Members of the ALP/Enigma family of PDZ-LIM proteins play a role in cytoskeletal anchorage and mutations in at least one member of this family are associated with human cardiomyopathy. Here, we describe the analysis of the Caenorhabditis elegans alp-1 gene. alp-1 is predicted to encode the entire nematode ALP/Enigma protein family, consisting of one ALP-related protein with a single LIM domain and three Enigma-like proteins containing four LIM domains. We demonstrate that the ALP-1 proteins are expressed in muscle cells, where they localize to actin anchorage and muscle attachment sites. We show that the PDZ domain of the ALP-1 proteins is sufficient to target the protein to the dense bodies, which are important actin anchorage sites in C. elegans body wall muscle. We demonstrate that the C. elegans ALP/Enigma proteins are also localized to cell–cell junctions and to both epithelial and muscle cell nuclei. These findings suggest new roles for the ALP/Enigma protein family that may lead to the understanding of their involvement in cardiomyopathy. Developmental Dynamics 235:530–538, 2006. © 2005 Wiley-Liss, Inc.

Key words: muscle; cytoskeleton; Caenorhabditis elegans; PDZ domains; LIM domains

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INTRODUCTION

Sites of actin anchorage within muscle cells are critical for muscle function, playing a role in muscle cytoarchitecture, force transmission, and transcellular signaling (for review, see Clark et al., 2002). Actin anchorage sites in muscle act as docking sites for multiple protein complexes involved in both tethering the contractile machinery and coupling it to the muscle cell membrane (for review, see Vigoreaux, 1984).

The Z-discs that border the sarcomeric contractile units of striated muscle have historically provided a popular model for dissecting the structure and function of actin anchorage sites in muscle cells (Carlsen and Knappies, 1962; Knappies and Carlsen, 1962; Franzini-Armstrong and Porter, 1964). Although several proteins reside at Z-discs, the most extensively characterized of these is α-actinin (Ebashi and Ebashi, 1965; Maruyama and Ebashi, 1965). α-Actinin is an antiparallel homodimer that crosslinks actin filaments (Takahashi and Hattori, 1989) and provides a platform for the assembly of protein complexes at these sites of actin anchorage (for review, see Clark et al., 2002). Mutations affecting α-actinin function result in human dilated cardiomyopathy (Mohapatra et al., 2003).

The α-actinin–associated LIM protein (ALP) and Enigma (ALP/Enigma) protein family is a recently described group of related proteins, many of which bind α-actinin (Xia et al., 1997; Pomies et al., 1999; Pashmforoush et al., 2001). Mammalian ALP/Enigma proteins are characterized by the presence of an amino-terminal PDZ domain followed by either one (ALP subfamily) or three (Enigma subfamily) carboxy-terminal LIM domains and are postulated to play a role in actin anchorage in muscle as well as nonmuscle cells. Both PDZ (PSD95, Discs-large, ZO-1) domains (Way and Chalfie, 1988; Woods and Bryant, 1991; Willott et al., 1993) and LIM (LIN-11, Is1-1, MEC-3) domains (Way
and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990) are known protein-binding interfaces (Schmeichel and Beckerle, 1994; for review, see Ponting et al., 1997). The ALP subfamily consists of four known members: ALP, RIL, CLP36/Elfin, and Mystique (Xia et al., 1997; Bashirova et al., 1998; Kotaka et al., 2000; Vallenius et al., 2000; Kotaka et al., 2001). In this group, ALP is the most well-characterized and has been shown to colocalize with α-actinin and to enhance the ability of α-actinin to crosslink actin filaments (Pashmforoush et al., 2001). Mice that lack ALP function develop cardiomyopathy (Pashmforoush et al., 2001). The ALP subfamily members, RIL and CLP-36, are expressed in nonmuscle cells such as epithelia where their roles remain obscure (Bashirova et al., 1998; Valle- nius et al., 1990) are known proteins, one ALP isoform and three Enigma isoforms, could result from expression of alternatively spliced transcripts. We have confirmed the existence of four T11B7.4-derived transcripts shown in Figure 1C by Northern blot and 3’ rapid amplification of cDNA ends (RACE) analyses of mixed-stage wild-type animals (Fig. 1B). Consistent with this data, we have identified four classes of cDNAs by direct sequencing of all available T11B7.4 cDNAs (kindly provided by Yuji Kohara and Jonathon Hodgkin). The shortest transcript (referred to as alp-1a) is 1,278 bp and encodes the C. elegans ALP homologue (Fig. 1C,D). We have investigated the role of the ALP/Enigma family in C. elegans. By searching the completely sequenced C. elegans genome, we identified a single gene, T11B7.4, that is predicted to encode both ALP and Enigma homologues. Further sequence analysis indicated that the production of four highly related proteins, one ALP isoform and three Enigma isoforms, could result from expression of alternatively spliced transcripts. We have confirmed the existence of four T11B7.4-derived transcripts shown in Figure 1C by Northern blot and 3’ rapid amplification of cDNA ends (RACE) analyses of mixed-stage wild-type animals (Fig. 1B). Consistent with this data, we have identified four classes of cDNAs by direct sequencing of all available T11B7.4 cDNAs (kindly provided by Yuji Kohara and Jonathon Hodgkin). The shortest transcript (referred to as alp-1a) is 1,278 bp and encodes the C. elegans ALP homologue (Fig. 1C,D). The remaining three transcripts of 1,938 bp, 2,316 bp, and 4,275 bp (referred to as alp-1b, c, and d, respectively) encode three Enigma-like proteins (Fig. 1C,D). Because these multi-LIM domain-containing Enigma-like proteins are transcribed from the alp-1 gene, we have kept their designations consistent with the C. elegans nomenclature; the three Enigma proteins are referred to as ALP-1B, ALP-1C, and ALP-1D.

