Identification and Differential Expression of a Novel Alternative Splice Isoform of the βA4 Amyloid Precursor Protein (APP) mRNA in Leukocytes and Brain Microglial Cells*

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The gene for the βA4-amyloid precursor protein (APP) consists of 19 exons which code for a typical N- and O-glycosylated transmembrane protein with four extracellular domains followed by the transmembrane domain and a short cytoplasmic domain. The βA4-amyloid sequence is part of exons 16 and 17. Several APP isoforms can be generated by alternative splicing of exons 7 and 8, encoding domains with homologies to Kunitz-type protease inhibitors and the MRC OX-2 antigen, respectively. The mechanism by which the pathological βA4 is generated is unknown, it is however a critical event in Alzheimer's disease and is distinct from the normally occurring cleavage and secretion of APPs within the βA4 sequence. We report here for the first time considerable APP mRNA expression by rat brain microglial cells. In addition we showed by S1 nuclease protection and polymerase chain reaction analysis of reverse transcribed RNA (RT-PCR) that T-lymphocytes, macrophages, and microglial cells expressed a new APP isoform by selection of a novel alternative splice site and exclusion of exon 15 of the APP gene. This leads to a transmembrane, βA4 sequence containing APP variant, lacking 18 amino acid residues close to the amyloidogenic region. The use of this novel alternative splice site alters the structure of APP in close proximity to the βA4 region and thus may determine a variant, potentially pathogenic processing of leukocyte-derived APP in brain.

Alzheimer's disease (AD) is characterized by the intracerebral deposition of large amounts of βA4-amyloid, a 4-kDa insoluble breakdown product of a larger transmembrane span-

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§ The abbreviations used are: AD, Alzheimer's disease; DS, Down's syndrome; APP, βA4-amyloid precursor protein; KPI, Kunitz-type protease inhibitor; bp, base pair(s); RT-PCR, polymerase chain reaction of reverse transcribed RNA; L-APP, leukocyte-derived APP; PMBL, peripheral mononuclear blood leukocytes; PHA, phytohemagglutinin; kb, kilobase(s).

MATERIALS AND METHODS

Isolation of Hematopoietic Cells—Fresh human peripheral mononuclear blood leukocytes (PMBL) were obtained from buffy coats of healthy or AD donors by Ficoll-Hypaque density centrifugation (24, 25). Cells were cultured at a density of 2 × 10⁶ cells/ml in

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Dulbecco's modified Eagle's medium (GIBCO/BRL, United Kingdom), containing 1 g/liter glucose, penicillin (50 units/ml), streptomycin (40 mg/ml), and 10% (v/v) fetal calf serum (GIBCO/BRL). Stimulation of the nonadherent resting T-lymphocytes was performed with 10 μg/ml phytohemagglutinin (PHA, Sigma) for different time intervals. B-cells were isolated from the PMBL fraction using a monoclonal antibody against the CD19 antigen coated with magnetizable polyurethane beads (Dynabeads Dynal, Norway). CD4+ and CD8+ T-cells were isolated using a monoclonal antibody coated with magnetizable polyurethane beads (Dynabeads, Dynal, Norway). Monoeytes were separated from lymphocytes by adherence to the plastic dishes. Adherent monocytes were either cultivated overnight in medium (RPMI 1640, 10% human AB serum), recovered from the dishes by vigorous pipetting, and resuspended in fresh medium and placed in rectangular Teflon bags (Biofolie 25, HERAEUS, Germany). At days 5 and 8, fresh medium was added to the bags. At the latter time point a 100% pure macrophage culture could be recovered by sedimentation of nonadherent cells (26). Macrophages were stimulated with either lipopolysaccharide (Salmonella abortus equi, generous gift of Chris Galanos, MPI für Immunologie, Freiburg, Germany; 100 ng/ml), or γ-interferon (600 units/ml, Bachem, Germany) for 8 h. Microglial cells were prepared from newborn rat brain as described previously and cultured for 2–4 weeks without further stimulation (27, 28).

Isolation of RNA—Total RNA was prepared as described (39). Poly(A)+ RNA was isolated by oligo(dT)-cellulose affinity chromatography (Pharmacia), following the manufacturer's protocol. Yield and quality of RNA preparations were determined by spectrophotometry and agarose gel electrophoresis (30).

