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The \textit{Caenorhabditis elegans} APC-related gene \textit{apr-1} is required for epithelial cell migration and \textit{Hox} gene expression

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Inactivation of the \textit{Caenorhabditis elegans} APC-related gene (\textit{apr-1}) has pointed at two separate functions of \textit{apr-1}. First, \textit{apr-1} is required for the migration of epithelial cells during morphogenesis of the embryo. In this process, \textit{APR-1} may act in a Cadherin/\textit{\alpha}-Catenin/\textit{\beta}-Catenin complex as a component of adherens junctions. Second, \textit{apr-1} is required for \textit{Hox} gene expression, most likely by positively regulating the activity of the Wingless signaling pathway. During embryogenesis, \textit{apr-1} is required for the expression of \textit{ceh-13 labial} in anterior seam and muscle cells and during larval development, \textit{apr-1} is necessary for the expression of \textit{lin-39 deformed} in the vulval precursor cells. Thus, \textit{APR-1} may positively regulate the activity of the \textit{\beta}-Catenin/Armadillo-related proteins \textit{HMP-2} in migrating epithelial cells and \textit{BAR-1} in the vulval precursor cells.

[Key Words: \textit{Caenorhabditis elegans}; cell migration; vulva; APC; Hox gene; Wingless]

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The human APC tumor suppressor gene is mutated in most cases of inherited colorectal cancer [for review, see Kinzler and Vogelstein 1996; Polakis 1997]. Humans carrying a germ-line mutation in one APC allele develop hundreds of colonic polyps at a young age, in which the second, wild-type APC allele has been lost spontaneously. Some of these polyps eventually accumulate additional mutations and progress to carcinomas. Spontaneous mutations in both APC alleles have also been observed in the majority of sporadic colon carcinomas. Biochemical studies have identified a number of APC-binding partners and suggested possible functions for APC. In particular, binding of APC to \textit{\beta}-Catenin [Rubinfeld et al. 1993] is thought to regulate the activity of \textit{\beta}-Catenin in the evolutionary conserved Wingless [Wnt] signal transduction pathway [for review, see Polakis 1997; Wodarz and Nusse 1998; Peifer 1999]. In both familial and sporadic forms of colorectal cancer, the vast majority of APC mutations that have been characterized result in carboxy-terminal truncations of the APC protein and eliminate part of the \textit{\beta}-Catenin-binding site [Polakis 1995]. Colon cancer cells that express truncated APC often exhibit elevated levels of cytoplasmic \textit{\beta}-Catenin and an increased activity of the Wnt signaling pathway, suggesting that wild-type APC may negatively regulate \textit{\beta}-Catenin function [Munemitsu et al. 1995; Korinek et al. 1997; Morin et al. 1997]. Supporting this model, mutations in the \textit{Drosophila} APC homologs dAPC1 and dAPC2 (or E-APC) result in the constitutive activation of the \textit{\beta}-catenin homolog \textit{armadillo} in different tissues [Ahmed et al. 1998; McCartney et al. 1999; Yu et al. 1999]. However, studies of \textit{Xenopus laevis} axis induction and early \textit{Caenorhabditis elegans} embryonic development have suggested an activating rather than an inhibitory function for APC in the Wnt signaling pathway [Rocheleau et al. 1997; Vlemingckx et al. 1997]. In addition to its role in Wnt signaling, it has been proposed that vertebrate APC may control the migration and adhesion of epithelial cells [Nathke et al. 1996; Wong et al. 1996; Pollack et al. 1997], cell cycle progression [Baeg et al. 1995], and cell death [Morin et al. 1996].

During \textit{C. elegans} development, activation of the Wnt signaling pathway induces the expression of \textit{Hox} genes in different cell types [Eisenmann et al. 1998; Jiang and Sternberg 1998; Maloof et al. 1999]. The \textit{C. elegans} \textit{Hox} genes are organized in an evolutionarily conserved gene cluster consisting of the four genes \textit{ceh-13, lin-39, mab-5}, and \textit{egl-5} that are the homologs of the \textit{Drosophila} \textit{Hox} genes \textit{labial, deformed, antennapedia}, and \textit{abdominal-B}, respectively [for review, see McGinnis and Krumlauf 1992; Lawrence and Morata 1994; Salser and Kenyon 1994]. They are expressed in distinct domains along the anterior-posterior (A/P) axis and specify cell fates in their expression domains by controlling diverse processes such as cell division, polarity, migration, adhesion, fusion, and death. The \textit{\beta}-catenin/armadillo-related
gene bar-1 is an essential regulator of the Wnt signaling pathway, leading to the induction of Hox gene expression during postembryonic development (Eisenmann et al. 1998; Maloof et al. 1999). In particular, bar-1 is required for the transduction of a Wnt signal that specifies the vulval equivalence group in the six hypodermal Pn.p cells P3.p through P8.p (P3–8.p). In response to this Wnt signal, P3–8.p express the Hox gene lin-39 and become vulval precursor cells (VPCs). The anterior Pn.p cells (P1.p and P2.p) and posterior Pn.p cells (P9.p–P11.p) that may not receive a Wnt signal do not express lin-39 and adopt a fusiform (F) cell fate; they dissolve their adherens junctions and fuse with the surrounding hypodermal syncytium hyp7 (Sulston and Horvitz 1977). In animals carrying a loss-of-function mutation in bar-1, P3–8.p frequently fail to express lin-39 and adopt an F fate instead of a VPC fate (Eisenmann et al. 1998).