One feature that distinguishes the C. elegans Enigma isoforms (Fig. 1D) from the vertebrate Enigmas (Fig. 1A) is the presence of four, rather than three, LIM domains. Interestingly, phylogenetic analysis demonstrates that the N-terminal LIM domain (LIM1) of the C. elegans Enigmas is most closely related to the LIM domain in the vertebrate ALP proteins, whereas LIM2, LIM3 and LIM4 of the C. elegans proteins are related to LIM1, LIM2, and LIM3 of the vertebrate Enigmas (Fig. 1E). In Drosophila melanogaster, a single gene also encodes both ALP and Enigma isoforms and the predicted proteins are very similar to those in C. elegans (Fig. 1E). This analysis supports the view that the T11B7.4 gene products are the C. elegans ALP/Enigma homologues and reveals an intriguing link between ALP and Enigma family members.

### RESULTS

**C. elegans Has a Single ALP/Enigma Gene**

As described above, the ALP/Enigma family in vertebrates consists of at least seven members, including four ALP proteins and three Enigma proteins (Fig. 1A). We have investigated the ALP/Enigma protein family in C. elegans. By searching the completely sequenced C. elegans genome, we identified a single gene, T11B7.4, that is predicted to encode both ALP and Enigma homologues. Further sequence analysis indicated that the production of four highly related proteins, one ALP isoform and three Enigma isoforms, could result from expression of alternatively spliced transcripts. We have confirmed the existence of four T11B7.4-derived transcripts shown in Figure 1C by Northern blot and 3’ rapid amplification of cDNA ends (RACE) analyses of mixed-stage wild-type animals (Fig. 1B). Consistent with this data, we have identified four classes of cDNAs by direct sequencing of all available T11B7.4 cDNAs (kindly provided by Yuji Kohara and Jonathon Hodgkin). The shortest transcript (referred to as alp-1a) is 1,278 bp and encodes the C. elegans ALP homologue (Fig. 1C,D). The remaining three transcripts of 1,938 bp, 2,316 bp, and 4,275 bp (referred to as alp-1b, c, and d, respectively) encode three Enigma-like proteins (Fig. 1C,D). Because these multi-LIM domain-containing Enigma-like proteins are transcribed from the alp-1 gene, we have kept their designations consistent with the C. elegans nomenclature; the three Enigma proteins are referred to as ALP-1B, ALP-1C, and ALP-1D.

