Similar Alternative Splicing of a Non-homologous Domain in βA4-Amyloid Protein Precursor-like Proteins*

Rupert Sandbrink‡‡, Colin L. Masters§, and Konrad Beyreuther†

From the ‡Center for Molecular Biology Heidelberg (ZMBH), University of Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany and the §Department of Pathology, University of Melbourne, Parkville, Victoria 3052, Australia

The βA4-amyloid protein precursor (APP) is a transmembrane glycoprotein that is the source of the characteristic βA4-amyloid deposits found in Alzheimer brains. It is expressed in several isoforms generated by alternative splicing of exons 7, 8, and 15, of which the leukocyte-derived APP mRNAs lacking exon 15 are significantly expressed in non-neuronal tissues, but not in neurons. The recent finding of APP-like proteins prompted us to analyze alternative splicing of the nearest relative of APP, the amyloid protein precursor homologue (APPH) or amyloid precursor-like protein 2 (APLP2). We were able to show that there are two alternatively spliced inserts, i.e. the Kunitz protease inhibitor domain and a 12-amino-acid-encoding region on the NH₂-terminal side of the transmembrane domain, which is part of the region of highest divergence between APP and APLP2/APPH. Analysis of the tissue-specific differential expression of the resulting four APLP2/APPH mRNA isoforms revealed that isoforms lacking the latter, non-homologous insert are highly expressed in non-neuronal tissues, but only weakly in neurons. While this resembles the tissue-specific alternative splicing of exon 15 of APP, expression of the Kunitz protease inhibitor-encoding exon of APLP2/APPH is abundant in both neuronal and non-neuronal tissues and thus differs from APP. Because of the similar regulation of alternative splicing of exon 15 of APP and the described APLP2/APPH insert, and because of structural similarities of the sequences and the predicted secondary structures, a functional homology of alternatively spliced isoforms of APP and APLP2/APPH is suggested.

A characteristic feature of Alzheimer's disease is the cerebral deposition of βA4-amyloid (1, 2). The βA4 peptide consists of maximally 43 residues and is derived from a larger transmembrane glycoprotein termed the βA4-amyloid protein precursor (APP)* (3–5) by a partially characterized metabolic pathway (6–8). The major part of APP molecules, however, undergoes proteolytic cleavage within the βA4 region to give rise to soluble, non-amyloidogenic secretory products (9, 10). Several isoforms of APP have been reported that are generated by alternative splicing and named according to their length in amino acids. The domain and exon structure of APP770, the full-length APP isoform, is given in Fig. 1 (11). This isoform contains a Kunitz protease inhibitor (KPI) domain of 56 amino acids, encoded by exon 7 (12–14). Additionally, it contains a 19-amino-acid domain encoded by exon 8 and skipped in APP751, with homology to the MRC OX-2 antigen (15). Exon 7-encoding APP isoforms were found to be the major primary translation products in peripheral organs (12–14). Both these exons are missing in APP695, the most abundant APP transcript in brain (12, 16–18). On the RNA level, an APP714 isoform with exon 8, lacking exon 7, was also detected (17–18).

The aforementioned lengths of the APP isoforms are correct under the assumption that all exons other than exon 7 and 8 are being expressed. Recently, however, another alternatively spliced site was identified, involving the 54 bp of exon 15. This exon codes for 18 amino acids preceding the NH₂-terminus of the βA4 region of APP by 16 amino acids. The APP transcripts excluding exon 15 were first discovered in peripheral leukocytes and immunocompetent cells of the brain and are, therefore, denoted leukocyte-derived APP (L-APP) mRNAs (19, 20). By means of a quantitative polymerase chain reaction assay from reverse-transcribed RNA (RT-PCR), it was then shown that L-APP mRNA isoforms are ubiquitously expressed with the exception of neurons (21).

In peripheral rat tissues, L-APP mRNA isoforms represent between 25% (skeletal muscle) and ~70% (spleen, pancreas) of total APP transcripts. All four possible APP mRNA isoforms without exon 15 were shown to exist, i.e. L-APP752, L-APP733, L-APP696, and L-APP677. In the rat central nervous system, where L-APP expression accounts for about 4% of total APP mRNA, non-neuronal tissues like meninges, plexus choroides, and brain vessels also showed significant expression of L-APP transcripts. While primary cultured glial cells equally contain high portions of L-APP mRNA, primary cultured neurons were shown not to express detectable levels of L-APP transcripts (21).

A distinct physiological role for the ubiquitously distributed APP has not yet been determined. Several putative functions have been ascribed for the transmembrane and/or secreted APPs (discussed in Ref. 20). Apart from the putative function of the KPI insert (exon 7) as protease inhibitor, however, the functional significance of alternative splicing of exon 8 and 15 has not been elucidated yet.