The hmp-2 gene encodes another C. elegans β-Catenin homolog that is required for epithelial cell migration and elongation during morphogenesis of the embryo (Costa et al. 1998). HMP-2 does not appear to act in a Wnt signaling pathway, but rather, it functions as a component of the Cadherin/β-Catenin/β-Catenin complex at the adherens junctions of epithelial cells in the epidermis (which is called hypodermis in C. elegans).

We have taken a genetic approach to study the role of the C. elegans APC-related gene apr-1 (Rocheleau et al. 1997) during embryogenesis and vulval development. Unlike the truncations found in human (Polakis 1995), mouse (Su et al. 1992), and Drosophila APC (Ahmed et al. 1998), the apr-1 mutation described in this work is predicted to disrupt the synthesis of the entire APR-1 protein. Our analysis of the apr-1 loss-of-function phenotype points at two separate functions of apr-1. First, apr-1 is required for the migration and elongation of hypodermal cells that enclose the embryo during morphogenesis. In this context, APR-1 appears to function as a component of the hypodermal adherens junctions together with HMR-1 Cadherin, HMP-1 α-Catenin, and HMP-2 β-Catenin. Second, apr-1 controls the expression of Hox genes during embryogenesis and vulval development, most likely by positively regulating the activity of the Wnt signaling pathway. In both processes, APR-1 may be necessary for the activity of β-Catenin/Armadillo-related proteins.

Results

Isolation of an apr-1 loss-of-function mutation

A database search of the complete C. elegans genome sequence revealed the presence of a single APC homolog that had been previously named apr-1 (Rocheleau et al. 1997). APR-1 and human APC1 both contain seven Armadillo repeats in their amino-terminal domains that are 31% identical and a conserved PDZ-binding motif at their carboxyl termini. The putative β-Catenin/Armadillo-binding site in the central region of APR-1 shows weaker sequence similarity when compared with human APC1.

To isolate a loss-of-function mutation in apr-1, a library consisting of ~5 × 10⁵ mutagenized animals was screened for deletions in the apr-1 genomic region using a nested PCR assay (see Materials and Methods). A single allele, apr-1(zh10), was isolated from this screen and used for the subsequent experiments. apr-1(zh10) animals contain a deletion of 1414 bp that removes the first, second, and most of the third exon (Fig. 1A). The apr-1(zh10) deletion is likely to completely eliminate gene function, as it removes the ATG translational initiation codon as well as the part of the 5′ promoter region that contains a likely transcriptional start site with a TATA box (Bensimhon et al. 1983).

The following observations indicate that loss of apr-1 function causes a zygotic lethal phenotype. The progeny of apr-1(zh10)/+ heterozygous parents contained no viable apr-1(zh10) homozygous animals as determined by PCR analysis [n = 48] or by scoring the segregation of a linked unc-29(e1072) mutation (see Materials and Methods). The lethality associated with apr-1(zh10) could be rescued by germ-line transformation with a plasmid containing a 7.3-kb genomic fragment of the apr-1 locus (Fig. 1A). Furthermore, apr-1(zh10) homozygous animals only survived to adulthood if they carried an extrachromosomal array with the rescuing apr-1 transgene.

Figure 1. Genomic structure of the apr-1 locus. (A) A 7.3-kb fragment [apr-1wt] containing all the sequences necessary to rescue the apr-1 loss-of-function phenotype is shown. Exons are indicated by thick lines and are numbered. The positions of the ATG initiation and TAA stop codons are shown. Exon-intron boundaries were confirmed by RT–PCR analysis [data not shown]. The extent of the 1414-bp deletion in apr-1(zh10) animals is indicated by the line underneath the genomic structure. The arrowheads indicate the position of the primers O95 and O101 that were used for the PCR analysis shown below. (B) Single-animal PCR analysis of a wild-type larva (+/+), a heterozygous apr-1(zh10)/unc-29(e1072)/lin-10(e1439) larva [zh10/+], an apr-1(zh10) unc-29(e1072)-arrested embryo [zh10(zh10) embryo] and an apr-1(zh10) unc-29(e1072)-arrested L1 larva [zh10/zh10 larva]. (M) Molecular weight standards, the numbers to the left of this lane indicate the length of relevant markers in kilobase pairs [kb]. The arrows at right indicate the 2.9-kb product from the wild-type and the 1.5-kb product from the zh10 allele.
To determine at what stage of development apr-1(zh10) mutants die, we monitored the development of a known number of eggs laid by apr-1(zh10)/+ heterozygous parents. A total of 77 ± 4% of the eggs hatched and developed into viable animals of the genotype apr-1(zh10)/+ or +/+, 15 ± 3% arrested during embryogenesis and 8 ± 3% arrested shortly after hatching during the first larval (L1) stage (n = 450). All arrested embryos and L1 larvae were homozygous for apr-1(zh10) as determined by PCR analysis (n = 28, Fig. 1B). Thus, approximately two-thirds of homozygous apr-1(zh10) mutants arrest during embryogenesis and the remaining third during the L1 stage. To test whether apr-1(zh10) L1 larvae may have escaped from the embryonic lethality because of rescue by maternal apr-1 activity, we examined apr-1(zh10) germ-line mosaic animals [see Material and Methods]. However, because loss of apr-1 function in the germ line causes a sterile phenotype, we were unable to examine the contribution of maternal apr-1 activity to embryogenesis.

