APLP2, a member of the Alzheimer precursor protein family, is required for correct genomic segregation in dividing mouse cells

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Introduction

The amyloid precursor-like protein 2 (APLP2) belongs to the Alzheimer peptide precursor family. A possible role in pre-implantation development had been suggested previously, and was investigated further by creating a large deletion in the genomic locus. While heterozygous mice developed normally, homozygous embryos were arrested before reaching the blastocyst stage. One-cell embryos which contained protein of maternal origin underwent a limited number of cleavages. The progressive disappearance of the protein at stages 4 and beyond correlated with the appearance of extensive cytopathological effects. Nuclear DNA contents of the arrested embryos departed widely from the normal 2–4C value, thus suggesting a role for the protein in replication and/or segregation of the embryonic genome. Embryonic mortality was not due to the untimely initiation of programmed cell death, and it occurred before the stage at which apoptotic cells normally appear. The same abnormal distribution of DNA contents was seen in primary cultures of Aplp2 +/− embryonic fibroblasts following transfection of an expression vector for Aplp2 antisense RNA with green fluorescent protein (GFP) expressed from a co-transfected construct. Daughter cells derived from a GFP-positive cell showed abnormal DNA contents both >4C and <2C, thus indicating a role for the protein in the mitotic segregation of the genome and establishment of the proper nuclear structure.

Keywords: APLP2 protein/Alzheimer peptide precursor/mitotic segregation/nuclear structure
then generated an intragenic 11.35 kb deletion, which abolishes the expression of all the known isoforms. No obvious developmental defect was noted in the heterozygotes, which consistently were produced with normal Mendelian ratios. In sharp contrast, homozygotes failed to reach the blastocyst stage.

Results

Generation of the Aplp2-deleted mice

The replacement targeting vector pYY-V8 (Figure 2) was designed to create a null mutation in the Aplp2 (Cdebp) gene. It contains a 3 kb fragment covering exons 5 and 6, with the adjacent and intervening intron sequences linked to a neo cassette and to 2.9 kb of sequences from intron 14. The size and location of the expected deletion (11.35 kb corresponding to eight exons and seven introns) were chosen in such a way that none of the isofrom mRNAs could possibly be generated from the mutated allele. WW6 embryonic stem (ES) cells were electroporated with linearized pYY-V8 DNA. Positive/negative selection was applied in medium containing both G418 and ganciclovir. A first screen by Southern blot hybridization after BamHI cleavage (see below) detected 18 homologous recombinants among 78 survivors analysed. They exhibited identical genomic structures, with the neo cassette inserted between exon 4 on the 5′ side and exon 15 on the 3′ side. Long-distance PCR amplification (primers p44 and p45; Figure 2B) yielded the expected 8.5 kb fragment hybridizing with the neo probe. Results of PCR analysis were confirmed by subsequent Southern blot analysis of tail DNA using two probes, A and B, on both sides of the deletion, and one, C, corresponding to the neo sequences (Figure 2). Probe A detected the expected 2.7 kb recombed BamHI fragment, which also hybridized with the neo probe (not shown), BglII generated a fragment of 7.3 kb reacting with probes A and B, BclI, a 12 kb fragment hybridizing with probes A and B, and EcoRI, a fragment of 7.3 kb detected by probe B.

Absence of homozygous mutants in the progeny of heterozygous mating

To find evidence for a possible role for Aplp2 in development, crosses between heterozygotes were performed to generate homozygous animals. Litters were significantly smaller, yielding an average of seven pups as compared with nine in crosses with wild-type mice, with one-third of the mice homozygous for the wild-type allele, and two-thirds, heterozygous (Table I). The proportion of heterozygous and wild-type and the lack of the homozygous genotypes are consistent with the notion that homozygous embryos die before birth. If this is the case, death must occur at an early embryonic stage, since the same distorted allelic distribution was found among embryos dissected at about mid-gestation (see below).