Recently, cDNAs were isolated coding for proteins related to APP. From a mouse brain library, a cDNA was cloned that encodes a protein whose predicted amino acid sequence is 42%
Alternative Splicing of APLP2/APPH

The family of APP-like proteins. The domain structures of human APP, human APLP2/APPH (in all other figures referred to as APP), murine CDE1-binding protein, rat sperm membrane protein YWK-II, and murine APLP1 (initially termed APLP) are illustrated. Regions of high homology are indicated by diagonal hatching or dotted. SP denotes the presumed signal peptide (cross-hatched), KPI the Kunitz protease inhibitor domain. βA4, the βA4-amylloid region of APP (black bar), while TM and dark striped bars indicate the putative transmembrane domains. For APP, boundaries of regions encoded by individual exons are shown. The conserved cysteine-rich region and the less conserved acidic region are concentrated within two regions of the extracellular part of the protein. The cytoplasmatic domain, while the region on the NH₂-terminal side of the transmembrane domain shows the highest divergence.

More recently another full-length cDNA of this emerging multigene family was identified. This molecule, which also shares the overall domain organization with APP, was termed amyloid precursor-like protein (APLP) (23) or amyloid precursor-like protein 2 (APLP2) (24) (Fig. 1). As identified by Sprecher et al., it is 763 amino acids in length and encodes a KPI domain and residues 613-624, which are part of the region on the NH₂-terminal side of the transmembrane domain which shows the highest divergence.

As recently another full-length cDNA of this emerging multigene family was identified. This molecule, which also shares the overall domain organization with APP, was termed amyloid precursor-like protein (APLP) (23) or amyloid precursor-like protein 2 (APLP2) (24) (Fig. 1). As identified by Sprecher et al., it is 763 amino acids in length and encodes a KPI domain and residues 613-624, which are part of the region on the NH₂-terminal side of the transmembrane domain, while the region on the NH₂-terminal side of the transmembrane domain shows the highest divergence.

The homology between APP and APLP2/APPH is even higher than between APP and APLP1. As with APLP1, there is no significant similarity in a region NH₂-terminal to the transmembrane domain.

A partial-length cDNA had been isolated from a rat sperm library even before this (25) that appears to be identical with the COOH-terminal part of rat APLP2/APPH (Fig. 1). The sequence of the encoded protein YWK-II is highly homologous to human APLP2/APPH including a very high similarity regarding the region on the NH₂-terminal side of the putative transmembrane domain.

Besides APLP1, another murine cDNA encoding for an APP-like protein was recently identified, which was called CDE1-binding protein (26). By comparing its sequence to human APLP2/APPH, we realized that both sequences are highly homologous to each other. Of the 511 amino acids encoded by the published murine sequence, ~90% are identical to human APLP2/APPH. However, the first nucleotide of the murine sequence corresponds to position 551 of the coding sequence of human APLP2/APPH. As minor differences, there seem to be two missing nucleotides around position 16 and a surplus nucleotide at position 1479. More importantly, there is a deletion of 168 nucleotides at position 70/71 which exactly represents the KPI sequence. And, there is also another region of 12 amino acids which seems to be deleted. This is encoded by nucleotides 1919-1945 (residues 613-624) of APLP2/APPH, and this sequence is also part of the YWK-II protein. This region was also deleted in several human APLP2/APPH cDNA clones analyzed (23, 24).

Taken together, the described sequences of human APLP2/APPH, murine CDE1-binding protein, and rat YWK-II seem to represent the species-specific versions of the same protein. The differences between the murine and the human APLP2/APPH sequences indicate the existence of at least two alternatively spliced regions, residues 509-364 of APLP2/APPH representing the KPI domain and residues 613-624, which are part of the region on the NH₂-terminal side of the transmembrane domain in which APP and APLP2/APPH most strongly differ. This seems to constitute a striking parallel to the above described alternative splicing of APP. Therefore, we performed a detailed analysis of alternative splicing and tissue-specific expression of APLP2/APPH mRNAs in the rat.

Materials and Methods

Tissue Preparation and Primary Cell Culture—Perfused tissues were obtained from 2- or 8-month-old rats as described (21). Primary neuronal cultures and a mixed culture of neurons, astrocytes, and some microglial cells were prepared from septal cells of E18 rat brain and analyzed at 8 days in vitro. Cell culture conditions and preparation of astrocyte-enriched cultures have also been described (21).

