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apl-1, a *Caenorhabditis elegans* gene encoding a protein related to the human β-amyloid protein precursor

ISABELLE DAIGLE AND C. LI*

Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215

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ABSTRACT **The major component of senile plaques found in the brains of Alzheimer disease patients is the β-amyloid peptide, which is derived from a larger amyloid precursor protein (APP). Recently, a number of APP and APP-related proteins have been identified in different organisms and constitute the family of APP proteins. We have isolated several cDNAs encoding an APP-related protein in the nematode *Caenorhabditis elegans* and have designated the corresponding gene as *apl-1*. The *apl-1* transcripts undergo two forms of posttranscriptional modification: trans-splicing and alternative polyadenylylation. *In vitro* translation of an *apl-1* cDNA results in a protein of approximately the expected size. Similar to the *Drosophila*, human, and mouse APP-related proteins, APL-1 does not appear to contain the β-amyloid peptide. Because APP-related proteins seem to be conserved through evolution, the *apl-1* gene from *C. elegans* should be important for determining the normal function of human APP.**

The β-amyloid peptide (β-AP) is the major component of dense plaques, which are characteristically found in brains of Alzheimer disease (AD) patients (1, 2). β-AP is an ≈40-aa peptide derived from the β-amyloid protein precursor (APP; ref. 3). Mutations in the *APP* gene, located on chromosome 21 (4, 5), have been correlated with early-onset familial AD (6, 7), suggesting that disruption of the *APP* gene may be one of the causative factors in AD. However, familial AD has also been linked to genes located on chromosomes 14 (8–11) and 19 (12, 13). Two *APP*-related genes, *APLP1* and *APLP2* (also known as *APPH*; ref. 17), which have been localized to chromosomes 19 (14, 15) and 11 (16), respectively, encode proteins that, along with APP, are members of the APP family of proteins.

APP is a transmembrane protein whose single membrane-spanning region separates a large N-terminal extracellular domain from a much smaller C-terminal cytoplasmic domain. The β-AP spans part of the extracellular and transmembrane domains of APP (3) and is not present in *APLP1* (14) or *APLP2* (16, 17). APP can be either secreted, a process that would cleave APP within the β-AP and prevent β-AP formation (18–21), or processed through an endosomal–lysosomal pathway (22, 23), which could presumably generate β-AP (24–28). In humans, several alternatively spliced transcripts of the APP gene have been found in different tissues (29); however, the normal function of the various proteins encoded by the *APP* and *APP*-related genes is poorly understood.

An *APP*-related gene, *Appl*, has also been identified in *Drosophila* (30). Deletion of the *Appl* gene leads to behavioral defects in phototaxis that can be partially rescued by injection of the human APP_{695} cDNA (31), suggesting that a function of APP_{695} and *Drosophila* APPL is conserved.

Because of the apparent conservation of function between the human APP and *Drosophila* APPL proteins, we investigated whether APP-related genes were present in the nematode *Caenorhabditis elegans*. The use of *C. elegans* as a model system is valuable because of the simplicity of the organism and the ease with which it can be manipulated genetically and molecularly (32, 33). We report here the identification of a *C. elegans* cDNA† that encodes a predicted protein, APL-1, whose sequence contains the characteristic features of proteins in the APP family.

MATERIALS AND METHODS

Growth and Maintenance of Strains. Bacterial strains and λ phage were grown and maintained according to Sambrook *et al.* (34), and *C. elegans* animals (N2 var. Bristol) were grown and maintained according to Wood (35).

Isolation and Sequencing of cDNAs. All standard molecular techniques were performed as described in Sambrook *et al.* (34). A mixed-stage λZAP cDNA library (36) was screened as described by Bürglin *et al.* (37), using 4096-fold degenerate oligonucleotides (5′-ATYGGNTAYGARAAYCCNACN-TAYAAR-3′, where Y is T or C, N is G, A, T, or C, and R is A or G) corresponding to a highly conserved cytoplasmic region between APP and APP-related proteins. Prehybridization and hybridization were carried out at 37°C. Filters were washed at 40°C and exposed to film using intensifying screens at −70°C.