S1 Probes and Nucleotide Protection Analysis—An 826-bp fragment, spanning the KPI- and OX-2 coding domain from position +320 to position +1135 of the APP770 cDNA, was subcloned into the phase M13mp18 vector. Two protected fragments were synthesized by annealing 1 pmol of M13 phase-specific universal oligonucleotide primer to 250 ng of single-stranded DNA probe. The primer was used in combination with 1 unit of Klenow polymerase (Boehringer Mannheim) in the presence of 25 mm DATP, dGTP, dTTP, 2 mm dCTP, and 40 μCi of 32P-dCTP (3000 Ci/mmole, Amersham) for 15 min at 37°C. Extended products were digested with SstI (for probe 1), HindIII (for probe 2), PvuII (for probe 3), and with BamHI (for probes 4 and 5) for 45 min. The labeled single-stranded DNA probe was purified on a 5% denaturing polyacrylamide gel, cut out, and eluted in 300 mm NaCl, 30 mm Tris/HCl, pH 7.5, 5 mm EDTA, pH 8.0, at 65 °C for 20 min. S1 probes were ethanol-precipitated and resuspended in DEPC-H2O to 50,000 cpm/μl. This solution (10 μl amounts see figure legends) was hybridized overnight (75% formamide, 0.4 μM NaCl, 20 mM Tris/HCl, pH 7.4, and 1 mm EDTA) at Ta−5°C to probe 1 or at Ta+3°C (calculated for longest protected fragment) to probes 2–5. S1 nucleic digestion (Boehringer Mannheim, 1200 units/sample) was performed for 2 h at 37°C (0.3 μM NaCl, 5 mm Na2SO4, 50 mm NaAc, pH 4.5, 0.5 μg of denatured calf thymus DNA) and uncutting products were ethanol-precipitated. The digestion products were resolved on 5% denaturing sequencing gels (32). Autoradiography was performed using Kodak X-Omat AR films, and appropriately exposed x-ray films with signals in the linear response range were used for densitometry on a Henschel Exscript 400 densitometer.

Reverse Transcription of RNA and Polymerase Chain Reaction (RT-PCR)—5 μg of total or 1 μg of poly(A)+ RNA (T-lymphocytes of control case Fig. 4A, lane 13) were reverse-transcribed with SuperScript™ (200 units, GIBCO/BRL), using an APP-specific primer 5′-AATTCAGACGGCTGTGGC-3′ (nucleotides 5′-2320–2340-3′) of probe 1, which was isolated from the cDNA. Sequencing reactions were analyzed on 5% denaturing polyacrylamide gels (30).

Southern and Northern Hybridization—RNA was run in denaturing 1% agarose, formaldehyde gels, and blot-transferred to GeneScreen Plus (Du Pont) nylon membranes following the manufacturer's protocol (35). RT-PCR products were analyzed by 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Agarose gels were transferred to GeneScreen Plus (Du Pont) nylon membrane following the manufacturer's protocol. Filters with RNA and DNA were hybridized with a Sari/Real L-APP cDNA restriction fragment using [α-32P]dCTP (Amersham) and nick translation (Boehringer Mannheim) (36). The buffer used for prehybridization (1 h) and hybridization contained 0.5 μM NaHPO4, pH 7.4, 7% sodium dodecyl sulfate, 1% bovine serum albumin. Hybridization was performed overnight at 65°C, washed three times in 40 mM NaHPO4, pH 7.4, 1% sodium dodecyl sulfate at 65°C, and exposed to a Kodak X-AR film. Exposure times for Southern hybridization were approximately 1 h, for Northern hybridization 4 days at −80°C (Fig. 5A) or 18 h at −80°C (Fig. 5F).

RESULTS AND DISCUSSION

Human PMBL were isolated, and the nonadherent T-lymphocytes were stimulated for 5 days with PHA. Total RNA was prepared and subjected to S1 nucleic protection analysis with probe 1, which scans from exon 3 to exon 9 of APP770 cDNA (Fig. 1). Three protected fragments were obtained using probe 1; they corresponded to splice products which contained neither the KPI nor the MRC-OX2 exons (APP695 mRNA), the KPI exon alone (APP751 mRNA), or both the KPI plus the MRC-OX2 exons (APP770 mRNA) (Fig. 2A). The ratio of alternatively spliced APP mRNAs was approximately 4/7/2 of APP695/APP751/APP770. To study additional alternative splicing more proximal to the βA4 region in APP, we used S1 nucleic protection analysis with a probe spanning the region from exon 9 to exon 17, which codes for the COOH-terminal domain of the βA4 sequence and part of the cytoplasmic domain. Two protected fragments with probe 2 were observed (Fig. 2B, lane 3). The longer protected fragment corresponded to transmembrane coding APP695, APP751, and APP770 mRNA splice forms (5, 10–12). The second protected fragment, obtained with probe 2, was about 280 bases shorter and suggested the presence of a novel form of APP mRNA. Subsequently, probes 3–5 were used in S1 nucleic protection analysis to determine that the