Primers and APP cDNA Encoding Plasmids—The designation of PCR primers reflects the position of the primer in regard to the human APLP2/APPH cDNA sequence as published by Sprecher et al. (23). The following primers were used: sr11809, 5'-CACACACCACCTGCGGC-3' (nucleotides 51-71); sm1838, 5'-GGAGACGTACATATGAGAAGAATCC-3' (838-863); sm11099, 5'-ACCTCCCCCTCAGAACATGCATG3' (1099-1121); ar1809, 5'-CTCTCTCCTATTTCTGTCG-3' (1809-1830); ahm11099, 5'-ACATATCTGCTTGTCGAGACAGG-3' (1899-1920); ar11877, 5'-CCATCTGTGCTCTGACATGCAGTG3' (1877-1898); ar12242, 5'-CAAGCCGACACACCAGTCTAG3' (2360-2381). End-labeling of or1700, sm1838, and sm11099 was performed with polynucleotide kinase and [γ-32P]-ATP (Amersham or DuPont NE).
Fig. 2. RT-PCR assay to detect alternatively spliced APLP2/APPH mRNA isoforms. a, the two potential alternatively spliced inserts of APLP2/APPH are shown. Bottom, the series of PCR reactions employed in this study is depicted. The following primer pairs were used: PCR A, shl051/shl1099; PCR B, shl1099/orl1805; PCR C, shl838/orl1805; PCR D, shl1099/orl1877; PCR E, shl838/orl1877; and PCR F, sr1809/orl2342. Tm indicates the sequence coding for the transmembrane part of APLP2/APPH (gray). b, PCR products obtained with cortical and thymus cDNA preparations. Total RNA was extracted from perfused rat brain and thymus, and 2 μg were reverse transcribed and aliquots amplified for 35 cycles using the primer pairs described in a. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Cx, cortex; Thy, thymus; M, DNA molecular weight marker.

Using PCR analysis D and F, cDNA regions encoding more COOH-terminal parts of APLP2/APPH were analyzed. Two different PCR products were amplified with both primer pairs differing by ~40 bp. Again, it was proven by PCR analysis B that the occurrence of these two isoforms was due to the small region which the PCR products of both primer pairs D and F have in common. This region was defined to include the 36-bp sequence in which the described mouse and the human APLP2/APPH cDNA differ (expected length of PCR products: PCR D, 800 and 764 bp; PCR F, 555 and 519 bp).

Finally, with PCR analysis E, alternative splicing of both the KPI and this 36-bp region of APLP2/APPH was detected simultaneously. Three different bands representing four PCR products with the expected lengths were observed (1061 and 1015 bp (unresolved on this gel), 893 bp, and 857 bp).

**Sequencing Proves Alternative Splicing of Inserts Encoding the KPI Domain and the More COOH-terminal Located Region of 12 Amino Acids**—The four different isoforms detected by PCR analysis E (primer pair shl838 and orl1877) were further analyzed by sequencing. We therefore amplified these four APLP2/APPH cDNA fragments from a cDNA preparation of rat cardiac muscle and purified them by electrophoresis and subsequent electroelution. They were then reamplified using the same primer pair and their purity checked on the gel shown in Fig. 3a. Other aliquots were then used for thermal cycle sequencing, proving the presence of the KPI domain-encoding sequence (168 bp) in fragments 1 and 2 and its absence in fragments 3 and 4 (Fig. 3b). On the other hand, only fragments 1 and 3, but not fragments 2 and 4 contain a 36-bp insert at their 3′-end (Fig. 3c). This alternatively spliced sequence exactly represents the 36-bp region omitted in the murine CDE1-binding protein cDNA. Since the full-length APLP2/APPH isoform (corresponding to fragment 1) should consist of 763 residues as predicted from the human cDNA sequence (32), the other APLP2/APPH isoforms (fragments 2–4) were calculated to consist of 751, 707, and 695 residues. These four different isoforms were therefore denoted as APLP2/APPH763, L-APLP2/APPH751, APLP2/APPH707, and L-APLP2/APPH695.

In analogy to APP denotation, we introduced the prefix "L-" to selectively label those isoforms lacking the alternatively spliced 12-amino-acid region on the NH2-terminal side of the transmembrane domain. As will be shown later, alternative splicing of this insert exhibits extensive parallels to alternative splice patterns of APP transcripts regarding exon 15.

**Rat Sperm Membrane Protein YWK-II and Murine CDE1-binding Protein Are the Species-specific Versions of Human APLP2/APPH**—We then continued sequencing of the four described PCR products to obtain their complete sequence. Apart from the alternatively spliced regions, this sequence was identical for all four PCR fragments. The last 152 nucleotides are completely identical to the YWK-II cDNA sequence published. The protein sequence intermediate to the two alternatively spliced inserts is highly homologous to the human APLP2/APPH sequence (97%) and to the murine CDE1-binding protein (98%). We therefore conclude that rat APLP2/APPH as ana-
Alternative Splicing of APLP2/APPH

No APLP2/APPH Equivalent to the Alternatively Spliced Exon 8 of APP Was Observed—Our results indicate that APLP2/APPH and APP not only resemble each other in their domain structure, but also in alternative splicing. The question arises whether the alternatively spliced exon 8 of APP (expressed in APP770, L-APP752, APP714, and L-APP696) also has an equivalent expressed in one or more of the alternatively spliced APLP2/APPH mRNA isoforms. However, no homologue to exon 8 of APP was detected in any of the four rat heart APLP2/APPH transcripts analyzed. No further APLP2/APPH mRNAs were detected within this cDNA preparation nor in a large number of other rat organs and tissues including brain, as described later. Therefore, in contrast to APP, either the rat APLP2/APPH gene does not contain the MRC OX-2 domain-encoding exon, or this region is constitutively spliced during APLP2/APPH hnRNA processing.