Positive clones were isolated and excised from the λZAP vector according to the manufacturer's protocol (Stratagene) to yield pBluescript plasmids. DNA sequencing using the double-stranded dideoxynucleotide method (38) identified three APP-related clones (APL3.2, -6.2, and -1.2.2). To isolate additional clones, the cDNA library was rescreened with APL3.2, which was ^{32}P-labeled by random priming. For this second screen, prehybridization and hybridization were done at 65°C in 6× standard saline citrate (SSC)/5× Denhardt's solution/1% SDS/0.05% sodium pyrophosphate/salmon sperm DNA (100 μg/ml). Filters were then washed in 2× SSC/0.1% SDS/0.05% sodium pyrophosphate for 30 min at room temperature, followed by two 15-min washes at 68°C in 0.1× SSC/0.1% SDS/0.05% sodium pyrophosphate. Hybridizing plaques were visualized by autoradiography. Isolated clones were excised and sequenced as described above.

Isolation of RNA and Reverse Transcription–PCR. Worms were grown in liquid cultures and isolated by sucrose flotation (35). Total RNA from freshly isolated mixed-stage worms was extracted by the procedure of Chomczynski and Sacchi (39), as modified by Rosoff *et al.* (40). Reverse transcription was performed with a modified procedure of Rosoff *et al.* (40). Approximately 30 μg of total RNA was used for cDNA synthesis using Moloney murine leukemia virus reverse transcriptase and 30 ng of a primer (APP11, 5′-GAAGCATCGCAAGAGTTC-3′) located ≈1260 bp downstream of the 5′ end of the longest clone (APL23). A negative control was done using no RNA.

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Abbreviations: APP, amyloid protein precursor; β-AP, β-amyloid peptide; AD, Alzheimer disease.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U00240).

The PCR amplification was performed as follows: 5 μl of cDNA was mixed with 2.5 mM $MgCl_2$/1× PCR buffer (Promega)/all four dNTPs (each at 2 μM)/50 ng of 5′ primer [SL1 (5′-GTTTAATTACCCAAGTTTGA-3′) or SL2 (5′-GGTTTTAACCCAGTTACT-3′)]/25 ng of 3′ primer [APP6 (5′-CTTCTGAGTAATCATCCTCAT-3′), located ≈675 bp downstream of the 5′ end of APL23]/2 units of *Taq* polymerase (Promega) in a final volume of 50 μl. The amplification was done in a MJ Research thermal controller with the following parameters: 94°C for 1 min, 55°C for 1.5 min, and 75°C for 1 min for 5 cycles, followed by 30 cycles of 94°C for 1 min, 55°C for 1.5 min, and 75°C for 3 min. The samples were then heated to 95°C for 5 min and slowly cooled to 4°C (1°C/min). One-tenth of this reaction mixture was then reamplified in a similar manner. The final products were electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining. The amplified DNA was extracted from the gel using the MERmaid kit (Bio 101) and cycle-sequenced by the manufacturer's protocol using Vent polymerase (New England Biolabs).

Primer-Extension Analysis. Primer-extension analysis was performed as described by Boorstein and Craig (41). Twenty-five ng of ^{32}P-end-labeled primer (APP9, 5′-TGGTTGCGGTATCCACATGAA-3′), located ≈100 bp downstream from the 5′ end of clone APL23, was used. Samples containing 25 ng and 50 ng of total RNA were annealed to the primer and extended at both 42°C and 50°C. Control reactions were performed using no RNA or 50 ng of yeast tRNA as template. The resulting fragments were analyzed on a 6% polyacrylamide gel containing 7 M urea and visualized by autoradiography. Sequencing reaction products of clone APL23 using the same primer were also electrophoresed on the gel for size comparison.

***In Vitro* Transcription/Translation.** A *Hin*dIII–*Pst* I fragment of clone APL23, which contains the entire *apl-1* coding region, was subcloned into pGEM-4 and pGEM-3 (Promega) to provide clones that could be transcribed in the sense and antisense orientations, respectively. Approximately 2.5 μg of each plasmid was transcribed and translated *in vitro* using the SP6 TNT wheat germ lysate-coupled transcription/translation system (Promega). A control reaction was also performed using the vector DNA without an insert. Proteins were labeled in the translation step with 20 μCi of [^{35}S]methionine and cysteine (ICN, Tran^{35}S-label; >1000 Ci/mmol; 1 Ci = 37 GBq), separated by SDS/PAGE on an 8.5% gel, and visualized by autoradiography.