Loss of apr-1 function causes multiple morphogenesis defects in the embryo

During wild-type morphogenesis, contralateral pairs of hypodermal cells that are born on the dorsal side migrate ventrally along the left and right sides of the embryo until they reach the ventral midline and the hypodermis fully encloses the embryo, a process called ventral closure [Pries and Hirsh 1986; Williams Masson et al. 1997; Costa et al. 1998]. At the same time, dorsal hypodermal cells intercalate and then fuse to each other, thereby forming the dorsal syncytium [Podbilewicz and White 1994; Williams Masson et al. 1998]. After the cell migrations and fusions are completed, the hypodermis is organized in two rows of ventral and lateral epithelial cells on each side of the embryo plus a dorsal syncytium. Finally, the cells begin to elongate along the A/P axis until the length of the body has increased about fourfold.

apr-1(zh10) embryos that arrested before undergoing elongation ruptured on the ventral side, suggesting that apr-1 may be required for ventral closure [Fig. 2A,B]. To study the function of apr-1 in hypodermal cells, apr-1(zh10) embryos were fixed at various stages and stained with the mAb MH27 that recognizes the adherens junction marker JAM-1 [Francis and Waterston 1985; M. Köpken and J.D. Hardin, pers. comm.]. Alternatively, embryos carrying a jam-1::GFP reporter on the chromosomesomal integrated array jcIs1 were observed by four-dimensional time-lapse microscopy [Fig. 3; Mohler et al. 1998]. Four kinds of defects could be observed in apr-1(zh10) embryos.

First, the migration of hypodermal cells was disrupted. Of 22 apr-1(zh10) embryos observed by four-dimensional microscopy, 18 arrested before undergoing elongation. In 13 of these 18 embryos, hypodermal cells initiated ventral migration, but they did not reach the ventral midline and failed to enclose the embryo [Fig. 3A,B]. In the other five arrested embryos, the two pairs of anterior leading cells did reach the ventral midline and ventral closure was initiated, but the embryos ruptured on the anterior or posterior ventral side at about the 1.25-fold stage.

Second, in 7 of 10 apr-1(zh10) embryos, the dorsal pairs of hypodermal cells failed to intercalate [Fig. 3C,D]. If the remaining 8 embryos, dorsal intercalation defects also failed to undergo ventral closure.

Third, hypodermal cells did not elongate along the A/P axis. At a time point when wild-type embryos had
embryo (Fig. 2C; Sulston et al. 1983). In 81% of apr-1(zh10) embryos, the two anterior seam cells H2 and V1 were mislocalized \( n = 47 \). V1 was often shifted dorsally or ventrally (arrowhead in Fig. 2D) and H2 moved posterior to make direct contact with V2.

Similar ventral closure and elongation defects have been observed in hmr-1 mutant embryos and in hmp-1 or hmp-2 mutants that lack zygotic and maternal gene activity (Costa et al. 1998). On the other hand, hmr-1, hmp-1, or hmp-2 mutants do not exhibit dorsal intercalation defects or the misalignment of the anterior seam cells H2 and V1 that was observed in apr-1(zh10) mutants (Costa et al. 1998; our observation), suggesting that apr-1 may perform additional functions in the dorsal hypodermis and in anterior seam cells that do not require hmr-1, hmp-1, or hmp-2 activity. hmr-1, hmp-1, and hmp-2 encode proteins that are similar to vertebrate Cadherins, \( \alpha \)-Catenin, and \( \beta \)-Catenin, respectively, and are localized at hypodermal adherens junctions. We therefore asked whether APR-1 may also be a component of the hypodermal adherens junctions. Because the abundance of APR-1 was too low to allow the detection of endogenous APR-1 in immunohistochemistry experiments, multiple copies of wild-type apr-1 were introduced on an extra-chromosomal array \( [zhEx2] \) to increase APR-1 expression levels. In zhEx2 embryos, APR-1 was partially localized at adherens junctions, but some cytoplasmic staining could be detected as well (Fig. 2E,F). No staining could be observed with APR-1 preimmunserum and the addition of an excess of soluble APR-1 antigen completely blocked the staining shown in Figure 2E.

In summary, apr-1 is necessary for at least four different processes during morphogenesis of the embryo, for the ventral migration, dorsal intercalation, and elongation of hypodermal cells and for the alignment of the anterior seam cells H2 and V1.

**Fig. 3.** Ventral closure and dorsal intercalation defects in apr-1(zh10) embryos observed by four-dimensional microscopy. The migration of hypodermal cells in embryos carrying the adherens junction marker \( jcsIs1[jam-1::GFP] \) was observed by two-photon excitation microscopy and four-dimensional reconstructions were done as described (Mohler and White 1998; Mohler et al. 1998). Ventral views of a developing wild-type \( [A] \) and an apr-1(zh10) embryo \( [B] \), dorsal views of a developing wild-type \( [C] \) and another apr-1(zh10) embryo \( [D] \). The time point when JAM-1::GFP could first be detected was taken as reference point 0 and is shown in the top frames of A–D. The numbers at left of the following frames indicate the time points relative to the reference point in minutes. Note in B the failure of the ventral hypodermal cells to reach the ventral midline and in D the lack of intercalation between the dorsal hypodermal cells. The arrowheads in C and D indicate fusing cells. The embryos were descendants of apr-1(zh10) unc-29(e1072)/lin-10(e1439); jcsIs1[jam-1::GFP] mothers. In all embryos, anterior is up. Scale bar in D, 10 \( \mu \)m.

e elongated about two- to threefold, hypodermal cells in arrested apr-1(zh10) embryos had retained their square shape and the length of the embryos had not increased >1.25-fold (Fig. 3; data not shown).