RT-PCR Assay for Quantitation of APLP2/APPH mRNA Isoforms—For quantitation of alternatively spliced APLP2/APPH mRNAs by RT-PCR, the primer pair sm1838 and ar1877 was employed after radioactive labeling of ar1877 on its 5'-end with polynucleotide kinase and [γ-32P]ATP. Initially we tested the PCR amplification rate of the different products for different cycling times, using a cDNA pool from perfused rat hippocampus (Fig. 4). Four different bands were detected corresponding to APLP2/APPH763, L-APLP2/APPH751, APLP2/APPH707, and L-APLP2/APPH695 (Fig 4a). Thus, all four APLP2/APPH isoforms detected in rat cardiac muscle are also observed in rat hippocampus. The amount of radioactivity incorporated in the individual bands was then measured and the logarithms plotted as a function of the PCR cycle number (Fig. 4b). As illustrated, the slopes of the curves remain constant through 32 cycles with a similar amplification efficiency for all four APLP2/APPH mRNA isoforms (17, 21). Therefore, the initial relative amounts of the different APLP2/APPH transcripts in the sample should be identical to the numerical values as determined for the corresponding PCR products. As was shown for a similar RT-PCR assay for quantitation of APP mRNA isoforms, the relative amounts of individual mRNA isoforms obtained by use of such a method are highly accurate and well reproducible (21).

All Four Alternatively Spliced APLP2/APPH mRNA Isoforms Are Ubiquitously Expressed in Peripheral Rat Tissues—For a detailed analysis of APLP2/APPH mRNA isoforms in peripheral tissues, the aforementioned quantitative PCR assay was used. The corresponding autoradiograms are shown in Fig. 5, a and b. In general, four different bands are visible. As described, the upper two bands correspond to the KPI domain-encoding APLP2/APPH transcripts, while the second from the cardiac muscle cDNA preparation. Initial amplification was performed as described (PCR analysis E, Fig. 2). PCR products were separated by agarose gel electrophoresis, individually electroeluted, and reamplified. Aliquots were compared by gel electrophoresis with the initial mixture of amplified cDNA fragments, b and c. sequence analysis of the individual APLP2/APPH cDNA fragments. Aliquots of the purified and re-amplified fragments 1–4 from a were sequenced by thermal cycle sequencing using sm1838 (b) or ar1877 (c) as sequencing primer. Alternatively spliced inserts are indicated and the derived sequences of the sense strand for the boundary regions given. T, C, G, and A denote the lanes of the corresponding sequencing reactions.
top and the lowest band are due to the two L-APLP2/APPH transcripts. All four APLP2/APPH mRNA isoforms are expressed in all peripheral tissues analyzed, including the two L-APLP2/APPH isoforms. This is very similar to L-APP expression which was recently also found to occur in all of these peripheral rat tissues (21). Quantitation of the four cDNA fragments revealed that in peripheral tissues L-APLP2/APPH expression varies between 35% (skeletal muscle) and 92% (epididymis, followed by uterus and ovary) of total APLP2/APPH mRNAs (Table I). Comparison with results obtained for selected unperfused tissues showed that the results are virtually independent of perfusion (data not shown). This is in agreement with the only very weak levels of PCR products observed for blood cDNA, which was also the case for pancreatic cDNA.

**FIG. 4.** Quantitation of APLP2/APPH mRNA isoforms using RT-PCR. a, PCR products of hippocampal cDNA for different numbers of PCR cycles. 2 μg of hippocampal RNA (Sprague-Dawley rat, 1 year of age) were reverse transcribed, the resulting cDNAs amplified for the stated number of cycles using unlabeled sm1838 and 32P-end-labeled orl1877 as PCR primers, and the products separated by denaturing polyacrylamide gel electrophoresis. b, incorporation of radioactivity as a function of the PCR cycle number. The bands from about equal for all bands analyzed. The amplification efficiencies remain constant through 32 cycles and were about equal for all bands analyzed.