RESULTS

Isolation and Sequencing of *apl-1* cDNAs. All members of the APP family of proteins contain a highly conserved sequence (NGYENPTYK) located in the cytoplasmic domain. To iso-

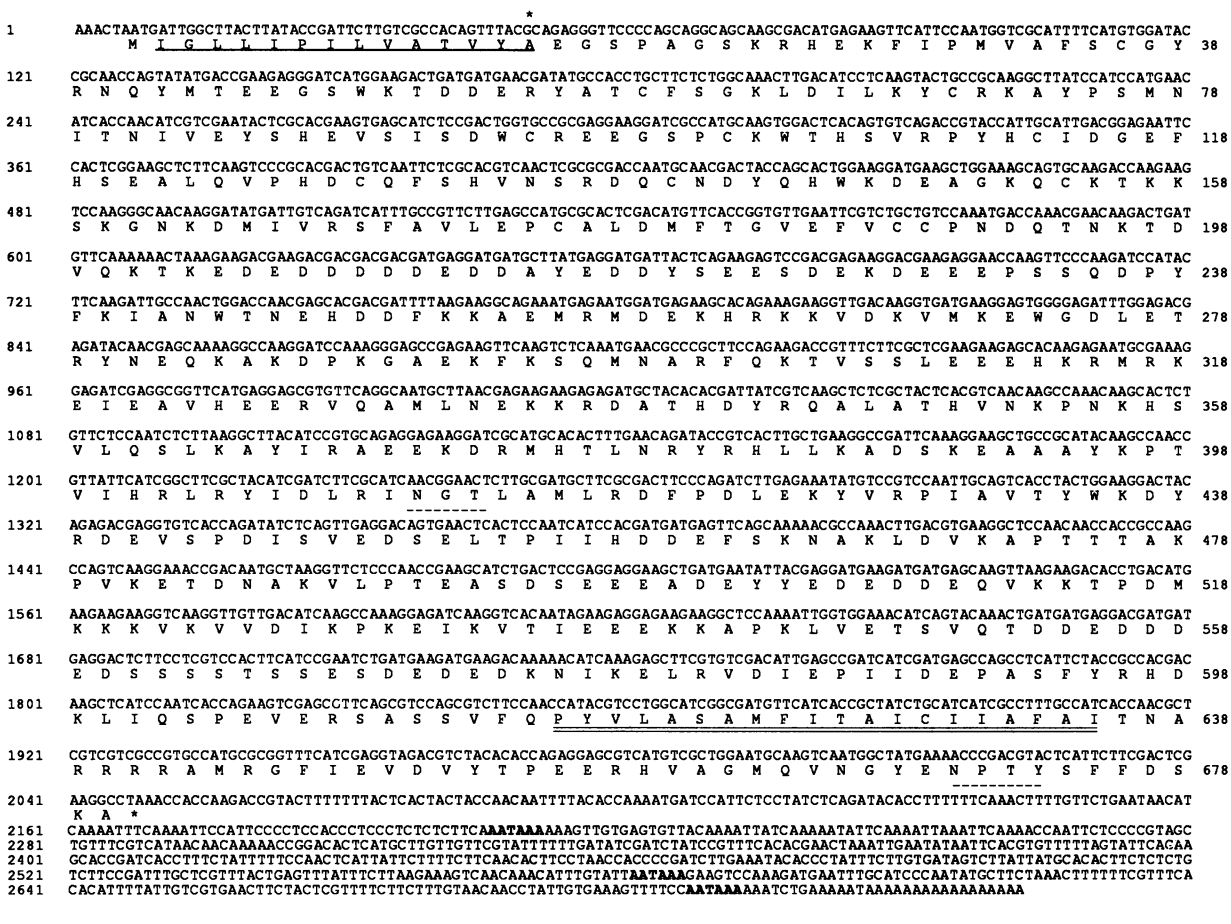

FIG. 1. (*A*) Nucleotide and predicted amino acid sequence of the *apl-1* cDNA from *C. elegans*. The nucleotide number is to the left, and the amino acid number is to the right. The predicted signal sequence is underlined, and the possible signal sequence cleavage site is marked by an asterisk. The putative transmembrane domain is double underlined; possible glycosylation (extracellular) and clathrin-coated vesicle targeting (cytoplasmic) sequences are underlined with a dashed line. The multiple polyadenylylation signals are in boldface type. (*B*) Three classes of *apl-1* transcripts. The coding region is identical for all cDNAs, but different clones appear to use different polyadenylylation signals. Some of the sequence observed 5′ of the polyadenylylation signals is presented.

late APP-related cDNAs from *C. elegans*, degenerate oligonucleotides corresponding to this sequence were used to screen ≈300,000 plaques of a mixed-stage *C. elegans* cDNA library. In an initial screen, three cDNAs encoding APP-related proteins were isolated. To isolate additional clones, the library was rescreened using one of the positive clones as a probe. All cDNA clones were mapped with restriction enzymes and sequenced at their 5′ and 3′ ends, which demonstrated that they were all related (data not shown).

We have designated the gene that encodes these cDNAs as *apl-1* for APP-like gene 1, and the predicted protein as APL-1. Clone APL23, the longest cDNA, contains a long open reading frame of 680 codons, followed by a stop codon (Fig. 1). The putative translation product of APL23 has a predicted molecular mass of 78.8 kDa. The probable APL-1 initiator methionine, which is followed by a potential signal sequence (42) and signal peptidase cleavage site (ref. 43; see Fig. 1*A*), is in a position similar to the initiator methionines in other APP-related