transmembrane protein. Therefore, D4.1 appears to be associated with the cytoplasmic face of septate junctions in *Drosophila* epithelial cells.

In light of the model for erythrocyte protein 4.1 function, namely that it serves to stabilize spectrin/actin interactions in the membrane skeleton, we were curious whether or not there is a general co-localization of D4.1 and actin in *Drosophila* epithelia. We therefore co-stained wing imaginal discs with anti-D4.1 monoclonal antibodies and rhodamine-phalloidin and then examined these preparations under the confocal microscope. In optical cross-sections of the highly folded hinge region of the wing imaginal disc, it is possible to examine the apical-basal polarity of cells at a high degree of resolution. As Fig. 5 shows, filamentous actin is largely localized to the most apical region of the cell, while the majority of D4.1 protein is clearly more basal. In addition, there is a significant amount of filamentous actin in the basolateral domain of these cells, but only background levels of D4.1 antibody staining. Anti-*Drosophila* spectrin antibodies have shown that spectrin has a similar apical and basal localization to that of actin, although the basolateral component is more predominant for spectrin (I. Rebay, R. Fehon, and S. Artavanis-Tsakonas, unpublished observations). Thus, the D4.1 protein does not appear to co-localize to a great extent with either filamentous actin or spectrin in the apical-basal axis of the cell.

Genetic analysis of D4.1/coracle

In situ hybridization to polytene chromosomes using a probe made from the degenerate PCR product places the D4.1 locus at cytological position 56C on the right arm of the second chromosome (data not shown). We have identified two point-mutant alleles of a previously undescribed gene, which we call *coracle* (*cor*), that we show encodes D4.1 by the following criteria. First, the *coracle* gene maps to position 2-86.9 based on recombination between *curved* (2-75.5) and *plexus* (2-100.5), which closely correlates with the cytological position of D4.1 at 56C from in situ hybridizations. Second, monoclonal antibodies that recognize the N-terminal portion of the D4.1 protein show abnormal staining patterns when used to detect D4.1 protein in embryos homozygous for either *coracle* allele (Fig. 6). cor^1 embryos show punctate, membrane-associated antibody staining and are in general less intensely stained than their wild-type siblings, while cor^2 embryos show almost no staining (Fig. 6A-C). Immunoblots performed using the same antibody detect a 55×10^3 M_r species in proteins isolated from homozygous cor^1 embryos (Fig. 6D) and while this species is absent from cor^2 embryos a new protein species of slightly greater apparent molecular weight is detected (data not shown). These smaller proteins suggest that the *cor* mutations result in truncated coding sequences that express only the N-terminal third of the 180×10^3 M_r wild-type protein. Consistent with this notion, a monoclonal antibody raised against a fusion protein from a more C-terminal region of the coding sequence (see Fig. 2A and Methods) did not recognize the smaller protein in embryos homozygous for either mutant allele (data not shown).

We confirmed the hypothesis that the *coracle* mutations affect the D4.1 coding sequence by direct sequencing and genetic rescue of the mutant alleles. To determine the genetic lesion that causes the *cor* mutations, the sequence of PCR-amplified genomic DNA from embryos or rescued homozygous adult flies (mutant genomic DNA was distinguished from the rescue transposon by the presence of intronic sequences) was compared to that of the parental strain and the original cDNA clones. We concentrated this analysis on sequences close to the junction between regions D and E (Fig. 2A),