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**C. elegans ALP/Enigma Isoforms Are Differentially Expressed During Development**

To define the expression patterns and subcellular localizations of the ALP-1 proteins, we generated genomic translational green fluorescent protein (GFP) fusion constructs under control of the predicted native alp-1 promoter. We took advantage of the fact that the alp-1a transcript contains a unique 3’ terminal exon, which enabled the separation of ALP-1A/ALP from the ALP-1BCD/Enigmas. Using the pPD117.01 vector (kindly provided by A. Fire), DNA encoding GFP was fused in frame to exon 14 to tag alp-1a (pUU42 construct) (Fig. 2A). The alp-1a::GFP transgene contains all of the 3’ genomic DNA sequences downstream of GFP to minimize alterations of potential regulatory elements. The resulting protein product from this transgene will be referred to as ALP-1a::GFP. We used the same approach to generate an Enigma::GFP-expressing construct; however, in this case DNA encoding GFP was fused in frame to exon 18 (pUU10 construct; Fig. 2B). Because the three Enigma isoforms share identical 3’ sequences, the Enigma::GFP transgene expresses GFP-tagged versions of ALP-1B, ALP-1C, and ALP-1D, which will be referred to as ALP-1BCD/Enigma::GFPs. We generated these translational fusions from genomic DNA such that each would express all four ALP-1 proteins, but only the ALP-1A would be GFP-tagged in pUU42 and ALP-1B, ALP-1C, and ALP-1D would be tagged with GFP in pUU10. We have examined the expression of these transgenes in a wild-type background to define the expression patterns and subcellular dis-
Fig. 1. Molecular characterization of alp-1. A: The ALP/Enigma protein family in vertebrates. For comparison, the vertebrate protein sequences are from *Mus musculus*. Numbers represent the amino acid length of each protein. Accession numbers: (sm)ALP (CAB53970), RIL (AAH30068), CLP-36 (O070400), Mystique (AAH24556), Enigma (AAH45538), ENH (NP_062782), and Cypher(1s) (AAO26189). B: alp-1 encodes 4 transcripts of 1.3 kb, 1.9 kb, 2.3 kb, and 4.3 kb as demonstrated by both 3' rapid amplification of cDNA ends (RACE; left) and Northern blot analysis (right). It should be noted that alp-1a transcripts with varying poly-A tail lengths were recovered using this method; these could account for the lower molecular weight bands that are unlabeled in the figure. C: alp-1 maps to LGIV near the *unc-22* gene cluster. Cosmid C02E5 contains the entire ALP/Enigma coding sequence plus approximately 4-kb flanking on each side of the first start and final stop codons. The alp-1 locus is highly complex, with two start sites, two different stop codons, and four transcripts resulting from alternate splicing. Exon colors correspond to their translated protein domains in (D). D: The *Caenorhabditis elegans* alp-1 gene encodes one ALP and three Enigma isoforms. Numbers represent the amino acid length of each protein. Accession numbers: ALP-1A (CAE52906), ALP-1B (CAE52905), ALP-1C (CAE52906), ALP-1D (NP_501533). E: Note that the ALP-1 Enigmas have four LIM domains and ALP-1C does not have the PDZ domain. C. elegans ALP/Enigma LIM domains are related to their vertebrate counterparts. Phylogeny was performed using ClustalW analysis with PAM250 residue weight. Colored lines in E correspond to colored LIM domains in A and D.
The GFP-tagged ALP and Enigma proteins display both overlapping and distinct patterns of expression and localization during embryogenesis. The ALP-1A::GFP is expressed early in muscle development where it first appears during the comma-stage in the characteristic pair of bands that line the lateral surfaces of the embryo, demarcating the nascent body wall muscle cells (Fig. 2C, arrows). This muscle-specific localization of ALP-1A::GFP first appears as the muscle cytoskeleton is being established and continues within developing musculature throughout embryogenesis where it is detected both cytoplasmically and in the body wall muscle nuclei (Fig. 2D, arrows). Throughout adulthood ALP-1A is most prevalent in the body wall muscle. In addition to the muscle expression, ALP-1A::GFP is expressed in the embryonic hypodermis. Just before embryonic elongation, ALP-1A::GFP is localized to periodic circumferential bundles in the hypodermal cell nuclei (a slightly twofold embryo is shown in Fig. 2G). Our sequence analysis has revealed a putative nuclear localization signal in ALP-1D (PKVKKEP, amino acids 497-505) (Kalderon et al., 1984), which could be responsible for this pattern of localization. At this stage, we also observe the proteins beginning to localize to circumferential bundles (Fig. 2H, arrowheads), which are required for an actin-myosin–mediated contractile process that drives morphogenesis of the embryo (Priess and Hirsh, 1986). ALP-1A::GFP localization at these circumferential actin bundles persists throughout morphogenesis during which the fusion protein also localizes to cell junctions in the hypodermal seam cells (Fig. 2E, arrows). Later in development, we detect ALP-1A::GFP expression at the cell–cell junctions of the hypodermal seam cells during larval stages as well (Fig. 2F, arrows).