Development of the Aplp2 −/− homozygous embryos is arrested before the blastocyst stage

Examination of the earliest developmental stages of the Aplp2-mutated embryos showed a fraction of morphologically abnormal embryos increasing in number between fertilization and the blastocyst stage, up to values close to the expected Mendelian proportion of homozygotes (Table II). These embryos showed a grossly distorted morphology, with a small number of cells of unequal sizes
surrounded by the zona pellucida (Figure 3). For 39 arrested embryos analysed at day 3.5, the average number of cells per embryo was 11.4 (± 0.03), as compared with 44.8 (± 2.4) for their normal littermates (nine blastocysts analysed). At that time, where normal embryos had undergone compaction, cells of the abnormal embryos were dissociated readily by incubation in Ca²⁺-free medium. They did not attach when cultivated in ES cell medium with or without feeders, and after 1 week in culture, most of them had become Trypan Blue-positive.

We then set up a PCR procedure for the genotypic analysis of individual embryos at these early stages. A set
Table I. Litter size and hereditary transmission

<table>
<thead>
<tr>
<th>Parent genotypes</th>
<th>No. of litters</th>
<th>Total progeny</th>
<th>Litter size</th>
<th>Genotypesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ × +/+</td>
<td>59</td>
<td>516</td>
<td>8.7 ± 0.12b</td>
<td>279</td>
</tr>
<tr>
<td>+/+ × +/−</td>
<td>42</td>
<td>292</td>
<td>6.9 ± 0.18</td>
<td>99</td>
</tr>
</tbody>
</table>

aTail DNA was analysed by Southern blot hybridization after BamHI cleavage (see Figure 2).
bAverage ± SEM; P < 0.01.

Table II. Development of morphological abnormalities among the early embryos from mating of two heterozygous parents

<table>
<thead>
<tr>
<th>d.p.c.</th>
<th>Developmental stage</th>
<th>No. of litters</th>
<th>No. of embryos</th>
<th>Litter size</th>
<th>Abnormal embryos</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1-cell</td>
<td>17</td>
<td>146</td>
<td>8.6</td>
<td>7</td>
<td>4.8</td>
</tr>
<tr>
<td>2.5</td>
<td>4- to 8-cell</td>
<td>28</td>
<td>196</td>
<td>7.0</td>
<td>18</td>
<td>9.2</td>
</tr>
<tr>
<td>3.5</td>
<td>blastocyst</td>
<td>19</td>
<td>152</td>
<td>8.0</td>
<td>28</td>
<td>18.3</td>
</tr>
</tbody>
</table>

We next asked whether the limited and irregular number of cleavages accomplished by homozygous mutant embryos reflected the possibility that division may occur in the absence of APLP2 or, alternatively, whether it resulted from the presence in the fertilized egg of protein of maternal origin.

The apparently normal development of the null mutant embryos up to stages 2–4 may be accounted for by the presence of protein of maternal origin

The distribution of the protein throughout the cell volume in pre-implantation blastomeres, both in wild-type and heterozygous embryos (Figure 4A), appears to be similar to that previously observed in mitotic somatic cells (Blangy et al., 1995). Since it is generally admitted that zygotic transcription starts in the mouse embryo at the two-cell stage, the protein present at early times after fertilization is likely to be synthesized from maternal RNA. Consistent with this conjecture is the observation that all the one- and two-cell embryos of litters generated by two heterozygous parents appeared equally labelled by immunofluorescence assay. When they progressed further to the advanced morula stages, a fraction of them showed progressive weakening of the label and, concomitantly, the first unequal cellular divisions (Figure 4B).

Is the arrest of homozygous embryos due to untimely programmed cell death?