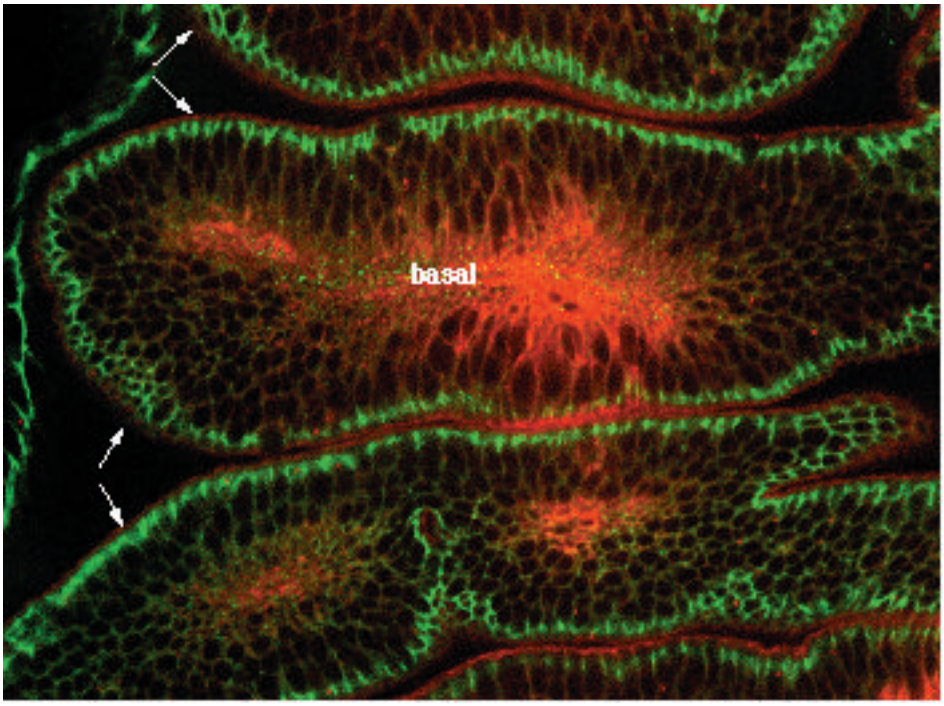


Fig. 5. D4.1 and filamentous actin do not co-localize in polarized epithelial cells. A confocal optical section of the wing imaginal disc epithelium that has been double-labeled with monoclonal anti-D4.1 (green) and rhodamine-phalloidin (red). This section shows the hinge region of the wing disc, where the epithelium is highly folded. Arrows indicate the apical surface of the epithelium in two of the folds. Rhodamine phalloidin, which binds to filamentous actin, stains a thin band at the very apical end of each cell in the region of the adherens junction, as well as a broad region basolaterally (basal). Anti D4.1 stain is restricted to a subapical domain of the cell membrane, in the region of the septate junctions. These two staining patterns do not overlap, as is indicated by the absence of yellow staining in the apical part of the cell.

because the size of the mutant D4.1 protein from immunoblots suggested that the disruption should fall within this region of sequence. We therefore sequenced the interval from base pair 941 to 2556, and detected only a single change relative to the parental chromosome for each allele (indicated in Fig. 1). Both changes are C to T transitions as expected for the EMS mutagen, and both result in nonsense codons which truncate the coding sequence near the 5′ end of region E in cDNA1. Because region E is unique to cDNA1, the two existing alleles, *cor*^{1} and *cor*^{2}, affect only the predominant splice form of D4.1. This result is consistent with the finding that monoclonal antibodies that recognize the 3′ end of coding region E show no reactivity on immunoblots or whole mounts of homozygous *cor*^{1} or *cor*^{2} mutant embryos (data not shown). We also note that the two mutations reside less than 50 bp apart within the region E coding sequence.

Final confirmation of the *coracle*/D4.1 identity was obtained from a genetic rescue experiment. To test whether or not the D4.1 gene could rescue the *coracle* mutations (which are embryonic lethal, see below), we placed cDNA1, the predominantly expressed D4.1 message, under the control of the *Drosophila* ubiquitin promoter (Lee et al., 1988) and introduced this construct into the germ line via P-element-mediated transformation (see Materials and Methods for details of this construct). The expression of this cDNA, driven by the ubiquitin promoter, was sufficient to rescue the embryonic lethality of both *cor* alleles so that homozygotes survived to fertile adults. We found no apparent deleterious effects of this expression, even though the ubiquitin/cDNA1 construct was expressed in tissues that normally do not express D4.1, such as the central nervous system (data not shown).