proteins. The APL-1 putative transmembrane domain separates a large extracellular domain (≈610 aa) that contains an unusually high content of acidic residues from a cytoplasmic domain of 45 residues. The extracellular domain also contains a possible N-linked glycosylation site (NXS/T). The predicted cytoplasmic domain includes a sequence (NPTY) that is involved in clathrin-coated vesicle targeting (23).

Different Transcripts Are Produced by *apl-1* Through the Use of Alternative Polyadenylylation Signals. DNA sequencing of the 3′ ends of eight *apl-1* cDNA clones revealed three classes of cDNAs that differ as a result of the use of alternative polyadenylylation signals (Fig. 1*B*). Since multiple cDNAs from the three classes were isolated (two clones for each class), this suggests that transcripts using all three polyadenylylation signals are present *in vivo*. The presence of these transcripts *in vivo* has been confirmed by amplification of reverse-transcribed mixed-stage total RNA, which revealed amplification products corresponding to the expected sizes of transcripts using each of the polyadenylylation signals (data not shown). An additional two *apl-1* cDNAs containing all three polyadenylylation signals, but lacking a poly(A) tail, were also isolated, suggesting that additional 3′ untranslated sequence and polyadenylylation signals may exist. All isolated *apl-1* cDNAs appear identical within the predicted coding region.

***apl-1* Transcripts Have a 5′ Splice Leader Sequence.** To determine whether APL23 represented a full-length cDNA, primer-extension analysis was performed using an oligonucleotide primer located 100 bp from the 5′ end of APL23. A single band corresponding to a position 37 or 38 bp upstream of the 5′ end of clone APL23 was observed when two temperatures (42°C and 50°C) for the annealing and extension and when different amounts of total RNA (25 ng and 50 ng) were used; no bands were observed when no RNA or yeast tRNA was used as a template (Fig. 2*A*). From these results, we conclude that clone APL23 is 37 or 38 bp short of full length.