We considered the possibility that the arrest in the development of homozygous embryos could be due to the triggering of programmed cell death (PCD). PCD occurs, mostly by apoptosis, at some defined periods of the development of the vertebrate embryo. Programming of cell death is
Role of APLP2 in chromosomal DNA segregation

Fig. 4. Distribution of the APLP2 in wild-type and mutant early embryos. Embryos were recovered at 3.5 d.p.c., fixed in 4% paraformaldehyde and treated for immunofluorescence analysis (see Materials and methods) using antibody Ab61 (Blangy et al., 1995), which recognizes APLP2 and not APP. (A) 1, immunofluorescence localization of the protein in wild-type mouse embryos at stages 1 and 2; 2, control without addition of Ab61. (B) Representative litters of embryos from two heterozygous parents. 1, stage 2; 2, morula. The two embryos indicated by stars show both unequal cell divisions and decreased immunofluorescence staining (overexposed photograph to show the residual label in the mutant embryos).

part of the formation of the proamniotic cavity in the implanted embryo (Coucouvanis and Martin, 1995), and the occurrence of cell death has been documented at earlier stages of development (El-Shershaby and Hinchliffe, 1974). Death of the Aplp2 −/− mice could be interpreted as the untimely initiation of the PCD programme, as was reported in cases of growth factor deprivation during development (reviewed by Coucouvanis and Martin, 1995). We therefore examined homozygous and heterozygous Aplp2 mutants and wild-type embryos at successive pre-implantation stages (Figure 5) using the TUNEL (TdT-mediated dUTP nick end-labeling) assay for the detection of the endonucleolytic cleavage of nuclear DNA characteristic of apoptosis. During normal development, positive cells were not found before the blastocyst stage. At that stage, some of the trophoderm cells were found engaged in apoptosis in all blastocysts. Among the embryos generated by two Aplp2 +/+ parents, however, about one-quarter did not show TUNEL-labelled cells. They were the same embryos that showed cytopathic effects. We therefore conclude that the arrest of development of the embryos lacking a functional Aplp2 gene is not due to an abnormal PCD signal and, moreover, that it is actually effective before the stage of normal development at which apoptosis takes place in the trophoderm.

Abnormal nuclear DNA contents in the arrested embryos suggest a defect in DNA replication and/or mitotic segregation

Embryos collected at 2.5–3.5 d.p.c. were fixed and stained with Hoechst 33258, and the DNA content of individual nuclei was estimated by in situ determination of fluorescence intensity (Figure 6). Normal blastocysts showed the expected complement of DNA, with most values comprised between 2C and 4C (S phase), and a small proportion of G1 (2C) or G2 (4C) nuclei. The nuclei of arrested embryos exhibited a much wider distribution, most of them scoring either below the 2C or above the
Fig. 6. Unequal distribution of nuclear DNA in arrested Aplp2 –/– embryos. Blastocysts collected at embryonic day 3.5 in crosses between two heterozygous parents were fixed and stained with Hoechst 33258. (A) Arrested embryos (left) and normal littermates (right); upper row, whole embryos; lower row, isolated blastomeres. All pictures were taken with identical exposure times: note the presence in the abnormal embryos of nuclei with either higher (arrows) or lower than normal (stars) fluorescence intensity. (B) Nuclear DNA contents were determined by fluorescence intensity reading performed on digitized images of individual nuclei. Nuclei of morphologically altered (homozygous) and normal (heterozygous and wild-type) embryos were measured separately. Data are presented for one of the normal and the two abnormal embryos of the same litter. The same results were obtained on a total of 10 litters from heterozygous parents. Control measurements were performed on G0-arrested circulating lymphocytes.

4C values. Such an effect of the Aplp2 deletion is suggestive of a role for one of the gene products in the replication and/or segregation of nuclear DNA.