The coracle mutant phenotype

To define further the function of the *Drosophila* protein 4.1 homologue, we analyzed the phenotype of *coracle* mutant embryos. It is important to note that because the existing *coracle* mutations truncate rather than completely remove the protein product, we cannot yet be certain of the null phenotype of this gene. Both alleles cause recessive embryonic lethality, and show defects in dorsal closure, with *cor*^{1} leaving a small scab on the dorsal side while embryos homozygous for *cor*^{2} display a much larger dorsal opening and abnormalities in head involution (Fig. 7B,C) Observations of living embryos and fixed whole-mount preparations have revealed no other obvious embryonic defects. The timing of this phenotype is consistent with the timing of abundant D4.1 expression (Figs 2, 3).

In addition to the recessive, embryonic-lethal phenotype just described, both alleles have a dominant phenotype. These alleles were isolated in a screen for suppressors of the *Ellipse* (*Elp*) mutation, a dominant allele of the *Drosophila* EGF-receptor homologue (*Egfr*) gene. *Elp* results in a rough eye due to improper spacing of the ommatidia and an overall reduction in ommatidial number (Fig. 7D). Thus, although neither *cor* allele alone shows any dominant phenotype, the transheterozygous combination between either *cor* allele and *Elp* results in suppression of the *Elp* rough eye to an almost wild-type pattern, with *cor*^{2} giving the stronger suppression (Fig. 7E). The addition of a single copy of cDNA1 driven by the ubiquitin promoter ‘rescues’ this suppression by *cor* (B. McCartney and R. Fehon, unpublished observations), and therefore produces the normal *Elp* rough eye phenotype, confirming that the suppression is due to reduction of *coracle* function and not genetic background or a closely linked mutation.