The ALP-1BCD/Enigma proteins are first detected during embryogenesis at the comma-stage in the cell–cell junctions of the embryonic hypodermis and in the hypodermal cell nuclei (a slightly twofold embryo is shown in Fig. 2G). Our sequence analysis has revealed a putative nuclear localization signal in ALP-1D (PKVKKEP, amino acids 497-505) (Kalderon et al., 1984), which could be responsible for this pattern of localization. At this stage, we also observe the proteins beginning to localize to circumferential bundles, similar to ALP-1A. At a later stage (fourfold), these circumferential bundles are more apparent (Fig. 2H, arrowheads),...
as is the late appearance of the ALP-1BCD/Enigma::GFPs in the developing body wall muscle (Fig. 2H, arrow). In a deeper optical section of the same embryo as in 2G, it is evident that the ALP-1BCD/Enigma::GFP fusion proteins localize to cell–cell junctions in the developing pharynx (Fig. 2F, arrow). At this stage, the pharynx is still developing, and the arcade cells that are required to attach the anterior part of the pharynx to the mouth have not yet become epithelialized (Portereiko and Mango, 2001). Once the pharynx begins to differentiate, we see an enrichment of the ALP-1BCD/Enigma::GFPs in the pharyngeal muscles and in specialized pharyngeal epithelial cells called marginal cells (Fig. 2J). This pharyngeal expression persists throughout development and is most prevalent in adults. Although both ALP-1A and ALP-1BCD/Enigma are localized in body wall muscle and pharynx, the expression patterns are somewhat complementary, in that ALP-1A is enriched in the body wall muscle while ALP-1BCD/Enigma is enriched in the pharynx. These inverse expression levels are retained throughout adulthood.

Both ALP-1::GFP constructs are expressed in the adult pharynx in wild-type animals. By extensive analysis of confocal microscopic optical sections, we determined that ALP-1A::GFP and the ALP-1BCD/Enigma::GFPs are expressed in both pharyngeal muscle and marginal cells, but are not detected in neurons (Fig. 2K,L). In muscle cells, ALP and Enigma proteins are localized to both the apical and basal surfaces of the cell. ALP and Enigma levels decline abruptly at the distal region of the pharyngeal–intestinal valve, and the signal does not extend into the intestinal epithelium.

**alp-1 Gene Products Localize to Sites of Actin Anchorage in Body Wall Muscle**

In adult wild-type animals, both ALP-1A/ALP (Fig. 3A) and the ALP-1BCD/Enigma (Fig. 3B) are present in body wall muscles. ALP-1A::GFP is detected in cell nuclei (Fig. 3A, arrowheads) and both proteins are present in a collection of puncta that correspond to actin tethering sites. Within *C. elegans* muscle, actin filaments are anchored at two distinct structures: the dense body and the dense plaque. The dense body is functionally analogous to the vertebrate Z-disc, demarcating the lateral boundaries of the sarcomere, and is marked by the presence of α-actinin (Francis and Waterston, 1985; Barstead et al., 1991). Each of the 95 *C. elegans* body wall muscles contains in excess of 300 dense bodies arranged in an obliquely striated pattern (Francis and Waterston, 1985). Dense plaques are sites of muscle–muscle adhesion at the muscle termini and lack α-actinin (Francis and Waterston, 1985, 1991). Both ALP (Fig. 2A) and Enigma (Fig. 2B) fusion proteins were detected where concentrations of α-actinin were observed (Fig. 3C–H), indicating that the ALP/Enigma proteins localize to the dense bodies of body wall muscle. Of interest, a subset of the Enigma puncta existed where no α-actinin was detected (Fig. 3F,H, arrowheads), illustrating a difference in the subcellular distributions of ALP and Enigma isoforms in muscle and suggesting that at least one of the three Enigma proteins might also be present at dense plaques. Confirmation of the differential localization of ALP and Enigma isoforms at sites of muscle–muscle adhesion was obtained by staining the transgenic animals with an antibody to vinculin that, in addition to its localization to the base of the dense bodies, is present at dense plaques (Barstead and Waterston, 1991). Although ALP-1A::GFP is excluded from the dense plaques (Fig. 3L–K, arrowhead), the ALP-1BCD/Enigma::GFPs colocalize with vinculin at the dense plaques (Fig. 3L–N, arrowhead).