Fourth, the lateral anterior hypodermis was frequently disorganized in apr-1(zh10) mutants. In wild-type embryos, 10 hypodermal cells \( [H0, H1, H2, V1–V6, and T] \) form the lateral row of seam cells on each side of the
swls1[ceh-13::GFP] embryos, GFP expression was absent in anterior muscle and seam cells, whereas the VNC precursors continued to express CEH-13::GFP in all but the first exon of apr-1 animals continued to express APR-1 as determined by antibody staining (data not shown).

Similar to the staining observed in embryos, some APR-1 staining was localized at the adherens junctions, whereas another fraction of APR-1 could be detected in the cytoplasm (Fig. 5C–F). The specific expression of APR-1 in the VPCs raised the possibility that apr-1 may act during vulval development.

apr-1 is necessary for the specification of the vulval equivalence group

We devised two different approaches to inactivate apr-1 function in the Pn.p cells without affecting apr-1 activity during embryogenesis. First, we used cre/loxP-mediated recombination to inactivate a rescuing apr-1 transgene (Kilby et al. 1993). To this end, loxP sites were introduced into the rescuing apr-1 construct such that cre-mediated recombination would result in the excision of all but the first exon of apr-1 (loxP > apr-1 > loxP; Fig. 6A). cre recombinase was expressed under control of the Pn.p cell-specific lin-31 promoter [lin-31::cre; Tan et al. 1998]. The loxP > apr-1 > loxP and lin-31::cre plasmids were introduced into apr-1(zh10) animals on two separate extrachromosomal arrays, yielding a strain of the genotype apr-1(zh10); zhEx11[loxP > apr-1 > loxP]; zhEx12[lin-31::cre] [see Materials and Methods]. In single animals from this strain, the expected recombination product could be detected by PCR analysis (Fig. 6A). As a second approach, we performed tissue-specific RNA-mediated interference [RNAi; Fire et al. 1991]. For this purpose, a plasmid carrying anti-sense apr-1 cDNA under control of the Pn.p cell-specific lin-31 promoter [lin-31::apr-1i; Fig. 6B] was introduced into wild-type animals on the integrated array gals44. No APR-1 protein was detectable in the VPCs of gals44; zhEx2[apr-1wt] animals, whereas the seam cells in the same animals continued to express APR-1 as determined by antibody staining (data not shown).

Both approaches yielded animals displaying essentially the same vulval phenotype, although with a different penetrance. In 15% of apr-1(zh10); zhEx11[loxP > apr-
apr-1 is required for the expression of the Hox gene lin-39 in P3–8.p

Because bar-1 regulates lin-39 expression in response to a Wnt signal [Eisenmann et al. 1998], we tested whether apr-1 may also be required to induce lin-39 expression in P3–8.p. The expression pattern of LIN-39 was determined by staining L2 and L3 larvae with LIN-39 antibodies [Maloof and Kenyon 1998]. In wild-type animals, P3–8.p expressed LIN-39, except for those L3 larvae in which P3.p had adopted an F fate [Fig. 7A, C, E]. In all 5 apr-1(zh10); zhEx12[lin-31::apr-1i] animals, no LIN-39 expression was detectable in Pn.p cells that had adopted an F fate. An example is shown in Fig. 7, B and D. Consistent with the increasing frequency of the F fate in Pn.p cells distal to the anchor cell, LIN-39 was undetectable in P3.p but strongly expressed in P6.p in most gals44[lin-31::apr-1i] animals [Fig. 7F]. Furthermore, the down-regulation of LIN-39 expression could be observed in late L1 larvae before Pn.p cells adopted an F fate (10–12 hr posthatching, data not shown), indicating that the loss of LIN-39 expression is a cause and not a consequence of the F fate. In summary, these data have suggested that apr-1 may act in a Wnt signaling pathway that induces LIN-39 expression in P3–8.p.

Discussion

Our genetic analysis of the APC-related gene apr-1 has revealed distinct functions of apr-1: First, apr-1 is required for the ventral migration, dorsal intercalation, and elongation of hypodermal cells during morphogenesis of the embryo [Fig. 8A]. Second, apr-1 regulates Hox gene expression in the developing P3–8.p.
Figure 6. Inactivation of apr-1 in the Pn.p cells causes P3–8.p to adopt an F fate. [A] Structure of the loxP > apr-1 > loxP transgene used for cre/loxP-mediated inactivation of apr-1 and of the predicted recombination product. The circular excision product is not shown. The arrowhead below the loxP > apr-1 > loxP construct indicates the position of the primers O101 (see Materials and Methods) and M13 reverse (Invitrogen) that were used for the PCR analysis shown at right. (+) The 1.3-kb recombination product obtained by PCR analysis of an L4 larva of the genotype apr-1(zh10); zhEx11[loxP > apr-1 > loxP]; zhEx12[lin-31::cre]; (−) an animal of the genotype apr-1(zh10); zhEx11[loxP > apr-1 > loxP] analyzed as negative control. The PCR conditions used did not allow amplification of the 6.5-kb product from loxP > apr-1 > loxP transgenes that had not undergone cre-mediated recombination. [M] Molecular mass standards, the numbers at left indicate the length of relevant markers in kilobase pairs. [B] Structure of the lin-31::apr-1i construct used for Pn.p cell-specific RNAi. mAb MH27 staining of the VPCs in wild-type (C), apr-1(zh10); zhEx11[loxP > apr-1 > loxP]; zhEx12[lin-31::cre] (D), and gals4d[lin-31::apr-1i] L2 larvae (E). The arrows point at the positions of the Pn.p nuclei as determined by parallel staining with DAPI [not shown]. Note that in the animal shown in D P3.p, P4.p, and P5.p and in the animal shown in E, all Pn.p cells except P6.p have adopted an F fate, because they failed to stain with the mAb MH27. See Table 1 for more examples and for the complete genotypes of the animals shown. Scale bar in E, 10 μm.