DISCUSSION

Previous studies of protein 4.1 have concentrated on its role as

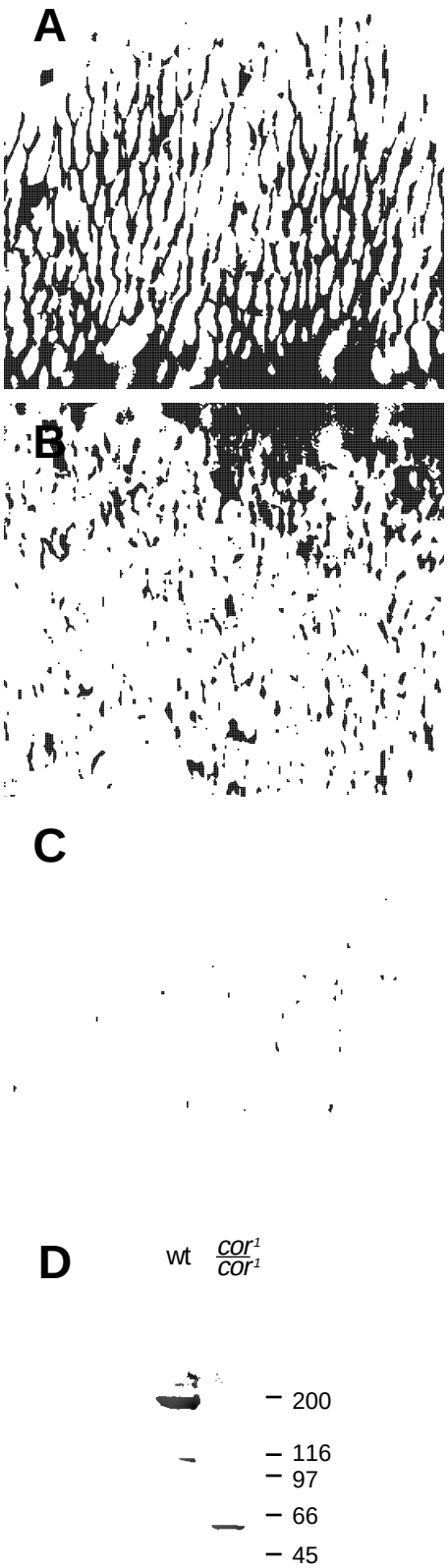


Fig. 6. The *coracle* mutations alter the D4.1 protein. (A-C) Optical sections of embryonic epithelial cells stained with monoclonal anti-D4.1. All embryos were stained in the same experiment to control for artifactual variations in staining intensity. (A) A phenotypically wild-type embryo (+/+ or $cor^1/+$); B) a cor^1/cor^1 mutant embryo, and (C) a cor^2/cor^2 embryo. Homozygotes were distinguished from their heterozygous siblings using balancer chromosome (CyO) carrying a *lacZ* reporter construct. Note the patchy staining and general decrease in staining intensity in B and C relative to A. (D) Immunoblot of proteins isolated from homozygous cor^1/cor^1 embryos and their phenotypically wild-type (wt) siblings (+/+ or $cor^1/+$). This blot was stained with monoclonal antibody C566.9C that recognizes the N-terminal region of D4.1 (Fig. 2). The wild-type embryos display the normal ~180×10^3 M_r D4.1 protein band, while the cor^1/cor^1 embryos lack this band and instead express a prominent 55×10^3 M_r band which is not detected in +/+ embryos. The cor^1/cor^1 lane is overloaded relative to the wild-type lane to increase the signal for the truncated form. Relative molecular mass size markers ($\times10^3$) are indicated at right.

a major component of the membrane skeleton in vertebrate erythrocytes. As a result, while much is known about protein 4.1 function in these specialized cells, little is known about its function in other tissues. To analyze the functions of protein 4.1 in non-erythroid cells, and to identify possible roles during development, we have identified and characterized the *Drosophila* homologue of protein 4.1. There is extensive structural similarity between the human and *Drosophila* genes, particularly in the N-terminal region, and a much lower degree of similarity with other members of the 4.1 family, including ezrin, the moesins, merlin, talin and two tyrosine-phosphatase genes. Because D4.1 is more similar to the human and *Xenopus* protein 4.1 genes than to any other family member, and because it has sequence similarities that are unique to protein 4.1 at the 3′ end, the gene that we report here appears to be a homologue of the protein 4.1 gene rather than another 4.1 family member.

D4.1 is associated with the septate junction

To analyze the cellular and developmental functions of D4.1, we have used antibodies to examine expression in embryonic and imaginal tissues. At the subcellular level, D4.1 is localized specifically to the cell membrane (Figs 3-6). Observation of epithelial cells at the light level reveals that D4.1 is expressed in a polarized manner toward the apical end of the cell, and immunoelectron microscopy shows that D4.1 localizes to the region of the septate junction in the apical-lateral domain of the cell. This type of junctional complex is found only in invertebrate cells, and may function analogously to tight junctions in vertebrate cells (Noirot-Timothée and Noirot, 1980; Wood, 1990).

Aside from its morphology, little is currently known about the septate junction. Recently, however, two other proteins associated with this prominent cellular junction have been identified in *Drosophila*. One, the product of the *discs large* (*dlg*) gene, appears to be composed of several domains that share homology with other genes, including a *src* homology 3 (SH3) region and a region that is similar to a yeast guanylate kinase (Woods and Bryant, 1991). The structure of dlg has two important implications for understanding the function of septate junctions. First, its homology to proteins that function in cGMP second messenger pathways, its function in regulat-