Segregation analysis in cell cultures
Experiments were designed to determine whether the role of the APLP2 protein was restricted to the pre-implantation embryo. Previous experiments performed on established cell lines had been inconclusive because of the frequent occurrence of abnormal nuclear structures and DNA contents in most immortalized mouse lines (our unpublished results). Further experiments were performed on two cell types which maintain diploid karyotypes in culture, ES cells and primary embryonic fibroblasts. Starting from the initial Aplp2 +/– targeted ES clones, we first attempted to select homozygous mutants by growth in medium containing elevated concentrations of G418 (400–3000 μg/ml), toxic for heterozygotes with only one neo' allele (Mortensen et al., 1992). Drug-resistant clones appeared with low frequencies, but out of 85 clones tested, none of them exhibited the Southern blot profile corresponding to the homozygous deletion (data not shown). This result is compatible with a lethal effect of the homozygous deletion in ES cells. However, to obtain a positive confirmation, further experiments were performed by antisense inhibition in diploid primary cultures of Aplp2 +/– heterozygous fibroblasts from mid-gestation embryos. Starting from the complete litters of two heterozygous parents, pure Aplp2 +/– cultures were selected in G418 medium. Morphology and growth patterns were indistinguishable from those of control wild-type fibroblasts, as expected from the fully normal phenotype of the heterozygous mice, but we hypothesized that these cells should be sensitive to antisense RNA inhibition. Transfection was performed with a 1:1 mixture of two plasmid constructs. pSVGFP expresses the green fluorescent protein (GFP) under the control of the early simian virus 40 (SV40) promoter, and in pCdebp-pbedc, the Aplp2 promoter (Yang et al., 1996) drives the transcription of the lacZ reporter fused to a region complementary to the 5' 1.1 kb of Aplp2 mRNA. Controls were performed by replacing this antisense expression vector by the same vector with lacZ sequences only. In cultures fixed at 1 day intervals, in situ (X-Gal) staining for β-galactosidase activity identified a Lac-positive fraction in cultures transfected with either the antisense or the control constructs amounting to 0.5–1%, the usual range after transfection of primary cultures. GFP fluorescence was seen in a comparable fraction of the cell population. Cultures were then stained with Hoechst 33258 and examined by fluorescence microscopy. As X-Gal staining interferes with the fluorescence of Hoechst 33258–DNA complexes, the GFP label was used to identify transfected cells, and their DNA contents were assessed by cytofluorometry.

Control cultures showed the 2C–4C distribution of DNA contents characteristic of cycling cells, both in the total population and in the GFP-positive fraction after cotransfection of the control plasmid pCdebp-lacZ (Figure 7). In contrast, DNA determination performed on GFP-positive cells in cultures that also received the antisense expression vector pCdebp-pbedc showed a number of cells with abnormal DNA contents. These abnormal nuclei were not seen before 48 h after transfection, the time at which
the transfected cultures had resumed their growth, thus suggesting that their appearance is in some way correlated with cell-cycle event(s). The possibility of an elementary pedigree analysis over the first few cellular generations after transfection was offered by the analysis of GFP staining patterns as a function of time. Whereas until 48 h after transfection, only isolated fluorescent cells were detected, examination of either the antisense or the control cultures at later times (72–96 h) showed small clusters of 2–4 positive cells, mitotic products from one transfected parent cell (Figure 8). One nucleus in each GFP-positive cluster had a >4C DNA content, while one or several nuclei exhibited DNA contents <2C. The segregation of DNA copies and regular partition of the nuclear structure during mitosis thus appear profoundly altered upon interference with APLP2 expression.

**Discussion**

To elucidate the role of the APLP2, we engineered mutant mice in which the gene is disrupted by a large deletion (11.35 kb). Heterozygous mice were apparently normal, but embryos homozygous for the disrupted locus underwent only a limited number of cleavages, corresponding to the period where they contain protein of maternal origin. From previous experiments performed on early mouse embryos (Blangy et al., 1991) as well as on a model viral replicon (Pierreffite and Cuzin, 1995; Pierreffite et al., 1996), we had proposed that the protein plays an essential role in the replication and/or maintenance of the genomes. The phenotype of the Aplp2-null mutants confirmed an important role for at least one of the protein isoforms in the dividing blastomeres. Experiments performed in primary cell cultures extended this role to one other cell type, the fibroblasts from mid-gestation embryos.