gene expression. During embryogenesis, apr-1 is required for the expression of the Hox gene ceh-13 labial in anterior cells (Fig. 8A) and during vulval development, apr-1 is necessary for the expression of the Hox gene lin-39 deformed in P3–8.p [Fig. 8B].

apr-1 is required for morphogenesis of the embryo

apr-1 plays multiple roles during morphogenesis of the embryo. The incomplete penetrance of the different phenotypes is probably due to a variable rescue by materially expressed apr-1. However, because loss of apr-1 function in the germ line causes complete sterility, we were unable to examine the contribution of maternal apr-1 activity to morphogenesis.

The ventral closure defect is caused by a disruption of hypodermal cell migration. In apr-1(zh10) embryos, hypodermal cells initiate ventral migration, but they stop before reaching the ventral midline. Moreover, in apr-1(zh10) larvae that arrest during the L1 stage the hypodermal Pn cells do not descend to the ventral midline, suggesting that apr-1 may also be required for the ventral migration of the Pn cells [data not shown]. What may be the role of apr-1 in hypodermal cell migration? One possible model predicts that APR-1 may act in a Cadherin/β-Catenin complex at the adherens junctions. It has been proposed that filopodial contacts must be made between the contralateral pairs of migrating cells before adherens junctions can be formed across the ventral midline and the cells are fixed in their new position (Costa et al. 1998; Raich et al. 1999). In particular, the two pairs of leading cells project actin-rich filopodia toward the ventral midline [Williams Masson et al. 1997]. APR-1 may be required together with HMR-1 Cadherin, HMP-1 α-Catenin, and HMP-2 β-Catenin to stabilize the transient filopodial contacts between the pairs of leading cells by attaching actin filaments to the newly forming junctions.

Dorsal intercalation does not depend on hmr-1, hmp-1, or hmp-2 activity, possibly because this process does not involve the formation of new adherens junctions. apr-1, on the other hand, may be required for the directed movement of dorsal hypodermal cells through a microtubule-dependent mechanism that has been proposed for vertebrate APC (Nathke et al. 1996; Pollack et al. 1997). In migrating vertebrate cells, APC was found in clusters near the ends of microtubules that extended into regions of actively migrating plasma membranes and at the sites where two cells first touched. This specific subcellular localization has suggested that APC may recruit microtubules into the extending membrane structures and thus stabilize the direction of cell migration. Although we have not observed a specific localization of APR-1 at the sites of actively migrating membranes, our data are consistent with an analogous role for apr-1 in directing cell movements during ventral closure and dorsal intercalation.

An alternative model explaining both the ventral closure and dorsal intercalation defects predicts that apr-1 may be required for the specification of hypodermal cell
apr-1 controls cell migration and Hox genes

Table 1. Pn.p cells lacking apr-1 activity frequently adopt an F fate

| Row | Genotype | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | Percent mutant | No. c |
|-----|----------|------|------|------|------|------|------|---------------|------|
| 1   | wild type| 39   | 0    | 0    | 0    | 0    | 0    | 0             | 61d  |
| 2   | apr-1, zhEx11[loxP>apr-1-loxP] | 33 | 1.4f | 0 | 0 | 0.7f | 0 | 1.4 | 140 |
| 3   | apr-1, zhEx11[loxP>apr-1-loxP],zhEx12[lin-31:cre] | 45 | 9 | 4 | 3 | 4 | 4 | 15 | 219 |
| 4   | gals44[lin-31::apr-li] | 72 | 60 | 44 | 17 | 35 | 35 | 86 | 57 |
| 5   | bar-1(0)h | 66 | 56 | 11 | 2 | 2 | 6 | 62 | 55 |
| 6   | bar-1(0), gals44[lin-31::apr-li] | 95 | 88 | 81 | 61 | 54 | 63 | 99 | 103 |

For each strain, L3 larvae were stained with mAb MH27 to detect the adherens junctions of unfused cells.

aEach column indicates the percent frequency at which Pn.p cells adopted an F fate instead of a VPC fate.

bThe number of animals scored for each strain.

cThe frequency at which animals with fewer than six VPCs were observed in each strain; animals in which P3.p had adopted an F fate were counted as wild-type.

dSimilar data for wild-type [N2] have been reported previously [Eisenmann et al. 1998].

The complete genotype of this strain was apr-1(zh10) unc-29(e1072), zhEx11[loxP>apr-1-loxP].

fIn two animals of this strain, P4.p and/or P7.p had adopted an F fate. This could be due to a spontaneous loss of the zhEx11 array in single Pn.p cells or to an incomplete rescue by the apr-1 transgene. In other strains carrying a rescuing apr-1 transgene, individual Pn.p cells adopted an F fate in 0.4%–0.8% of the animals (data not shown).

gThe complete genotype of this strain was apr-1 (zh10) unc-29(e1072), unc-119(e2498), zhEx11[loxP>apr-1-loxP], zhEx12[lin-31::cre, unc-119(+)].

hThe bar-1(ga80) null mutation was used in these experiments [Eisenmann et al. 1998].