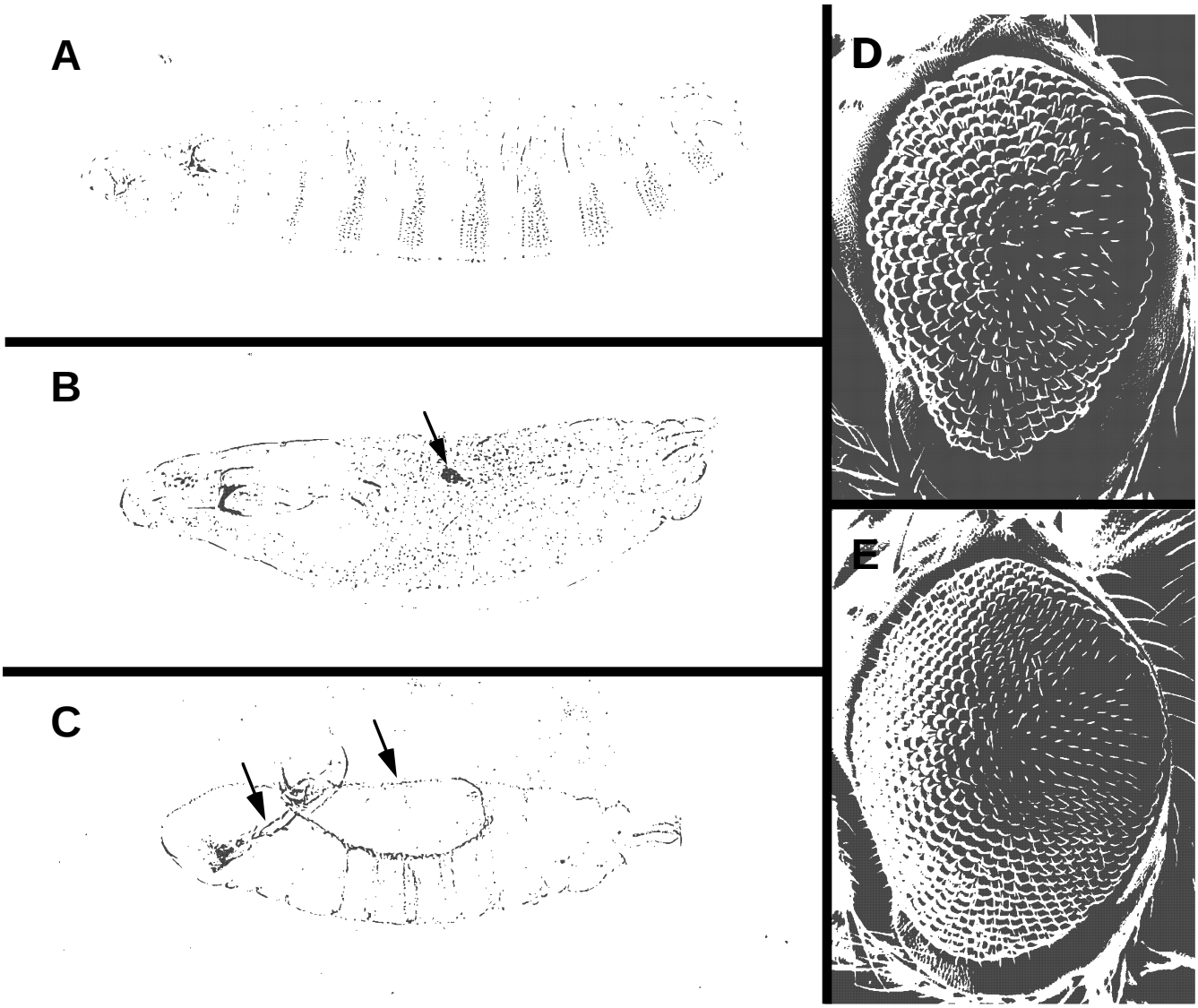


Fig. 7. The *coracle* mutations affect embryonic dorsal closure and interact with the *Ellipse* (*Egfr*^{Elp}) mutation. (A-C) Embryonic cuticle preparations from wild-type (A), *cor*^{1}/*cor*^{1} (B), and *cor*^{2}/*cor*^{2} embryos (C). Both alleles affect dorsal closure, as indicated by the dorsal cuticle defects (white arrows). In addition, *cor*^{2} mutant embryos display defects in head involution (black arrow). (D,E) Adult eyes from *Elp*/+ (D) and *Elp*/*b pr c cor*^{2} *px sp* (E) flies examined by SEM. The dominant *Elp* eye phenotype shown in D is suppressed when *Elp* is transheterozygous with the *cor*^{2} mutation (E), resulting in an almost normal ommatidial pattern. This suppression of *Elp* is 'rescued' to a typical *Elp* rough eye by the addition of the Ub-D4.1 transposon (data not shown).

ing cell growth in imaginal discs, and the dependence of this regulation on cellular interactions strongly suggest that septate junctions play a role in cellular interactions (Woods and Bryant, 1991). Second, recent results indicate that there are extensive regions of homology between dlg and ZO-1, the first identified component of the vertebrate tight junction (Stevenson et al., 1986), that extend over the entire length of the *dlg* protein (Willot et al., 1993). This structural similarity between components of the invertebrate septate junction and the vertebrate tight junction implies that these cellular junctions may be functionally similar, as has been suggested from ultrastructural analyses (Noirot-Timothée and Noirot, 1980), although further work will be required to determine the extent to which these proteins are functionally conserved. *dlg* mutations have also been reported to disrupt epithelial polarity (Woods and Bryant, 1991), indicating that the septate junction may also function in the maintenance of cellular polarity, as has been previously proposed for both septate (Wood, 1990) and tight junctions (van Meer and Simons, 1986).