**Fig. 5.** Tissue-specific expression of APLP2/APPH mRNAs. a and b, RT-PCR analysis of APLP2/APPH mRNA expression in peripheral tissues. CDNA preparation, amplification by PCR with a total number of 26 cycles, and analysis of PCR products were performed as in Fig. 4. A water control was performed which included all steps of RNA extraction, cDNA synthesis, and PCR, and no detectable signals were observed (data not shown). GI, gland. c, RT-PCR analysis of APLP2/APPH mRNAs in tissues of the central nervous system, in related tissues, and in primary cultured brain cells. Septal neurons were cultured for 8 days in vitro in a serum-free medium containing cytosine arabinoside, and mixed brain cells indicate septal cells cultured for the same period in a serum-containing medium without cytosine arabinoside. Sp., spinal; N. trig., trigeminal nerve; N. opt., optic nerve; Pl. chor. IV, choroid plexus from the IVth ventricle; Pl. brach., plexus brachialis.

isoforms was also observed for the trigeminal nerve; in contrast, to spinal cord, however, this tissue mainly expresses L-APLP2/APPH763 mRNA. In all neural tissues examined other than the described brain regions, which includes cranial, spinal, and peripheral nerves, significantly higher relative amounts of L-APLP2/APPH mRNAs were observed. For non-neuronal tissues such as meninges and choroid plexus, the APLP2/APPH splicing pattern resembles the typical expression pattern of peripheral organs. This includes expression of L-APLP2/APPH transcripts of about 70%.

Primary Cultured Astrocytes and Neurons Are Similar in Their Expression of KPI-APLP2/APPH mRNAs, but Differ in Regard to L-APLP2/APPH mRNA Expression—For comparison of neuronal with non-neuronal cells of the central nervous system, primary cultures of E18 rat septal cells were prepared. As shown in Fig. 5c, primary cultured septal neurons predominantly expressed APLP2/APPH763 mRNA (about 86%) (Table...
Radiolabeled and electrophoretically separated RT-PCR products (Fig. 5) were analyzed on a PhosphorImage and relative amounts calculated in percent. For each band, a corresponding background value was subtracted. 763, 751, 707, and 695 represent the corresponding APLP2/APP mRNAs. L-APPH denotes the sum of L-APLP2/APP763 and L-APLP2/APP695, while KPI represents the KPI domain-encoding mRNA isoforms (sum of APLP2/APP751 and L-APLP2/APP751).

| APLP2/APP mRNA isoforms | 763 | 751 | 707 | 695 | L-APPH | KPI |
|-------------------------|-----|-----|-----|-----|--------|-----|
| Salivary gland           | 12  | 65  | 2   | 20  | 85     | 78  |
| Tongue                  | 19  | 52  | 7   | 22  | 74     | 71  |
| Lung                    | 23  | 54  | 7   | 16  | 70     | 77  |
| Aorta                   | 22  | 49  | 3   | 26  | 75     | 71  |
| Heart                   | 44  | 23  | 19  | 43  | 45     | 68  |
| Stomach                 | 18  | 67  | 3   | 11  | 79     | 86  |
| Small intestine         | 14  | 71  | 3   | 12  | 85     | 83  |
| R ectum                 | 14  | 65  | 2   | 19  | 84     | 79  |
| Skeletal muscle         | 62  | 31  | 3   | 4   | 35     | 94  |
| Spleen                  | 18  | 65  | 4   | 13  | 78     | 83  |
| Thymus                  | 22  | 62  | 4   | 12  | 75     | 84  |
| Lymph node              | 22  | 60  | 4   | 15  | 74     | 81  |
| Liver                   | 13  | 71  | 3   | 13  | 84     | 84  |
| Kidney                  | 12  | 56  | 4   | 28  | 84     | 68  |
| Adrenal gland           | 31  | 54  | 6   | 9   | 63     | 85  |
| Thyroid gland           | 22  | 60  | 5   | 15  | 75     | 80  |
| Skin                    | 18  | 69  | 2   | 10  | 79     | 87  |
| U terus                 | 7   | 59  | 2   | 31  | 91     | 66  |
| Ovary                   | 7   | 63  | 3   | 27  | 90     | 71  |
| Testis                  | 7   | 54  | 6   | 33  | 87     | 61  |
| Epididymis              | 6   | 87  | 2   | 5   | 92     | 94  |
| Prostate gland          | 19  | 61  | 5   | 15  | 75     | 80  |
| Ves icular gland         | 14  | 67  | 3   | 16  | 83     | 81  |
| Bladder                 | 14  | 67  | 5   | 14  | 81     | 81  |

Cerebellum: 87, 4, 8, 1, 5, 91
Hippocampus: 83, 6, 9, 2, 8, 89
Cortex: 73, 9, 14, 4, 13, 81
Sensory cord: 50, 11, 44, 15, 26, 41
Cauda equina: 43, 44, 4, 9, 53, 88
N. trigeminus: 18, 14, 20, 49, 62, 31
N. opticus: 45, 20, 16, 18, 39, 66
Mesolonges: 23, 41, 10, 26, 67, 64
Choroid plexus IV: 19, 43, 8, 31, 74, 61
Retina: 22, 62, 4, 12, 75, 84
Large vessels: 27, 33, 9, 31, 64, 60
Plexus brachialis: 26, 36, 14, 25, 60, 62
Septal neurons: 86, 6, 7, 1, 7, 92
Mixed brain cells: 68, 23, 5, 3, 26, 91
Astrocytes: 15, 53, 5, 26, 79, 69

1. L-APLP2/APP mRNA isoforms were present in these cultures only up to ~7%, mainly as KPI-encoding transcript. Similar results were obtained for primary cultured neurons from other brain regions (data not shown). Therefore, the APLP2/APP mRNA expression pattern of neurons cultured in vitro highly resembles the splice pattern obtained for intact brain regions as described above.