Partial sequencing of a genomic clone corresponding to *apl-1* (unpublished results), however, revealed the presence of a 3′ splice acceptor site located 15 nt upstream of the 5′ end of clone APL23 (Fig. 2*B*). In *C. elegans*, at least 15% of the mRNAs appear to be trans-spliced to either a 5′ SL1 or SL2 leader sequence, each of which is 22 nt long (44–46). Thus the primer-extension data and the presence of an upstream splice acceptor site suggest that the *apl-1* transcripts are trans-spliced to either an SL1 or SL2 leader sequence. To determine whether the *apl-1* transcripts are trans-spliced, primers specific for SL1 and SL2 were used with an *apl-1*-specific primer to amplify cDNA prepared by reverse transcription of mixed-stage total RNA. A single amplification product that corresponds to the expected size (≈710 bp) was observed using the SL1, but not the SL2, primer (Fig. 2*C*); DNA sequencing

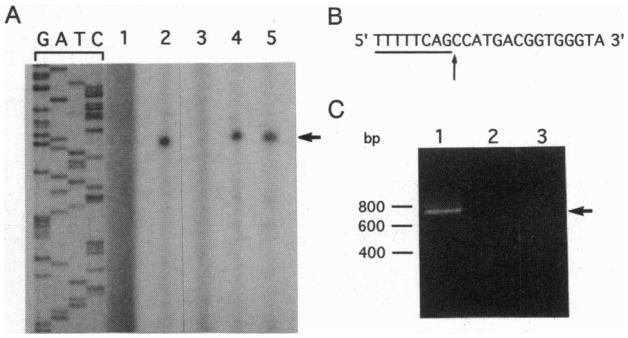

FIG. 2. *apl-1* transcripts have a 5′ SL1 splice leader sequence. (*A*) After primer extension using a primer annealed to mixed-stage total RNA, fragments were separated on a denaturing polyacrylamide gel and visualized by autoradiography. Lanes: 1 and 2, annealed and extended at 42°C; 3–5, at 50°C; 1, no RNA template; 2, 25 ng of total RNA used as template; 3, 50 ng of yeast tRNA; 4 and 5, 25 and 50 ng of total RNA, respectively. To determine the size of the fragments observed, sequencing reaction products using the same primer were electrophoresed concurrently and are shown to the left. APL23 is 37 or 38 nt short of full length. (*B*) Partial genomic DNA sequence containing the 3′ splice acceptor sequence (underlined), which is located 15 nt upstream of the 5′ end of APL23, is shown; the presumed splice site is indicated by an arrow. (*C*) PCR amplification from reverse-transcribed RNA using splice-leader-sequence-specific and *apl-1*-specific primers. Lanes: 1, amplification using the SL1- and *apl-1*-specific primers [a single band (arrow) is observed]; 2, amplification using SL2- and *apl-1*-specific primers; 3, amplification using SL1-, SL2-, and *apl-1*-specific primers and no cDNA, as a negative control. Size markers are indicated to the left.

confirmed that the product corresponds to the *apl-1* sequence (data not shown). The *apl-1* transcripts, therefore, appear to be trans-spliced to an SL1 leader sequence.

***In Vitro* Transcription/Translation of *apl-1*.** To demonstrate that the *apl-1* cDNA could express a protein of the predicted size, clone APL23 was first subcloned into a pGEM expression vector. This plasmid was then subjected to coupled *in vitro* transcription/translation in a wheat germ lysate. One specific product with an apparent molecular mass of 110 kDa was observed in the translation of clone APL23 (Fig. 3); no proteins were observed in the control samples. Although the size of the *in vitro*-translated protein differs from the predicted size of 78.8 kDa for the translation product of clone APL23, a similar anomalous migration has been reported by Dyrks *et al.* (47) when translating human APP_{695} cDNA *in vitro*; this

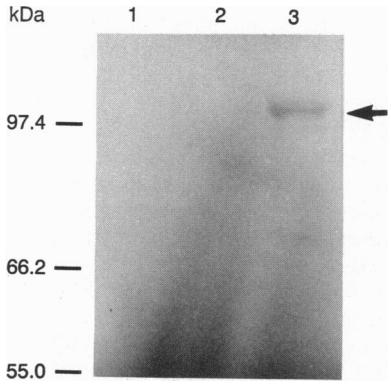

FIG. 3. *In vitro* transcription/translation of an *apl-1* cDNA (clone APL23). Translation in wheat germ lysate was done in the presence of [^{35}S]methionine and cysteine. Products were separated by SDS/PAGE and visualized by autoradiography. The migration of nonradioactive molecular mass markers is indicated to the left. Lanes: 1, pGEM vector alone (no insert); 2, clone APL23 in the antisense orientation; 3, translation of clone APL23, showing one specific band (arrow) corresponding to ≈110 kDa.