**Fig. 1.** Probes used in S1 nucleic protection analysis of APP mRNA. APP770 cDNA, regions scanned by S1 nucleic protection analysis. The hatched box denotes exon 7, encoding the domain with homology to KPI; the black box denotes exon 8 encoding the domain with homology to the MRC-OX2 antigen. Probe 1 is a restriction fragment from position +320 to +1135 of APP770 cDNA. Probe 2 is a Real restriction fragment from position +1109 to +2181, spanning the coding region of the carbohydrate, βA4 (black bar) and transmembrane (Tm) domains, and part of the cytoplasmic domain of APP cDNA. Probe 3 is derived from probe 2 by restriction with PvuII and, probe 4 and 5 with BamHI.
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APP751 mRNA (751) to 714 and 45 bases, APP695 mRNA (695) to 546 and 45 bases. B, S1 nuclease protection analysis of poly(A) RNA from PHA-stimulated (5 days) human T-lymphocytes (non-AD) with probes 2–5 gave rise to protected fragments of 1072, 828, 629, and 546 and 45 bases, respectively, for APP mRNA (arrows). Presence of the novel APP mRNA (L-APP) resulted in a second protected fragment which is about 260 bases shorter (arrows). Lane 1, undigested probe 2; lane 2, control hybridization of probe 2 with yeast tRNA (20 μg); lane 3, protected fragments of probe 2; lane 4, undigested probe 3; lane 5, protected fragments of probe 3; lane 6, undigested probe 5; lane 7, protected fragment of probe 5; lane 8, undigested probe 4; lane 9, protected fragments of probe 4. C, S1 nuclease protection analysis of total RNA from human macrophages and B-cells with probe 2. Macrophages gave the protected fragment of 1072 bases indicative of L-APP mRNA, as well as the shorter protected fragment indicative of L-APP (arrows). B-cells show only longer exposures (8 days), weak signals corresponding to APP and L-APP isoforms, than demonstrated here (36 h). S1, undigested probe 2; C0, control hybridization with yeast tRNA (20 μg); lane 1, macrophages (Mφ) (15 μg of total RNA) stimulated with lipopolysaccharide (LPS); lane 2, stimulated with γ-interferon (γIFN; 10 μg total RNA) for 8 h. 

Tissue Specificity of Novel Splicing Form—To determine whether the novel APP mRNA is expressed in other tissues, we applied PCR to cDNAs from various human, mouse, and rat tissues and cell lines. We used oligonucleotide primers flanking the sequence of exons 14–18; the corresponding PCR products were 462 and 408 bp, for cDNAs with and without exon 15, respectively. The 462-bp PCR product was detected in all tissues and cell lines examined, which were HeLa and neuroblastoma cells, astrocytoma, mouse cerebellar primary cell culture, human temporal cortex, and hippocampus of control and Alzheimer cases, kidney, skeletal muscle and fetal thymus tissue, and all of the T-lymphocytes. In contrast the 408-bp PCR product was only found in T-lymphocytes and rat microglial cells (Fig. 4A, lanes 18–23 and 4C). Macrophages were also shown to contain considerable amounts of the novel APP splice form, as demonstrated by S1 nuclease protection analysis (Fig. 2C). B-lymphocytes demonstrated weak APP as well as L-APP mRNA expression in the S1 nuclease protection analysis only after 8 days exposure time, compared with the 36-h exposure time shown in Fig. 2C, lane 3.
positive for L-APP mRNA (data not shown). It can be as-
firmed by Southern blot analysis with a human L-APP cDNA
probe comprising exons 14-17 (Fig. 4B). Both PCR products
in leukocytes were seen in leukocytes were APP-specific, since they
exon 14 was spliced by itself. L-APP mRNA is expressed in both subtypes of
phatic organs, such as spleen and lymph nodes were also
in regard to Alzheimer’s disease, there seems to be a reduction in the relative
expression level of L-APP mRNA in the two cases examined
a strictly relevant analysis.