Mixed septal cultures with a significant number of proliferating non-neuronal cells showed a different APLP2/APP splice pattern (Fig. 5c), in which the contribution of L-APLP2/APP mRNA isoforms was assessed to be ~26%. In cultures highly enriched for astrocytes, considerable amounts of L-APLP2/APP transcripts were observed contributing to ~79% (Fig. 5c). Mainly the KPI motif encoding L-APLP2/APP763 was detected. Hence, primary cultured astrocytes and neuronal cells are similar in regard to predominant KPI expression, but differ in alternative splicing of the more downstream, 12-amino-acid encoding region. This leads to predominant L-APLP2/APP expression in astrocytes, while only marginal amounts of L-APLP2/APP mRNA isoforms are expressed in neuronal cells.

### DISCUSSION

Among the two recently identified APP-like proteins, human APLP2/APP is the nearest APP relative identified so far. The striking degree of amino acid sequence similarity and the conservation of overall domain structure between human APP and human APLP2/APP prompted us to identify the rat version of APLP2/APP. We analyzed the partial cDNA sequence extending from nucleotide 865 to 1875 of the coding sequence, as presumed by comparison to the human APLP2/APP cDNA sequence. As we were able to show for rat brain and thymus by RT-PCR analysis covering the almost complete coding sequence of APLP2/APP, this partial cDNA encompasses the two principal sites of alternative splicing. Four different isoforms were detected, resulting from the combination of these two alternatively spliced inserts. By analyzing the composition and the tissue distribution of these rat APLP2/APP mRNA isoforms, we present for the first time a detailed analysis of alternative splicing of one of the recently identified APP-like proteins. We were able to show that the previously described murine CDE1-binding protein and rat sperm membrane protein YWK-II are the species-specific versions of APLP2/APP. The principal differences between the murine and the human APLP2/APP sequence can clearly be explained by alternative splicing of these two inserts.

These two alternatively spliced regions were characterized as follows. First, APLP2/APP mRNA contains a KPI-encoding region highly homologous to exon 7 of the APP gene. Expression of an MRC OX2-region homologous to exon 8 of the APP gene was not detected. The second alternatively spliced insert is a 12-amino-acid encoding sequence that is part of the region with the highest divergence in comparison to APP. Strikingly, APP mRNA also exhibits an alternative splicing of a similar sequence, exon 15, in exactly this non-homologous region. APP transcripts lacking this exon have been previously termed L-APP mRNAs. By analogy, we therefore denoted the four observed APLP2/APP mRNA isoforms as APLP2/APP763, APLP2/APP751, APLP2/APP767, and APLP2/APP695 transcripts; the latter two are lacking the KPI-encoding sequence. As with APP denotation, the numbers given correspond to the presumed number of residues. However, these APLP2/APP isoforms have to be distinguished from APP isoforms denoted with similar numbers (i.e., 695 and 751).

We then developed a quantitative RT-PCR assay to assess tissue-specific APLP2/APP splice patterns. Although there are interesting variations among the organs analyzed, two principal patterns of differential expression of APLP2/APP mRNAs were observed. In most of the non-neuronal tissues, both from peripheral organs and from the central nervous system, including meninges, brain vessels, choroid plexus, and primary cultured astrocytes, the KPI-encoding L-APLP2/APP751 mRNA was the predominant isoform, followed by APLP2/APP763 and L-APLP2/APP695 mRNAs. In brain regions and primary cultured neurons, however, a significantly different expression pattern was observed. APLP2/APP763 mRNA was in fact the most abundant isoform here. APLP2/APP767 was slightly more strongly expressed in these tissues than L-APLP2/APP751 and L-APLP2/APP695 mRNAs. Hence, L-APLP2/APP mRNA expression is high in peripheral organs, but low in neuronal tissues. KPI-encoding APLP2/APP mRNAs, however, are the predominantly expressed APLP2/APP transcripts in both neuronal and peripheral tissues.

When these results are compared with the corresponding APP expression patterns (21), alternative splicing of the 12-amino-acid-encoding APLP2/APP insert is found to be even
more similar to APP alternative splicing than that of the KPI-encoding region. With APP, both L-APP and KPI-encoding transcripts are highly expressed in non-neuronal tissues, while their expression in rat brain and in primary cultured neurons is quite low. Therefore, differential expression of L-APLP2/APPH mRNAs very much resembles L-APP mRNA expression.