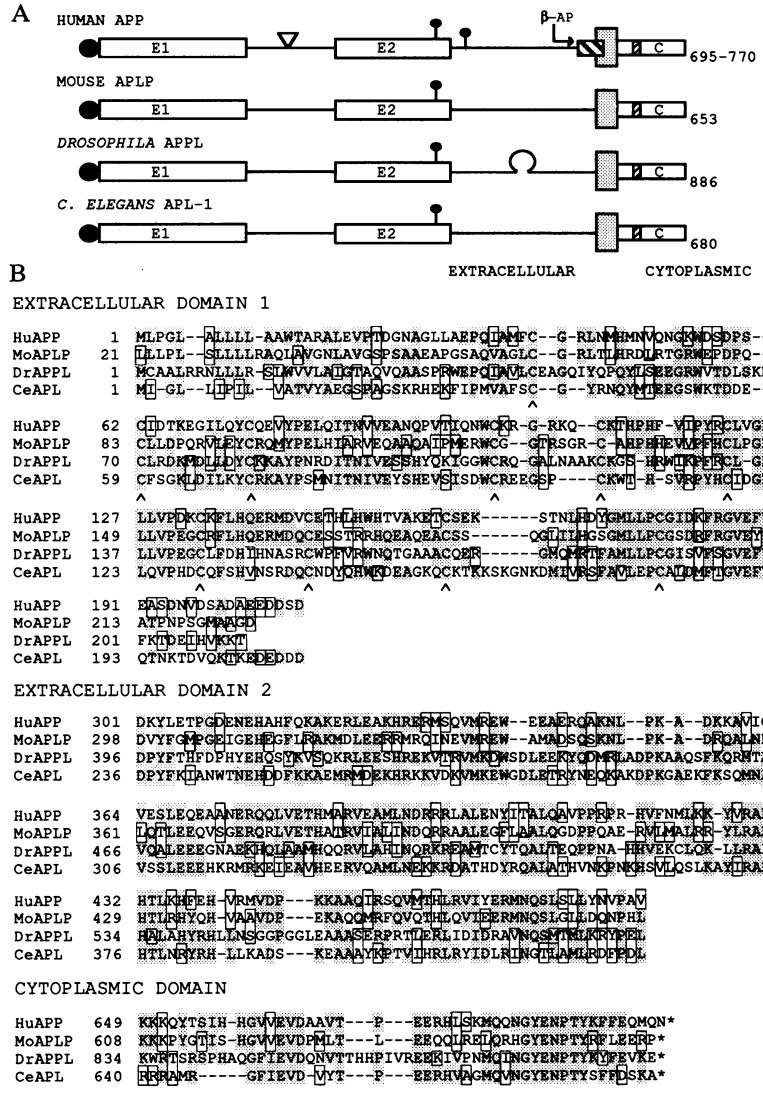

FIG. 4. Homology between APL-1 and other members of the APP-like family of proteins. (*A*) General characteristics of some members of the APP family. The open boxes represent the conserved extracellular regions E1 and E2 and cytoplasmic region C; the shaded boxes represent the putative membrane spanning regions. The signal peptide is shown as a solid circle at the N terminus. Glycosylation sites are shown as balloons; a clathrin-coated vesicle targeting sequence found in every APP and APP-like protein is shown as a hatched box in the C domain. Some of the human transcripts contain a Kunitz-type protease inhibitor sequence (marked as a triangle). (*B*) Amino acid sequence comparison. The regions of highest homology between the human APP_{695} (3), mouse APLP1 (APLP; ref. 14), *Drosophila* APPL (30), and *C. elegans* APL-1 are presented. The shaded residues are identical, and the boxed residues represent conservative changes. The 12 conserved cysteines are indicated by carets.

retarded migration has been attributed to poor binding of SDS due to the very acidic region in the extracellular domain of APP. Because APL-1 also contains a large number of acidic residues in its putative extracellular domain, the 110-kDa protein is likely to correspond to full-length APL-1.