Size of Novel APP Splicing Form—Analysis of leukocyte
eral human T-lymphocytes, the
CD44, CD88 T-lymphocytes (Fig. 4A). In regard to
exon 3 to exon 18. In
order to determine the full length of the corresponding novel
APP mRNA, Northern blot hybridizations were performed
Total RNA from activated human T-lymphocytes, the
CD44, CD88 subsets, and rat microglial cells were separated by agarose gel electrophoresis and blotted to a nylon filter. The filter was hybridized with a 1.05-kb EcoRI restriction fragment, comprising the 3’-part of the APP cDNA starting within exon 16. The hematopoietic cells showed a strong signal of APP mRNA in the range of 3.4 kb (Fig. 5, A and B). HeLa cells showed a roughly three times stronger expression of a similar 3.4-kb APP mRNA (Fig. 5A). Thus, L-APP mRNAs in hematopoietic cells are approximately the same size as transmembrane APP mRNAs and are distinguished only by the omission of exon 15. L-APP mRNA generates transmembrane APP isoforms, which are expected to be 18 amino acids shorter (54 bp) than the previously described APPs (3, 10-12).

Alternative Splicing of L-APP in Leukocytes—In order
to determine the relative amounts of L-APP mRNA versus total
coding APP mRNA in resting lymphocytes and lymphocytes
stimulated with PHA, we treated cells with PHA for various
times, isolated RNA, and conducted S1 nuclease protection
analysis with probe 2 (Fig. 6). A change in the ratio of the
two splice isoforms can be observed. Resting human T-lymphocytes showed approximately 40% (±5%; n = 2) L-APP mRNA compared with about 20% (±10%; n = 3) L-APP mRNA in short term stimulated human T-lymphocytes. After

The specificity of both signals in the RT-PCR was con-
figured by Southern blot analysis with a human L-APP cDNA probe comprising exons 14-17 (Fig. 4B). Both PCR products which were seen in leukocytes were APP-specific, since they
gave signals after stringent wash conditions (Fig. 4B, lanes 18-23). We refer to this new splice form as L-APP, since all
leukocytes examined showed L-APP mRNA expression. Lymph-
ocytes, such as spleen and lymph nodes were also positive for L-APP mRNA (data not shown). It can be as-
former human T-lymphocytes (lanes 17-23) showed an additional band of 408 bp with varying intensity, corresponding to the L-APP mRNA isoform. M, DNA molecular weight marker; lane 1, H2O in PCR analysis; lane 2, plasmid APP770 cDNA (10 pg); lane 3, total RNA (no reverse transcription). Lane 4, HeLa cells; lane 5, SY5Y neuroblastoma cells; lane 6, human astrocytoma biopsy; lane 7, mouse primary cerebellar culture; lane 8, fetal skeletal muscle of Down’s syndrome (DS); lane 9, fetal thymus of DS; lane 10, kidney of non-AD; lane 11, kidney of non-AD; lane 12, temporal cortex of non-AD; lane 13, fetal temporal cortex of DS; lane 14, hippocampus of non-AD; lane 15, hippocampus of AD; lane 16, temporal cortex of non-AD; lane 17, unstimulated T-lymphocytes of non-AD; lanes 18-23 are human T-lymphocytes stimulated with PHA for 5 days. Lane 18, non-AD; lanes 19 and 20, AD cases; lane 21, DS; lane 22, CD4 T-lymphocytes of non-AD; lane 23, CD8 lymphocytes of non-AD. E, Southern blot analysis of PCR products shown in Fig. 3. Lanes 2 and 4-16 showed the PCR product corresponding to APP cDNA as one signal on the autoradiograph. Lane 7 (mouse APP cDNA) showed a weaker signal, since the blot was washed with high stringency. L-APP cDNA is visible in lanes 17-23 as the second signal (arrows). C, PCR analysis of cDNA from rat microglial cells. Ethidium bromide-stained 2.5% agarose gel of PCR product of reverse-transcribed cDNA from isolated rat microglial cells of the central nervous system, which showed the two signals indicative of APP and L-APP mRNA. Lane 1, H2O in PCR; lane 2, APP770 cDNA (10 pg) in PCR; lane 3, cDNA from rat microglial cells.

several days of PHA treatment, the relative amounts of L-
APP increased again (Fig. 6, lanes 6 and 7). Thus we observed
a regulation of alternative splicing of L-APP during activation
of T-lymphocytes in vitro. We hypothesize that the regulation
of L-APP might therefore be important in the functional
transition of leukocytes during their stimulation through in-
flammatory agents in vivo.