Table 2. Vulval Lineages of Wild-type, gals44[lin-31::apr-li], and lin-39[0] animals

| Row | Genotype | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | Phenotype |
|-----|----------|------|------|------|------|------|------|-----------|
| 1   | wild type| S S or F | F | F | LLTN | TTTT | NTLL | S S | non-Vul |
| 2   | gals44[lin-31::apr-li] | F | F | LLLT | TTTT | F | F | Vul |
| 3   | gals44[lin-31::apr-li] | F | F | LLLT | OOOO | F | SOO | Vul |
| 4   | gals44[lin-31::apr-li] | F | F | F | TTTT | F | F | Vul |
| 5   | gals44[lin-31::apr-li] | F | F | F | OOOO | F | F | Vul |
| 6   | gals44[lin-31::apr-li] | F | F | F | F | F | F | Vul |

The cleavage planes of the third round of cell divisions are indicated as follows: (T) Transverse division; (O) oblique division; (L) longitudinal division; (N) cell that did not divide during the third round of division; (S) cell that fused with hyp7 after the first round of cell division; (F) cell that never divided. The shaded area indicates cells that adopted induced [1°, 1°-like, or 2°] cell fates.

eWhen inspected under a dissecting microscope, 80% of gals44[lin-31::apr-li] animals displayed a Vul phenotype (n > 200).

fData from Clark et al. (1993).
Inactivation of apr-1 in the Pn.p cells causes P3–8.p to adopt an F cell fate, similar to mutations in the Hox gene lin-39 (Clark et al. 1993; Wang et al. 1993). In both cases, apr-1 is necessary for the expression of the respective Hox genes (Fig. 8A,B).

A Wnt signal that is transduced by the β-catenin-related gene bar-1 induces expression of lin-39 in P3–8.p (Eisenmann et al. 1998). Thus, apr-1 may act in the Wnt signaling pathway in P3–8.p. Inactivation of apr-1 in the Pn.p cells by RNAi does not cause all six of the P3–8.p cells to adopt an F fate, suggesting that apr-1 may not be absolutely required for Wnt signaling. Pn.p cells that are distal to the anchor cell more frequently adopt an F fate and down-regulate lin-39 expression than those proximal to the anchor cell. A similar bias in the frequency of F fates has been observed in bar-1 mutants (Eisenmann et al. 1998). One possible explanation for this finding is that proximal Pn.p cells may be closer to the source of the Wnt signal.

Figure 8. Models for the different functions of APR-1. (A) During morphogenesis of the embryo, APR-1 may perform at least two separate functions. First, (left) APR-1 may act together with the adherens junction proteins HMR-1 Cadherin, HMP-1 α-Catenin, and HMP-2 β-Catenin in migratory hypodermal cells during ventral closure and elongation of the embryo. Second (right), APR-1 may act in the Wnt signaling pathway that controls the expression of the HOX protein CEH-13 in the anterior seam and muscle cells. (B) During larval development, APR-1 may be required for the transduction of a Wnt signal that induces expression of the HOX protein LIN-39 in P3–8.p to specify the VPC fate. The three β-Catenins WRM-1 (Rocheleau et al. 1997), HMP-2 (Costa et al. 1998), and BAR-1 (Eisenmann et al. 1998) may act redundantly in the transduction of Wnt signals.
Wnt signal in the central body region and thus require less apr-1 or bar-1 activity than distal Pn.p cells. Moreover, because loss of bar-1 function results in an incompletely penetrant vulval phenotype, one of the other two β-catenin homologs [hmp-2 or wrm-1] may be partially redundant with bar-1 in the transduction of a Wnt signal in P3–8.p. Consistent with this hypothesis, inactivation of apr-1 in animals carrying a bar-1 loss-of-function mutation appears to further decrease the activity of the Wnt signaling pathway.

Less is known about a putative Wnt signal that may control ceh-13 expression during embryogenesis. However, the ceh-13 gene contains a putative LEF-1/Tcf-binding site that plays a role in the transcriptional activation of ceh-13 in the E lineage [R. Köhler and F. Müller, pers. comm.]. Because the Wnt signaling pathway controls the activity of the LEF-1/Tcf family of transcription factors [Brunner et al. 1997; van de Wetering et al. 1997], ceh-13 expression may be regulated by Wnt signals. Furthermore, expression of the ceh-13 homolog labial in the Drosophila midgut is controlled by a Wnt signal [Bienz 1994]. These observations together with our data suggest that apr-1 may be required to transduce a Wnt signal that induces ceh-13 expression in anterior cells of the embryo [Fig. 8A]. It is not clear which of the three C. elegans β-catenin/armadillo homologs may participate in the transduction of this putative Wnt signal. hmp-2 mutant embryos do not exhibit defects in the alignment of seam cells [Costa et al. 1998] and they continue to express ceh-13 until the terminal stage (our observation), bar-1 mutant embryos do not exhibit an embryonic phenotype [Eisenmann et al. 1998] and no wrm-1 mutation has been described to date [Rocheleau et al. 1997]. However, two or all three of the β-catenin/armadillo-related genes might act redundantly.

APR-1 is necessary for β-Catenin/Armadillo function

Vertebrate and Drosophila APCs bind to β-Catenin/Armadillo proteins and promote their proteolytic degradation [Munemitsu et al. 1995; Polakis 1997]. Mutations that remove the β-Catenin-binding site of APC cause the cytoplasmic accumulation of β-Catenin, resulting in the constitutive activation of the Wnt signaling pathway. Thus, APC is thought to antagonize β-Catenin/Armadillo function.