Comparison of APL-1 to Other Amyloid-Related Proteins. A family of APP proteins, including human APP (3) and APLP2 (16, 17), *Drosophila* APPL (35), and mouse APLP1 (14), has been identified. Three main regions of sequence homology have been described among members of this family: two regions in the extracellular domain (E1 and E2) and one in the cytoplasmic domain (C; Fig. 4*A*). APL-1 shares extensive sequence homology with the APP family in these three conserved regions (Fig. 4*B*). The extent of similarity between APL-1 and other members of the family is approximately the same as the extent of similarity among them. The similarity between APL-1 and APP_{695} is 46% for E1, 49% for E2, and 71% for the highly conserved C domain.

APL-1 shares several characteristics with members of the APP family: (*i*) APL-1 contains a large number of negatively charged residues in its extracellular domain. (*ii*) APL-1 contains the stretch of completely conserved residues, GVEFVCCP, found at the C-terminal end of E1. (*iii*) The positions of 12 cysteine residues in E1 are conserved in APL-1. (*iv*) APL-1 contains a consensus sequence for N-linked glycosylation at the C-terminal end of E2. (*v*) The cytoplasmic domain of APL-1 contains the sequence (NPXY) implicated in clathrin-coated vesicle targeting (23) and 14 of the 20 aa implicated in the interaction of APP with guanine nucleotide binding protein G_o (48). The transmembrane domain of APL-1 does not share any extensive similarity with members of the APP family, other than the hydrophobicity of its residues and the presence of four positively charged residues at its C terminus (14). As with human APLP2 (16, 17), mouse APLP1 (14), and *Drosophila* APPL (30), APL-1 does not contain a sequence similar to the β-AP; in addition, like mouse APLP-1 and *Drosophila* APPL, APL-1 does not appear to contain the Kunitz-type protease inhibitor domain found in some human transcripts (3, 16, 29).

DISCUSSION

A family of APP proteins has been identified in diverse organisms, including humans, mammals, and *Drosophila*. We have identified an APP-related gene, *apl-1*, in the nematode *C. elegans*. The *C. elegans* APL-1 protein is a member of the APP family of proteins. In addition to sequence similarities, some important features are also conserved between all members of the family, suggesting that these similarities may be of functional significance. For instance, the presence of 12 conserved cysteines in the extracellular domain of the proteins suggests that there is conservation in structure, and the conserved NPXY sequence found in the cytoplasmic domain suggests that every member of the family may be processed similarly by the clathrin-coated-vesicle-mediated lysosomal pathway.

The *apl-1* transcripts undergo two forms of posttranscriptional processing: trans-splicing and alternative polyadenylylation. Although trans-splicing is common among many *C. elegans* mRNAs, the functional significance of acquiring an SL1 leader sequence is unknown. The multiple polyadenylylation signals used by *apl-1* result in 3′ untranslated regions of different lengths. Since variations in 3′ untranslated regions can affect mRNA stability (49), this processing may serve to control APL-1 expression. The human *APP* gene produces different transcripts from a single gene by alternative splicing and alternative polyadenylylation. We have not yet isolated *apl-1* cDNAs that differ in the coding region.

Two classes of *APP*-related genes are present in mammals: one whose translation product contains the β-AP sequence (3, 50) and one whose translation product does not (14, 16, 17). Only one class of *APP*-related genes, the one whose translation product does not contain the β-AP sequence, has been identified in *Drosophila* (30) and *C. elegans*. These data suggest that other *APP*-related genes are also present in *Drosophila* and *C. elegans* or that gene duplications have occurred during the evolution of higher animals. The β-AP generated from the other class of APP-like proteins, therefore, may serve some specific role in vertebrates. It is of obvious significance to determine whether an *APP*-related gene that encodes a β-AP-containing protein is present in *C. elegans*. To our knowledge, *C. elegans* is the simplest organism in which an APP-related protein has been found thus far. Elucidation of the role of the *APP*-related genes in *C. elegans* may give us information that is relevant to the function of APP in humans.

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