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Expression of β-Amyloid Precursor Protein in the Porcine Ovary

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ABSTRACT—cDNA cloning and 5'-RACE experiments were conducted on β-amyloid precursor protein (APP) using porcine ovary mRNA. The isolated cDNA clone and the clones generated by 5'RACE spanned 3,051 bp containing the complete open reading frame of APP which consisted of 770 amino acid residues. The amino acid sequence of porcine APP was 97.8, 97.1, and 97.4% homologous to those of human, mouse, and guinea pig APPs, respectively. The expression of APP in several porcine tissues was examined by Northern blot analysis. The ovaries and adrenal glands showed a strong expression of the APP mRNA, as did the granulosa cells from small and large follicles of the porcine ovary. RT-PCR analyses using two primer sets revealed that the porcine ovary expressed at least four types of APP mRNAs. Western blot analysis was conducted using the extract of granulosa cells and the fluid of ovarian follicles, and the results indicated that the follicular fluid contained soluble APP in relatively high content. These results suggest that APP undergoes proteolytic processing and/or degradation within the follicles during follicular development.

INTRODUCTION

β-Amyloid precursor protein (APP) is a ubiquitous transmembrane glycoprotein present in virtually all studied cell types (Hendriks and Broeckhoven, 1996). The APP gene has been shown to be a housekeeping gene with a promoter that lacks a TATA box, and to display a high G+C content, two features typical for a housekeeping gene promoter (Salbaum *et al.*, 1988). Previous studies have also demonstrated that alternative splicing of the primary transcript results in production of a family of eight proteins (Sandbrink *et al.*, 1994). All these distinct APP isoforms are generated from mRNA splicing events involving exons 7, 8, and 15 (Sandbrink *et al.*, 1993). APP695 is the predominant splicing product of the APP gene in the brain, whereas APP770 and APP751 are major products in peripheral tissues (Sandbrink *et al.*, 1994). In addition, less abundant leucocyte-derived APPs (denoted as L-APPs) have been identified in peripheral cells (König *et al.*, 1992; Sandbrink *et al.*, 1994).

APP has been extensively studied in connection with the formation of β-amyloid (Aβ). This 40- or 42-amino acid residue peptide is derived from the protein through aberrant proteolytic processing, and its deposition in the brain parenchyma and cerebral vasculature is a histopathological characteristic of Alzheimer's disease (Hendriks and Broeckhoven, 1996; Selkoe, 1997). Very recently, β-secretase, one of the crucial processing enzymes for generation of Aβ, was identified (Yan *et al.*, 1999; Sinha *et al.*, 1999). Increased production of Aβ is generally thought to result in local microglial and astrocytic activation, with concomitant release of cytokines and acute-phase proteins (McGeer and McGeer, 1995). By means of such changes or by direct Aβ neurotoxicity, local neurons and their processes can be injured. Because Aβ is known to induce apoptosis, the neuronal apoptosis-mediated increase in Aβ could be causative of further initiation of neuronal cell death (Hugon *et al.*, 1999). Recently, intracellular accumulation of wild-type APP was also demonstrated to induce degeneration of postmitotic neurons via the apoptotic pathway (Uetsuki *et al.*, 1999).

The physiological role of APP is still obscure, although several putative functions have been suggested, including participation in cellular interaction (Schubert *et al.*, 1989; Schubert and Behl, 1993; Beer *et al.*, 1995), receptor activity (Kang *et al.*, 1987; Nishimoto *et al.*, 1993), cell growth promotion (Saitoh *et al.*, 1989; Whitson *et al.*, 1989; Milward *et al.*, 1992), inflammatory and immunological reaction (Mönning *et al.*, 1990; Mönning *et al.*, 1992), and serine proteinase inhibition (Van Nostrand *et al.*, 1991). Considering that all these biological events indeed take place in the ovary, we are interested in studying the role(s) of APP in this reproductive organ. As a first step to examine how APP is involved in dis-

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crete biological processes in association with ovarian function, we conducted a molecular cloning study of APP using porcine ovary. The current data indicated that at least four APP mRNA isoforms are present in the ovary. Further, the follicular fluid of the porcine ovary was shown to contain soluble APP. These results suggest that APP undergoes proteolytic processing and/or degradation within the follicles during follicular development, leading to intrafollicular accumulation of the degraded product of APP.