Our data and a prior analysis of maternal apr-1 activity in early embryonic development by RNAi [Rocheleau et al. 1997] have indicated an opposite function for APR-1. In all cases that were studied, loss of apr-1 function resulted in essentially the same phenotype as loss of β-catenin function. In particular, we have found that APR-1 is necessary for HMP-2 function during morphogenesis of the embryo and BAR-1 function in the transduction of a Wnt signal in P3–8.p.

How could this apparent reversal of APR-1 activity in comparison with APC in other species be explained? One possibility is that, although APR-1 is most similar to vertebrate and Drosophila APC, apr-1 may not be a functional APC homolog. Because no second gene encod-

ing a protein with significant homology to APC has been found in the C. elegans genome, β-Catenins in C. elegans may not be subject to the same kind of negative regulation by APC as in other organisms. On the other hand, the activating function of APR-1 may reflect a unique requirement of the C. elegans β-Catenin homologs.

Another model predicts that APC may activate as well as inhibit β-Catenin/Armadillo function. The apr-1 loss-of-function mutation described in this work may disrupt both activities, and loss of the activating function may prevail over loss of the inhibitory function. In contrast, the carboxy-terminal truncations found in vertebrate and Drosophila APC [Su et al. 1992; Polakis 1995; Ahmed et al. 1998] may selectively disrupt the inhibitory function of APC, resulting in the constitutive activation of β-Catenin/Armadillo. Supporting this hypothesis, overexpression of human APC in Xenopus laevis embryos induces axis duplication, as does overexpression of β-Catenin or Xwnt-8, suggesting that human APC may also have an activating function in the Wnt signaling pathway [Vleminckx et al. 1997]. Such a dual model for APC may also explain why mutations that result in the complete loss of the APC protein are usually not found in human cancer cells, as loss of the entire APC protein might inhibit rather than activate β-Catenin function and may not lead to colon cancer.

Materials and methods

General methods and strains

C. elegans strains were cultured at 20°C as described in Brenner [1974]. Wild type refers to C. elegans variety Bristol, strain N2. Pn.p cell lineages were determined by direct observation of the cell divisions using Nomarski optics, as described previously [Stemberg and Horvitz 1986]. Unless noted otherwise, the mutations used have been described [Riddle et al. 1997] and are listed below.

LGI, unc-119(e2498); LGIII, swIs1[ceh-13::GFP] [Wittmann et al. 1997], gafs44[lin-31::apr-1] [see below], jcls1[jam-1::GFP] [Mohler et al. 1998], LGX, bar-1(ga80) [Eisenmann et al. 1998], LG not determined, zhls2[apr-1::GFP] [see below].

Isolation of the apr-1(zh10) deletion

A library consisting of ~4.8 x 10^5 mutagenized F1 animals was screened for deletions in the apr-1 locus using the strategy described [Jansen et al. 1997] except for the following modifications: Wild-type animals were treated with 75 mM EMS, allowed to recover for 16 hr and five P0 animals were plated on each of 960 NGM plates (6-cm diam.) such that each plate contained on the average 500 mutagenized F1 animals. The cultures were grown for 7 days before harvesting one-third of the animals for DNA isolation. To identify the plate containing the apr-1(zh10) deletion, DNA extracted from the individual cultures was combined in pools of 12 cultures and 50 ng of DNA from each pool was subjected to PCR analysis with the primers 096 [5’-CGTAAACGTAGGAGCACCAGCAG-
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GAGG-3' and O100 [5'-CAATACGACAGTTCGACGACG-3'] for the first amplification step followed by a second amplification with the nested primers O95 [5'-CGACATGAGTCGGTATAGCAGGCTTAG-3'] and O101 [5'-AAGAGATGGTAAGGACT-3']. The remaining animals from the culture containing the zh10 deletion were subjected to three rounds of selection until a single animal of the genotype apr-1(zh10)/+ had been identified. Before phenotypic analysis, the apr-1(zh10) mutation was backcrossed at least 10 times against unc-29(e1072), him-5(e1490) animals.

Strain construction

Standard methods were used to construct double and triple mutants [Brenner 1974]. The apr-1(zh10) deletion was cis-linked to the unc-29(e1072) mutation and followed by PCR amplification with the primers O95 and O101 (see above) or by observing the closely linked Unc phenotype. Strains heterozygous for the apr-1(zh10) mutation were balanced either with unc-29(e1072)/+ or apr-1(zh10) mutation and followed by PCR amplification as indicated in the Figure legends 1–4. apr-1(zh10) unc-29(e1072)/+ lin-10(e1439) animals segregated <1% viable Unc animals (>500) that were most likely recombinants between apr-1 and unc-29, because they were heterozygous for the apr-1(zh10) mutation as determined by PCR analysis. Strains that were homozygous for apr-1(zh10) contained a rescuing apr-1 transgene on the extrachromosomal array zhEx11 (see below).