**ALP-1 PDZ Domain Is Sufficient for Localization to Body Wall Muscle Dense Bodies**

The vertebrate ALP/Enigma PDZ domain associates directly with α-actinin, suggesting a possible mechanism by which ALP/Enigma proteins might be localized to particular subcellular locations (Xia et al., 1997; Faulkner et al., 1999; Zhou et al., 1999; Bauer et al., 2000; Kotaka et al., 2000; Nakagawa et al., 2000; Vallenius et al., 2000; Huang et al., 2003; Au et al., 2004). We have directly evaluated the ability of the ALP-1 PDZ domain to target to actin anchorage sites in *C. elegans*. We generated a PDZ domain-encoding construct under the control of the heat-shock promoter hsp-16.2 (hs-PDZ). In addition, we fused DNA encoding three myc tags in frame to follow the hs-PDZ in animals. Upon heat-shock, the 31-kDa hs-PDZ::MYC protein is stably expressed for several hours (Fig. 4A).

Analysis of the hs-PDZ animals indicates that the PDZ domain alone is sufficient for targeting to adult body wall muscle dense bodies where it colocalizes with α-actinin (Fig. 4B–D). The hs-PDZ domain does not localize to body wall muscle dense plaques that lack α-actinin (Fig. 4E–G). Surprisingly, the PDZ domain is not sufficient for any pharyngeal localization, despite that the promoter is expressed in those tissues (data not shown).

**DISCUSSION**

We have determined that the entire ALP/Enigma protein family is encoded by a single gene in *C. elegans*. Analysis of the structure and function of the *C. elegans* ALP-1 proteins may further our understanding of the roles of ALP and Enigma in vertebrates where they play central roles in striated muscle (Pashmforoush et al., 2001; Zhou et al., 2001; Vatta et al., 2003).

**Predicted and Unexpected Roles for the *C. elegans* ALP/Enigma Proteins**

Our analysis of the ALP-1 proteins in *C. elegans* has revealed both predicted and unexpected patterns of localization. We have demonstrated that the *C. elegans* ALP/Enigma proteins, like their vertebrate counterparts, localize to sites of actin anchorage in muscle. Both ALP-1A::GFP and ALP-1BCD/Enigma::GFP are also found in nuclei, with the ALP-1BCD/Enimas localizing to epithelial nuclei during embryogenesis and the ALP-1A protein localizing to body wall muscle nuclei throughout development. In future work, it will be of interest to probe the physiologic importance of the nuclear accumulation of ALP/Enigma. With respect to the cytoplasmic pools of ALP/Enigma, both ALP-1A and
Fig. 3. *alp-1* gene products are expressed sites of cell–cell and cell–matrix attachments in body wall muscle. ALP-1A/ALP::GFP transgenic adults (A,C,D,E,I,J,K) and ALP-1BCD/Enigma::GFP transgenic adults (B,F,G,H,L,M,N). A: ALP-1A/ALP::GFP is expressed in body wall muscle (arrows indicate muscle cell nuclei). B: ALP-1BCD/Enigma::GFPs are expressed in body wall muscle (arrow points to dense plaque between neighboring muscle cells). C: ALP-GFP is localized to periodic punctate structures in adult body wall muscle. D: ALP-GFP transgenic animal stained with an antibody against alpha-actinin. E: Merged image of C and D. ALP-GFP (green) and alpha-actinin (red) colocalize at dense bodies. F: Enigma-GFP is localized to periodic punctate structures in adult body wall muscle. G: Enigma-GFP transgenic animal stained with an antibody against alpha-actinin. H: Merged image of F and G. Enigma-GFP (green) and alpha-actinin (red) colocalize at dense bodies. (Arrow indicates a dense plaque where Enigma-GFP is present, but alpha-actinin is absent.) I: ALP-GFP transgenic animal stained with an antibody against alpha-actinin. J: ALP-GFP transgenic animal stained with an antibody against vinculin. K: Merged image of I and J. ALP is excluded from the vinculin-rich dense plaque (arrow). L: Enigma-GFP transgenic animal stained with an antibody against GFP. M: Enigma-GFP transgenic animal stained with an antibody against vinculin. N: Merged image of L and M. Enigma-GFP and vinculin colocalize at the dense plaque (arrow). GFP, green fluorescent protein. Scale bars = 50 μm.