The subcellular localization of D4.1 protein to septate junctions implies that it may be important in establishing and/or maintaining these cellular junctions. This is consistent with our observations on the tissue and temporal specificity of D4.1 expression in developing embryos. The correlation between D4.1 expression and the appearance of septate junctions during embryonic development is quite striking: septate junctions first appear at the end of germ band retraction (Eichenberger-Glinz, 1979), just after D4.1 protein is first expressed. Similarly, all tissues, both embryonic and imaginal, that we have shown to express D4.1 also have septate junctions

(Noirot-Timothée and Noirot, 1980; Tepass and Hartenstein, 1993). Specifically, while all of the ectodermally derived epithelia express D4.1 and have septate junctions of the pleated type (that is, display the characteristic cross septae), the midgut, Malpighian tubules and central nervous system express neither. Instead, cells of the midgut and Malpighian tubules display a morphologically different junction called the smooth septate (or continuous) junction (Noirot-Timothée and Noirot, 1980; Tepass and Hartenstein, 1993) which is not associated with D4.1. While these correlative data suggest that D4.1 may play a role in the formation or function of pleated septate junctions, further studies using null *coracle* mutations are necessary to elucidate the precise cellular functions of D4.1. It is also worth noting that D4.1 expression is strikingly different from that of *dlg*, which is expressed early in development well before the first septate junctions are morphologically detectable and in tissues that lack septate junctions altogether, such as the CNS (Woods and Bryant, 1991).

D4.1 function may differ from that of erythrocyte protein 4.1

As we have previously stated, most of our current understanding of protein 4.1 function is based on studies of the erythrocyte isoforms of protein 4.1, which differ due to alternative splicing from the predominant forms expressed in most other tissues. In particular, two alternatively spliced exons fall within the spectrin/actin binding domain, and while the predominant erythrocyte isoform contains only one of these exons (Motif I, Tang et al., 1988, 1990; 21 aa splice, Conboy et al., 1991b), other tissues predominately express isoforms that contain either both exons or neither exon. This observation raises the possibility that the erythrocyte isoform has a rather specialized function, as has previously been suggested (Tang et al., 1990). Thus, while the presumed function of erythrocyte protein 4.1 may be to mediate interactions between the transmembrane protein glycophorin and the cytoskeletal proteins actin and spectrin, this may not be true of isoforms expressed in other tissues.

One means of identifying functionally conserved domains is by comparing primary sequence between different species. We have shown that the greatest sequence conservation between the human and *Drosophila* 4.1 genes is in the N-terminal 350 aa of these proteins. Erythrocyte protein 4.1 has been shown to interact with glycophorin through this N-terminal domain (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985), the same region that is conserved in all members of the protein 4.1 gene family (Rees et al., 1990). Studies of these other family members indicate that all are associated with the cell membrane, strongly suggesting that this conserved N-terminal domain serves to localize these proteins to the membrane, perhaps by interactions with transmembrane proteins like glycophorin. In support of this notion, studies have shown that the N-terminal domain of ezrin, one member of the protein 4.1 gene family, is sufficient for membrane localization (Algrain et al., 1993). Our studies of D4.1 show that the N-terminal domains of the human, *Xenopus* and *Drosophila* 4.1 proteins are highly conserved (Fig. 1) and that D4.1 is also associated with the cell membrane (Figs 3-6). In addition, our preliminary results indicate that this localization is mediated by the N-terminal domain (R. Lamb and R. Fehon, unpublished observations). In contrast, we have not found significant homology between any sequences within D4.1 and the region of human 4.1 that is believed to mediate interactions between spectrin and actin. Furthermore, comparison of the subcellular localizations of D4.1 and filamentous actin in epithelial cells reveals that these proteins do not co-localize noticeably within the cell. Taken together, these results suggest that the *Drosophila* protein 4.1 homologue may not function to mediate spectrin/actin interactions as does erythrocyte protein 4.1. In particular, our data imply that D4.1 may be important for junctional structure, a role that is clearly not relevant to erythrocytes. Interestingly, studies of human 4.1 in non-erythroid cells have reported that it is also associated with the membrane in regions of cell-cell contacts (Leto et al., 1986; Shimizu et al., 1992), indicating that studies of D4.1 function will be important to understanding the functions of non-erythroid protein 4.1 in human tissues.