Molecular biology

The rescuing wild-type apr-1 construct (apr-1wt) was obtained by ligation of a 7.3-kb-long PCR product amplified from wild-type genomic DNA with the primers 5'-CAATACGACAGTTCCGACGACG-3' and 5'-TTTGCTCACCCTGGACGACT-3' into the vector pGEM-T (Invitrogen). To construct the apr-1::GFP reporter, a 4.8-kb SalI-BamHI fragment isolated from apr-1wt was cloned into the Sall and BamHI sites of the GFP vector pD95.67 (gift of A. Fire, Carnegie Institution, Baltimore, MD). The plasmid loxp apr-1 > loxp was obtained by inserting synthetic double-stranded oligonucleotides containing the loxp site 5'-ATAACTCTGATAATGATGC-TATAGGAAATTT-3' (Kimby et al. 1993) into the BpuAI and SphI sites of plasmid apr-1wt. A clone carrying the two loxp sites in a parallel orientation was identified by sequence analysis. For the construction of the lin-31:cre plasmid, a 1.1-kb cDNA fragment containing the entire cre recombinase ORF was isolated by PCR amplification with the primers 5'-TTTCTGGCCG-GCCCGCTTAATCGCCCATCTGCGACGAGC-3' and 5'-TTTGCTCACCCTGGACGACT-3' from the plasmid pCAGGS-cre [Araki et al. 1995], digested with Xhol and NotI and cloned into the Sall and NotI sites of plasmid pB253 (Tan et al. 1998). The plasmid lin-31::cre was obtained by cloning a 1.3-kb Xhol fragment of the apr-1 cDNA (nucleotides 85–1445) the coding sequence into the Sall site of pB253 in the antisense orientation.

Antibody production, immunofluorescence, and microscopy

A 1.3-kb apr-1 cDNA fragment was isolated from a partial cDNA clone by PCR amplification with the primers 5'-TTTG-GATCCGACGCAGATCGACGACT-3' and 5'-TTTCTGGCCG-GCCCGCTTAATCGCCCATCTGCGACGAGC-3' and cloned into the BamHI site of the Escherichia coli expression vector pD86 (Stueber and Bujard 1982) to produce an APR-1 fragment consisting of amino acids 551–983 with an amino-terminal His tag. The His6-APR-1 fusion protein was produced in the E. coli strain M15, purified on NTA agarose according to the manufacturer's instructions (Qiagen), and used to immunize rats and rabbits. Antisera were affinity purified against the recombinant APR-1 fragment immobilized on PVDF membranes [Immobilon].

Staining of embryos and staged larvae with rat anti-APR-1 antibodies was carried out as described [Miller and Shakes 1995], except that fixation was done in ice-cold methanol/acetone [1:1] for 5 min followed by air-drying. Rabbit anti-LIN-39 antibodies were preadsorbed against a total extract from lin-39(n1880) animals before use, and LIN-39 staining was performed as described [Maloof and Kenyon 1998]. CEH-13::GFP was observed in living embryos mounted on agarose pads in M9 solution. In all staining experiments, the mAb MH27 [Francis and Waterston 1985] was included to label the adherens junctions. As secondary reagents, fluorescein-conjugated donkey anti-rabbit [Jackson laboratories], Alexa488-conjugated goat anti-rabbit [Molecular probes], and rhodamine-conjugated donkey anti-mouse antibodies [Jackson laboratories] were used. DAPI was included during the incubations with secondary antibodies at a concentration of 0.5 µg/ml to label the nuclei of the cells. Fluorescent images were recorded by confocal laser scanning microscopy (Leica TC 4D or Bio-Rad MRC 600) or with a cooled CCD camera (Hammamatsu C4742-95) on a Leica DM/IR B inverted microscope. Embryos carrying the jclsl1 array were observed by four-dimensional microscopy using two-photon excitation as described [Mohler and White 1998, Mohler et al. 1998].

Transgenic animals

Transgenic animals were generated by microinjection of DNA into the distal arms of the gonad as described [Mello et al. 1991]. Where indicated, extrachromosomal arrays were integrated into the genome by y-irradiation of L4 larvae. Animals carrying integrated arrays were backcrossed at least five times before further analysis.

To rescue the lethal and vulvaless phenotypes caused by the apr-1(zh10) mutation, the plasmid apr-1wt was injected at a concentration of 100 ng/µl along with 100 ng/µl sur-5::GFP plasmid pTG96 [Yochem et al. 1998] as transformation marker into apr-1(zh10) unc-29(e1072)/+ lin-10(e1439) animals yielding the extrachromosomal array zhEx3. Transformants were identified with a dissecting microscope under fluorescent light illumination. apr-1(zh10) unc-29(e1072); zhEx3[apr-1wt] animals developed into healthy adults that were sterile, presumably because apr-1 is required for oogenesis and the multicopy apr-1 transgene was not active in the germ line. The sterility caused by apr-1(zh10) could be rescued when a low concentration of a rescue construct was injected together with an excess of genomic DNA to allow germ-line expression of the transgene as described below for the zhEx31 array. The zhEx2 array was obtained by injecting 100 ng/µl plasmid apr-1wt along with 50 ng/µl plasmid pRF4, which contains the semidominant marker rol-6(sa1006) into N2 animals. The plasmids apr-1::GFP, lin-31::cre, and lin-31::apr-1 were each injected at a concentration of 100 ng/µl together with 20 ng/µl wild-type unc-119 plasmid [Maduro and Pilgrim 1995] into unc-119(e2498) animals to yield the arrays zhIs2, zhEx12, and gaIs44, respectively. The plasmid loxp apr-1 > loxp was linearized with NotI and injected at a concentration of 5 ng/µl together with 200 ng/µl yeast genomic DNA and 5 ng/µl transformation marker pTG96 linearized with Eagl into apr-1(zh10) unc-29(e1072)/lin-10(e1439) animals. The resulting array zhEx11 rescued the sterility, lethality, and vulvaless phenotypes of apr-1(zh10) animals. For each construct, at least three independent lines were obtained and a representative line was kept for further analysis.
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