Fig. 4. The ALP-1 PDZ domain is sufficient for localization to dense bodies but not dense plaques. A: Western blot of whole *Caenorhabditis elegans* lysates probed with anti-MYC and anti-actin antibodies. Lane 1: wild-type animals, lane 2: pdz::myc transgenic worms at the noninducing temperature of 22°C, lane 3: pdz::myc transgenic worms after heat-shock. B: Heat-shock-PDZ-myc–expressing adult animal stained for the myc epitope. C: Same animal as in B stained for alpha-actinin. D: Merged image of PDZ-MYC (green) and alpha-actinin (red) shows that the myc-tagged PDZ domain is colocalized with alpha-actinin in the dense bodies of the adult body wall muscle. E: Heat-shock-PDZ-myc–expressing adult animal stained for the myc epitope. F: Same animal as in E stained for vinculin. G: Merged image of PDZ-MYC (green) and vinculin (red) shows that the myc-tagged PDZ domain is colocalized with vinculin in the dense bodies but is excluded from the dense plaques of the adult body wall muscle. Scale bars = 5 μm.
ALP-1BCD constructs are enriched at the dense bodies of body wall muscle where they colocalize with α-actinin and vinculin. In addition, the ALP-1BCD/Enigmas localize to distinct muscle–muscle adhesion structures, the vinculin-rich dense plaques. Whereas the PDZ domain alone is sufficient for localization to dense bodies, it is not located at these dense plaques. This finding suggests that either dense plaque localization of ALP-1B and ALP-1D is not dependent on the PDZ domain but on some other region of the proteins, or that the PDZ-deficient ALP-1C protein the sole Enigma variant concentrated at dense plaques.

Both ALP-1A::GFP and ALP-1BCD/Enigma::GFP are expressed in the pharynx where they are concentrated at apical and basal actin anchorage sites. This finding is in contrast to the hypodermal epithelium, where the ALP-1 proteins localize only to apical adhesion structures and are excluded from basal sites (data not shown). Despite the predicted interaction of the PDZ domain with α-actinin, the PDZ domain alone is not sufficient for pharyngeal localization of the ALP-1 proteins (data not shown). This finding suggests that the ALP-1 proteins have an α-actinin–independent function in the pharynx, that a different domain is responsible for pharyngeal localization, or that the PDZ domain can only act in context with the full-length protein in the pharyngeal muscle and pharyngeal epithelial cells.

**Evolutionary Conservation of ALP/Enigma Proteins**

Overall, ALP/Enigma proteins display conserved structural features and functional properties from invertebrates to humans. However, one notable difference in the invertebrate Enigma proteins is the number of C-terminal LIM domains. Whereas the vertebrate proteins contain three C-terminal LIM domains, both *C. elegans* and *Drosophila melanogaster* Enigmas exhibit four LIM domains. The functional consequences of this diversity in LIM number are not understood, but it might be anticipated that invertebrate Enigmas will also interact with proteins that associate with the ALP LIM domain, because the additional LIM domain is most similar to LIM domains in ALP subfamily members. An additional difference in the *C. elegans* Enigma subfamily is the presence of a non-PDZ domain-containing isoform, the ALP-1C protein. In vertebrates, it has been postulated that the PDZ domain is required for the subcellular localization of the protein by means of an interaction with α-actinin (Pomies et al., 1999). Because ALP-1C does not contain a PDZ domain, this isoform may have an α-actinin–independent function. The structure of ALP-1C suggests that it could serve as an endogenous competitor that sequesters protein partners of the Enigma LIM domains and, thus, modulates the activity of other family members. Alternatively, the ALP-1C protein may be required during a specific stage of development, similar to LET-413 where the PDZ domain is dispensable during embryogenesis (Legouis et al., 2003).

In both *C. elegans* and *Drosophila*, ALP and Enigma proteins are encoded by a single locus and protein variants are generated by means of alternative splicing. In contrast, in mammals, individual genes encode each member of the ALP/Enigma family. Of interest, in the human genome each ALP-like protein is paired with an Enigma-like protein in the nearby chromosomal neighborhood. On the human genetic map, ALP is located at 4q35 with ENH nearby at 4q22, CLP-36 is located at 10q22-26.3 with Cypher/ZASP at 10q22.3-23.4, and RIL is paired with Enigma at 5q31.1 and 5q35.3, respectively. This consistent pairing of an ALP with an Enigma in vertebrates lends support to the idea that, in the invertebrates *C. elegans* and *D. melanogaster*, the ALP/Enigma gene provides an “evolutionary snapshot” of the ancestral gene before duplication and mobilization events that expanded the complexity of the gene family.