Functional Implications—We were able to demonstrate for
the first time APP mRNA expression in the microglial cells. Microglial cells are of mononuclear phagocyte lineage and are thought to migrate into the brain during development of the
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FIG. 5. Northern blot analysis of APP/L-APP mRNA expression. A, blots were hybridized with a SacI/Rsal L-APP cDNA fragment. Human T-lymphocytes, CD4+ and CD8+ T-lymphocytes, and HeLa cells (20 μg of total RNA, each) exhibited a major band of about 3.4 kb for APP and L-APP mRNA after exposure for 4 days. B, blots were hybridized with a 1.05-kb EcoRI APP cDNA fragment. T-lymphocytes and rat microglial cells (20 μg of total RNA, each) exhibited comparable amounts of APP mRNA to the stimulated T-lymphocytes, with one band of about 3.4 kb after exposure for 36 h.

FIG. 6. S1 nuclease protection analysis with probe 2 of total RNA from human T-lymphocytes which were stimulated for various times with PHA. Lane 1, undigested probe 2; lane 2, control hybridization of probe 2 with yeast tRNA (20 μg). Lanes 3–6, protected fragments of probe 2 for T-lymphocytes treated with PHA for 0 min (lane 3) (40 μg of RNA), 30 min (lane 4) (20 μg of RNA), 14 h (lane 5) (20 μg of RNA), 4 days (lane 6) (20 μg of RNA), 6 days (lane 7) (20 μg of RNA), 8 days (lane 8) (20 μg of RNA).

FIG. 7. Location of alternative transmembrane APP mRNA splice sites. Those exons of the APP gene participating in alternative splicing of transmembrane APPs are indicated. Exon 7 codes for the KPI domain, and exon 8 codes for the MRC OX-2 domain. Inclusion of exon 7 results in APP751 and inclusion of exon 8 in APP714; inclusion of exons 7 and 8 gives APP770. APP695 is generated by exclusion of exons 7 and 8. Usage of the novel alternative splice site at exon 15 generates L-APP with exclusion of exon 15.

central nervous system (37). They share a number of surface antigens with peripheral macrophages and are thus clearly distinguished from all other glial cells as a class of potential immunoeffector cells in the brain. They have therefore been referred to as the "resident macrophages of the brain" (37). Activation of microglial cells during neuro-degeneration in the brain results in active production of APP as determined by APP immunoreactivity and immunoprecipitations of APP2. The precise role and fate of this new contributor of APP to the APP pool in brain has to be shown in further studies. Microglial cells are characterized by a high respiratory burst activity potential (28). In the light of the proposed role of redox potential as one possible factor for amyloidogenesis, microglial cells are an attractive brain cell to study APP synthesis and degradation pathways in more detail.

L-APPs are the first splice products of the APP gene for which a highly restricted expression pattern has been established. L-APP is generated by exclusion of a short exon in a region not previously thought to be involved in alternative splicing. L-APP mRNA represents a substantial fraction of total APP mRNA in leukocytes and microglial cells and is readily detected with S1 nuclease protection analysis. It has still to be determined if the new splice product contains the KPI and OX2 exons as would be expected in leukocytes (see Fig. 2A). Alternative splicing of APP could then give rise to at least three L-APPs (Fig. 7). Expression of L-APP and the ubiquitous APP forms APP695, APP751, and APP770 appears to be correlated with the functional state of leukocytes. L-APP is high in nonadherent and low in adherent leukocytes. The presence of a novel APP isoform (L-APP) in lymphocytes or cells of the mononuclear phagocyte system such as macrophages or microglial cells might be necessary to allow the rapid transition of the dual functions of nonadherence and adherence in the immune system (38). This is in accordance with the proposed role of APPs in cell adhesion and attachment in the nervous system and in the periphery (39).

Exclusion of exon 15 changes the molecular conformation of APP at a site that might be critical for recognition and binding of APP processing and possibly βA4-amyloid gener-

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ating proteases. Future protein expression of L-APP and sequencing of a proteolytic breakdown products will determine if the physiological cleavage of APP, which occurs inside the amyooidogenic region and prevents amyloid formation, is conserved in L-APP. β44 deposits in AD are infiltrated by microglial cells, which are, however, absent in early lesions (40, 41). Whether the microglial cells in brain are a substrate for a potentially amyooidogenic processing of APP isoforms has yet to be determined. The investigation of L-APP expressed in situ will help to follow the fate of microglial APP/L-APP and to clarify the suggested pathogenetic role of microglial cells in AD.

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