Aplp2 is expressed as a series of four differentially spliced isoforms (Sandbrink et al., 1994b); isoform 763 includes exons 7 and 14; 751 contains exon 7 but not 14; 706 contains exon 14 but not 7; and 694 contains neither exon (Thinakaran et al., 1995; Yang et al., 1996). In the rat, all isoforms were found to be expressed ubiquitously in peripheral tissues as well as in the central nervous system, with, however, variable levels of expression in different tissues. Two of them, isoforms 694 and 751, encode the chondroitin sulfate glycosaminoglycan-modified forms preferentially accumulated in defined regions of the central nervous system (Thinakaran et al., 1995). These different proteins with distinct localizations may exert distinct functions in the organism. The mutation that we generated eliminates all the known alternative splicing sites. None of the four isoforms can be produced, necessitating further studies to determine which protein(s) is required for genomic segregation and nuclear partition.

Death of the Aplp2 +/− embryos does not appear to be due to apoptosis. In the normal embryo, a significant proportion of cells undergo PCD (El-Shershaby and Hinchliffe, 1974; Coucouvanis and Martin, 1995). Under the conditions we used, PCD was only observed after the beginning of trophoblast and inner cell mass differentiation, thus constituting a convenient marker for an early stage of blastocyst formation. This stage was not reached by the mutants lacking Aplp2 expression. They also did not undergo compaction, and were not able to attach in culture, thus reinforcing the conclusion that trophoblast differentiation did not take place. Only the presence of protein of maternal origin could apparently ensure the progression of the homozygous embryos through the first cleavages, and its expression from the zygotic genome thereafter was absolutely required.

Taken together with the site-specific DNA-binding property of the protein (Blangy et al., 1991; Vidal et al., 1992), these results are indicative of a role either in chromosome replication or segregation (or both). A direct analysis of segregation was undertaken, by fluorescence in situ hybridization with chromosome-specific probes. However, it was made difficult by the small number and abnormal structure of nuclei in either arrested embryos or antisense-transfected fibroblasts. Further investigation on the function of the gene in embryonic and adult cells will in fact require its targeted inactivation at specified differentiation stages, by conditional systems such as Cre-dependent deletions (reviewed by Porter, 1998). At the present time, a segregation defect appears as a most likely possibility and this conclusion is reinforced by one additional observation. It was found consistently that the total DNA content of arrested homozygous morulae was close to that expected after four rounds of DNA replication, even though most of the cells showed DNA contents either above or below the normal 2C–4C range (Figure 7).
Fig. 8. Abnormal nuclear structure and DNA contents in cells transfected with Aplp2 antisense expression vector. Same experiment as in Figure 7. 1a,b: control cells transfected with a mixture of plasmid pSVGFP and pCdebp-lacZ DNA. 2a,b and 3a,b: GFP-positive cells after co-transfection of the antisense expression vector pCdebp-pbedc. Daughter cell nuclei show >4C (arrows) and <2C (stars) DNA contents. 1a-3a: GFP protein fluorescence. 1b-3b: Hoechst 33258 fluorescence of the same cells.

6). The same conclusion is also suggested by studies conducted on Aplp2 +/− heterozygous embryo fibroblasts in which expression of the remaining allele has been inhibited by an antisense transcript. Since the GFP marker allowed us to analyse in these experiments the progeny of individual transfectants, the regular finding of pairs of daughter cells with nuclear DNA contents lower and higher, respectively, than the expected 4C value, was direct evidence for an incorrect mitotic segregation. This conclusion is also consistent with our previous observations that a binding site for the protein in the episomal genome of bovine papillomavirus type 1 is necessary for its transient replication in transfected cells and its establishment in stable transformants (Pierrefite and Cuzin, 1995; Pierrefite et al., 1996). Finally, it is also relevant to note that the presenilin proteins recently were suggested to play a role in chromosome segregation (Li et al., 1997). These proteins are functionally related to APP, with a possible topological interaction in the cell membrane. One might therefore speculate that they are also functionally connected with APLP2.