**Conclusions**

We have demonstrated that *alp-1* encodes the entire ALP/Enigma protein family in *C. elegans*. The ALP-1 proteins localize to specific sites of cell adhesion including the body wall muscle dense bodies and dense plaques and epithelial cell junctions. We have shown that the PDZ domain is sufficient for subcellular localization to only a subset of these structures. The *C. elegans* ALP/Enigma proteins are also localized to cell–cell junctions and to both epithelial and muscle cell nuclei. These findings suggest new roles for the ALP/Enigma protein family that may lead to the understanding of their roles in human disease.

**EXPERIMENTAL PROCEDURES**

**Strains and Genetic Manipulations**

*C. elegans* strains were maintained according to standard methods (Brenner, 1974). For experimental procedures, all animals were grown at 22°C unless otherwise noted. Bristol N2 was used as wild-type and the following additional strains were used in this study: UU10 [N2;ex.pU10], UU12 [N2;ex.pU14], UU15 [(pqIs1), UU16 [(pqIs2)], and UU18 [(pqIs4)]. Strains UU10 and UU12 were generated by germline injection of the pUU10 and pU42 plasmids (see below) and maintained as extrachromosomal arrays. Strains UU15, UU16, and UU18 were generated by integrating pUU47, pUU42, and pU10, respectively, with γ-irradiation using standard protocols (Mello and Fire, 1995), and out-crossed a minimum of four times before analysis.

**cDNAs and Mutant Sequencing**

In addition to sequencing all of the available corresponding cDNAs to the T11B7.4 gene (kindly provided by Y. Kohara and J. Hodgkin), we isolated additional cDNAs using the 5′/3′ RACE procedure (Frohman, 1993), with SMART RACE (Clontech). RACE products were both directly sequenced and shotgun cloned into pCR2.1 (Invitrogen). Alternative splicing was determined from the cDNA and RACE product sequences, intron–exon boundaries were assigned by comparing the compiled cDNA sequences with genomic. We have informed the Wormbase administrators of this information and the database now reflects our analysis [http://www.wormbase.org].
Northern Blot Analysis

Total RNA was isolated from a mixed stage population of animals by extraction with phenol/guanidium thiocyanate, and subsequently purified using Qiagen RNeasy. Northern blot hybridization was performed as described previously (McKeown et al., 1998). RNA probe used is homologous to the region corresponding to exons 6, 7, and 8 in Figure 1C.

Tagged Constructs and Transgenetic Lines

For the GFP constructs, genomic DNA was amplified from the C02ES cosmid using the Expand Long Template PCR System from Boehringer Mannheim with Buffer 2. Primer sequences are available on request. Products were cloned into a TA vector intermediate (pCR2.1 Invitrogen) and then into the pPD117.01 GFP vector (kindly provided by A. Fire). Because of the large size of the inserts, constructs were verified by restriction mapping and sequencing of the inserts, constructs were verified by restriction mapping and sequencing of the cloning junctions. For the inducible PDZ construct, pUU47, the PDZ domain-encoding region was amplified by PCR from genomic DNA and cloned into the pPD49.78 vector (kindly provided by A. Fire) that contains the hsp-16.2 heat-inducible promoter. In addition, the pPD49.78 vector (kindly provided by A. Fire) that contains the hsp-16.2 heat-inducible promoter. In addition, the sequence encoding three tandem MYC (1:500; clone 9E10, University of Utah Antibody Core Facility), and anti-Mouse-HRP was used for secondary detection and visualized by ECL.

Western Blot Analysis

Worm lysates were made by collecting adult animals directly into 2× Sample Buffer and boiling for 10 min. Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the methods of Laemmli (1970), with the modification that 0.13% bis-acrylamide was used. Ten percent gels were used to analyze the PDZ:myc protein. Western blot analysis was performed as described previously (Beckerle, 1986). Primary antisera were used as follows: anti-actin (1:10,000; C4, ICN Biomedicals), anti-MYC (1:10,000; clone 9E10, University of Utah Antibody Core Facility), and anti-Mouse-HRP was used for secondary detection and visualized by ECL.

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REFERENCES

Freyd G, Kim SK, Horvitz HR. 1990. Novel cysteine-rich motif and homeodomain in...