In primary cultured septal neurons, L-APLP2/APPH transcripts contributed to -7% of total APLP2/APPH mRNAs. Using the same cDNA pool, L-APP mRNAs had previously been assessed to represent less than 1% of total APP transcripts. Hence, in neuronal cells, the relative amount of L-APP mRNAs is significantly lower than the corresponding contribution of L-APLP2/APPH transcripts. This was also the case in peripheral organs: the median value of the L-APLP2/APPH mRNA portion in the 24 peripheral tissues was determined as 79%, while the median value of L-APP mRNA in the same tissues was only 47%. Nevertheless, the “L-APP position” and the “L-APLP2/APPH position” of a given peripheral organ in this list of tissues seems to be quite conserved. For instance, skeletal muscle exhibits the lowest relative amount of both L-APLP2/APPH (35%) and L-APP (25%) transcripts. Surprisingly, the alternatively spliced inserts resulting in L-APP and L-APLP2/APPH formation are part of exactly that region of about 130 residues on the NH2-terminal side of their transmembrane domain in which APP and APPWAPLPB most strongly differ. Thus, the question of common features in the

**Fig. 6.** Comparison of amino acid sequence and predicted secondary structures of rat APP and APLP2/APPH. Only the part consisting of the non-homologous region, the transmembrane domain, and the intracellular, COOH-terminal domain is illustrated. a, amino acid sequence. Alignment according to the GAP program (Genetics Computer Group program package version Unix-7.2) using the algorithm of Needleman and Wunsch. Identical (x) and strongly (: ) or less well (.) related amino acids are indicated. On the left and on the right side, the number of the corresponding residue is given. Single underline, transmembrane domains; double underline, alternatively spliced inserts; bold, proline residues.

b, predictions of secondary structure. Prediction according to the Protean program (DNASTAR) using the indicated algorithms. Black bars denote those regions for which the given type of secondary structure is predicted. The transmembrane domain (Tm), and the alternatively spliced inserts are indicated; Res., residue. Small vertical arrowheads denote proline residues.
Amino acid sequence and secondary protein structure arises which could indicate a function regulating conformation. We therefore compared the APP and APLP2/APPH sequence of this divergent region plus the transmembrane and the intracellular, COOH-terminal domain (Fig. 6c). The alternatively spliced inserts do not only differ in length (18 versus 12 residues), but also exhibit a significantly different distance to the beginning of the transmembrane region. However, in both APP and APLP2/APPH these inserts represent the COOH-terminal end of a proline-rich region (9 prolines in APP, 8 in APLP2/APPH). Predications for the corresponding secondary structures are illustrated in Fig. 6b (proline-residues marked by small arrowheads). For the highly homologous transmembrane and COOH-terminal domains of APP and APLP2/APPH, a very similar secondary structure and hydrophilicity is anticipated. For both proteins, the insert region is predicted to be of substantial hydrophilicity. Between the transmembrane region and the alternatively spliced insert, for both APP and APLP2/APPH, a significant α-helical region is expected (Chou-Fasman prediction). With the APLP2/APPH insert shifted to the NH₂-terminus, this α-helical region is also NH₂-terminally shifted. Both proline-rich regions (inserts plus adjacent regions on the NH₂-terminal side), however, contain several stretches with a high turn potential. This is emphasized by the prediction method of Garnier-Robson: the insert domains are expected to contain the most COOH-terminal turns of this part rich in turn regions, followed by an extended domain with only a low probability for turn regions.

The described features of the protein sequences and the predicted secondary structures indicate a similar spatial organization of the non-homologous regions of APP and APLP2/APPH and the alternatively spliced inserts within these domains. Because of this and the similarities in the tissue-specific regulation of APP and APLP2/APPH alternative splicing, a related function of this domain is suggested. It is well conceivable that the common structural features of the non-homologous domain are an indication of the regulation or modulation of a yet unknown functional activity of these proteins. We also postulate that deletion of this insert leading to L-APP and L-APLP2/APPH isoforms will result in similar changes of the structural characteristics of these proteins, suggesting a functional homology of the individual alternatively spliced isoforms of APP and APLP2/APPH. In view of the special position of neuronal alternative splicing (virtually all transcripts encode the alternatively spliced insert), the low frequency of the insert lacking L-APP and L-APLP2/APPH isoforms might well be closely related to typical neuronal function. The relevance of this characteristic neuronal feature for the degeneration of neurons in Alzheimer's disease should be further evaluated.

Revealing the restriction and differential expression not only of APP, but also of other members of the family of APP-like proteins contributes to our efforts to understand the physiological function of these proteins. This should help to elucidate the role of APP and its related proteins in the neuropathogenesis of Alzheimer's disease. A careful and profound analysis of similarities and differences between APP and the other members of this family might turn out to be a tool for understanding the special role of APP in the etiology of this disease.