Materials and methods

Selection of homologous recombinants
From Aplp2 (Cdebp) C57BL/6 genomic clones (Yang et al., 1996), a 3 kb fragment including exons 5 and 6 and a 2.9 kb fragment containing intron 14 were prepared and inserted between XbaI, and the BamHI and HindIII sites, respectively, of vector pSSC-9 (Chauhan and Gottesman, 1992). The resulting construct (Figure 2) has two tk genes at both ends of the homologous sequence, and a neo′ gene replacing a 11.35 kb
region of the App2 gene that contains eight exons and seven introns. The WW6 ES cell line (Ioffe et al., 1995) was cultured on mitomycin C-treated STO feeder cells in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 10% fetal bovine serum (FBS; HyClone) and 1000 U/ml leukaemia inhibitory factor (Gibco-BRL).

pYY-V8 DNA was linearized by digestion with SptI prior to electroporation, which was performed on $10^6$ ES cells with 10–20 μg of linear DNA using the Bio-Rad Gene Pulser apparatus at 400 V and 250 μF. The cells were then plated onto 100 mm plates, and G418 (Gibco-BRL) (200 μg/ml) and 1 mM ganciclovir (Syntex) were applied 48 h later. The double-resistant colonies were picked after 8–10 days.

**Screening of recombinants**

Genomic DNA prepared from the drug-resistant clones and from mouse tails was tested by Southern blot analysis by standard methods (Sambrook et al., 1989) with probes A and B subcloned from genomic DNA (see Figure 2) and a vector probe corresponding to the neo coding sequences. Amplification with the Taq polymerase was performed according to the specifications of the manufacturer (Boehringer Mannheim). Long-distance PCR amplification was performed using the Expand™ Long Template PCR System (Boehringer Mannheim) on 1 μg of ES cell DNA.

PCR conditions for 4–5 kb DNA fragments: min 94°C, (10 s 94°C, 10 s 52°C, 2 min 68°C) for 10 cycles; (10 s 94°C, 10 s 52°C, 8 min 68°C) for 10 cycles; (10 s 94°C, 10 s 52°C, 8 min 30 s 68°C) for 25 cycles; and 7 min 68°C. For 7–10 kb DNA fragments: min 94°C, (10 s 94°C, 10 s 52°C, 8 min 68°C) for 10 cycles; (10 s 94°C, 10 s 52°C, 8 min 30 s 68°C) for 25 cycles; and 7 min 68°C. The position and orientation of the oligonucleotide primers are indicated in Figure 2. Nucleotide sequences are: pn5, 5'-CAAAACACACACGAGGGCCATTT-3'; p44, 5'-GCACCAGCGCTGGCACACG-3'; p45, 5'-GCACCAATGTCCCGGTGC-3'; p46, 5'-CGAGGGCCATTACAGTCCCCCGTC-3'; p47, 5'-GAAGCCATGCTGAATGAC-3'; p48, 5'-CTTGGTTTCGACGACACGAC-3'; p49, 5'-CATCGTGAACCTCTTTGTGCT-3'; p50, 5'-CGAGGTTGGTCCAGAAGCAG-3'.

**Generation of mutant mice**

ES cells 8-W54 and 8-W76 were injected into blastocysts from MF1 mice and transferred to pseudopregnant females as described (Robertson, 1987). Embryos were recovered at E0.5–E3.5 and individual embryos were thus generated, which subsequently were maintained by crossing BALB/c and with B6D2 partners, yielding in each case agouti offspring indicative of germline transmission. A total of four mutant families can be traced in chimeras. This work was made possible by grants from Association pour la Recherche sur le Cancer to F.C. and M.R.

**References**


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