The coracle gene encodes D4.1 and is necessary for dorsal closure

Numerous previous studies of *Drosophila* development have shown that the genes that encode different components of a single complex developmental process often give rise to a common phenotype when mutated (St Johnston and Nüsslein-Volhard, 1992). We have observed that embryos homozygous for either cor^1 or cor^2 fail to hatch, apparently due to a failure in dorsal closure (Fig. 7). Two other mutations in *Drosophila*, *zipper* and *scab* also have been shown to affect dorsal closure, although their phenotypes are not identical (Nüsslein-Volhard et al., 1984; I. Dawson, unpublished observations). While *scab* has not yet been characterized molecularly, *zipper* is known to encode a nonmuscle myosin heavy chain that accumulates at the leading edge of the lateral epidermis as it encloses the yolk (Young et al., 1993). Given that *coracle*, *zipper* and *scab* all affect the same morphogenetic process, dorsal closure, it is tempting to speculate about possible cooperative interactions between D4.1 and the products of these genes. Indeed, previous studies of human protein 4.1 have shown that it can interact with muscle and nonmuscle myosin (Pasternack and Racusen, 1989; Racusen and Pasternack, 1990), though we do not yet have evidence of such interactions between their *Drosophila* homologues. Alternatively, these genes could function more or less independently on different components of this complex morphogenetic process.

Other functions of coracle/D4.1

The existing *coracle* mutations were originally identified for their ability to dominantly suppress the *Ellipse* (*Elp*) mutation. *Elp* is a dominantly hypermorphic allele of the *Drosophila* EGF-receptor homologue (*Egfr*), a member of the transmembrane receptor tyrosine-kinase gene family, that has been shown to be involved in signal transduction events necessary for cell fate decisions throughout *Drosophila* development (Schejter and Shilo, 1989). At the moment, we do not know whether this observed genetic interaction between *coracle* and *Egfr* represents a direct interaction between their products, a more general effect on cellular function or polarity, or an indirect interaction. However, previous studies have demonstrated that the mammalian EGF receptor tyrosine kinase and other receptor tyrosine kinases do phosphorylate protein 4.1 and other members of the 4.1 gene family, that the kinase/substrate interactions are specific, and that phosphory-

lation affects the functions of 4.1 family proteins (Subrahmanyam et al., 1991; Fazioli et al., 1993). In addition, a new member of the 4.1 gene family, merlin, has been shown to have a tumor-suppressor function in humans, possibly due to an involvement in receptor tyrosine kinase-mediated signal transduction (Trofatter et al., 1993; Rouleau et al., 1993; Kinzler and Vogelstein, 1993). Consistent with the notion that D4.1 could be phosphorylated by *Egfr* or another tryosine kinase, our sequence analysis has revealed three consensus tyrosine-kinase substrate domains within the D4.1 sequence, although we do not yet know if D4.1 is phosphorylated. In addition, D4.1 is abundantly expressed by all cells of the eye imaginal disc. Given these biochemical/structural data and the observed genetic interactions between *coracle* and *Egfr*, it will be of significant interest to examine further the relationship between D4.1, a cytoplasmic protein that is closely associated with the membrane, and developmentally important cellular interactions mediated by transmembrane receptor proteins such as the EGF receptor. The identification of *coracle* as the locus that encodes the D4.1 gene now provides us with tools to identify cellular and developmental functions of this evolutionarily conserved protein.

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