Note Added in Proof—While the manuscript was in proof, we sequenced all the missing parts of the rat version of APLP2/APPH. The sequence data will appear in the EMBL/GenBank and DDBJ Nucleotide Sequence Databases under the accession number X77234 and are in press (Sandbrink, R., Masters, C. L., and Beyreuther, K. (1994) Biochim. Biophys. Acta, in press). Because of a deletion of two codons and an insertion of four codons within the acidic domain of rAPLP2/APPH as compared with its human and murine homologues, the four different rAPLP2/APPH isoforms raised by alternative splicing are now expected to consist of 765, 753, 709, and 697 amino acids.

REFERENCES

1. Gleuener, G. G., and Wong, C. W. (1984) Biochem. Biophys. Res. Commun. 120, 420–426
2. Masters, C. L., Simms, G., Weinmann, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985a) Proc. Natl. Acad. Sci. U. S. A. 82, 4245–4249
3. Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grassee, K. H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature 325, 733–736
4. Goldgaber, D., Leeman, M. I., McBride, O. W., Saffiotti, U., and Gajdusek, D. C. (1987) Science 235, 877–880
5. Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruno, G. A. P. St George-Hyslop, P., van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M., and Neve, R. L. (1987) Science 235, 880–884
6. Shoji, M., Golde, T. E., Cheung, T. T., Ghise, J., Estus, S., Shaffer, L. M., Cai, X. D., McKay, D. M., Tintner, R., Franpione, Y., and Younkin, S. G. (1992) Science 256, 129–132
7. Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaaszewski, B. L., Lieberberg, I., Koo, E. H., Schenk, D., Teplow, D. B., and Selko, D. J. (1992) Nature 359, 222–225
8. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindelhurst, C., McCormack, H., Woltfer, R., Selko, D. J., Lieberberg, I., and Schenk, D. (1992) Nature 359, 325–327
9. Siodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A., and Price, D. (1990) Science 248, 493–496
10. Esch, F. S., Reins, P. S., Beatrice, E. C., Blechere, R. W., Culwell, A. R., Oltersdorf, T., McBride, D., and Ward, P. J. (1990) Science 244, 1122–1124
11. Yoshihaki, S., Sasaki, H., Ohara, K., Furuya, H., and Sakaki, Y. (1990) Gene (Amst.) 87, 267–269
12. Tanzi, R. E., McClatchey, A. I., Lamperti, E., Villa-Komaroff, L., Gusella, J. F., and Neve, R. L. (1988) Nature 331, 529–530
13. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shojiro, S., and Ito, H. (1988) Nature 311, 450–453
14. Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hau, G., Greenberg, B., Davis, K., Wallace, W., Lieberberg, I., Fuller, F., and Cordell, B. (1988) Nature 331, 525–527
15. Clark, M. J., Gagnon, J., Williams, A. F., and Arnley, A. N. (1985) EMBO J. 4, 113–118
16. Neve, R. L., Finch, E. A., and Dawes, L. R. (1988) Neuron 1, 689–677
17. Golde, T. E., Estus, S., Ustak, M., Younkin, L. H., and Younkin, S. G. (1990) Neurov 4, 293–297
18. Kang, J., and Muller-Hill, B. (1990) Biochim. Biophys. Res. Commun. 166, 1192–1200
19. Koenig, G., Moening, U., Czech, C., Prior, R., Bonati, R., Schreiter, G. U., Bauer, J., Masters, C. L., and Beyreuther, K. (1992) J. Biol. Chem. 267, 10804–10809
20. Moening, U., Koenig, G., Banati, R. B., Mechler, H., Czech, C., Gehrmann, J., Schreiter, G. U., Masters, C. L., and Beyreuther, K. (1992) J. Biol. Chem. 267, 20856–20860
21. Sandbrink, R., Masters, C. L., and Beyreuther, K. (1994) J. Biol. Chem. 269, 1510–1517
22. Wang, W., Leun, K., Magendanz, M., Gusella, J. F., Tanzi, R. E., and Solomon, F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10758–10762
23. Sprecher, C. A., Grant, F. J., Grimm, G., O’Hara, P. J., Norris, F., and Potter, D. C. (1993) Biochemistry 22, 4451–4456
24. Wasco, W., Guruhugavatula, S., Paradis, M. D., Romano, D. M., Siosid, S. S., Hymon, B. T., Nave, R. L., and Tanzi, R. E. (1993) Nature Genet. 5, 95–100
25. Yan, Y. C., Bai, Y., Wang, L., Miao, S., and Koide, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 97, 5455–5458
26. Vidal, F., Blangy, A., Rassoulzadegan, M., and Cuzin, P. (1992) Biochem. Biophys. Res. Commun. 180, 1236–1241
27. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
28. Saiak, R. K., Gelfand, D. H., Stoffel, S., Schafir, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. (1988) Science 239, 487–491