MATERIALS AND METHODS

Materials

Porcine ovaries were obtained from a local slaughterhouse within 30 min of the animals' death and transported to the laboratory on ice.

Preparation of follicular fluid and granulosa cells

Follicular fluid and granulosa cells were prepared as described previously (Kimura *et al.*, 1998).

cDNA cloning of porcine APP770

A probe was prepared by reverse transcriptase-polymerase chain reaction (RT-PCR). Two primer sets were designed from the sequences of human (NM000484, M28373), mouse (U84012, X15210), and guinea pig APP (X97631). The sequences of the first set were 5'-CATCTTCACTGGCACACCGT-3' (nucleotides 439 to 457) and 5'-CCTCTCTTTGGCTTTCTGGA-3' (nucleotides 1191 to 1172), and those of the second set were 5'-ATGTCCCAGGTCATGAGAGA-3' (nucleotides 1216 to 1235) and 5'-AGAACCTGGTCGAGTGGTCA-3' (nucleotides 1967 to 1949). Three micrograms of total RNA extracted from porcine granulosa cells was reverse-transcribed using a Superscript II Preamplification System (Life Technologies, Inc., Rockville, MD). The PCR was conducted under the condition of 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min. The products corresponding to nucleotides 439-1191 and 1216-1967 were sequenced and the mixture of the two fragments was used as a probe.

Poly(A)$^+$RNA was prepared from porcine ovary by the guanidine isothiocyanate-cesium chloride method (Chirgwin *et al.*, 1979) and oligo(dT)-cellulose column chromatography. The cDNA was synthesized using a cDNA synthesis module (Amersham Pharmacia Biotech, Buchinghamshire, England), and was then inserted into a λgt 10-EcoRI vector arm (Life Technologies, Inc.). A GIGAPACK II GOLD packaging kit (Stratagene, La Jolla, CA) was used for in vitro packaging. 8.0 $\times 10^5$ plaques were screened by plaque hybridization. Positive clones were isolated and sequenced using an ABI automatic sequencer, model 377 (Perkin-Elmer/Applied Biosystems, Foster City, CA).

The sequence of the 5'-end was obtained by the 5'-rapid amplification of cDNA ends method (Frohman *et al.*, 1988) using the 5'RACE System (Life Technologies, Inc.). The primers used were as follows: 5'-TGGGCAACACACAA-3' (RT; nucleotides 564 to 551) for the RT reaction; 5'-CGCTTACAAACTCGCCAACT-3' (RA-1; nucleotides 373 to 354) for the first PCR; and 5'-TAAGCAGCGGTACGGAATCA-3' (RA-2; nucleotides 354 to 335) for the second PCR. The PCR was performed under the condition of 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min.

Northern blot analysis

Twenty micrograms of total RNAs isolated from the porcine liver, adrenal glands, testis, and ovaries, and from porcine granulosa cells were electrophoresed on a formaldehyde/agarose gel and transferred to a Nytran membrane (Schleicher & Schuell, Dassel, Germany). The blot was hybridized for 16 h with a ^{32}P-labeled probe at 42 °C in 50% formamide, 5 × Denhardt's solution, 5 × SSPE, 1% SDS, and 100 μg/ml herring sperm DNA. The probe was the same as used in screening. The membrane was washed at 50°C in 0.1% SDS/0.1 × SSC and exposed to Kodak X(OMAT)AR film.

Analysis of alternative splicing products by RT-PCR

Alternative splicing was tested by RT-PCR. Two primer sets were used, one with the sequences 5'-GAGTCTGTGGAAGAGGTGGT-3' (841SS; nucleotides 841 to 860) and 5'-TGACCTGGGACATT-CTCTCG-3' (1228AS; nucleotides 1228 to 1209), and the other with the sequences 5'-TGCCGTCTCTGACTGAAACC-3' (1781SS; nucleotides 1781 to 1800) and 5'-CCGTCTTGATGTTGGTCAAC-3' (1990AS; nucleotides 1990 to 1971). The PCR was carried out under the condition of 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The products were fractionated on a 5% polyacrylamide gel and detected with ethidium bromide staining.

Western blot analysis

Samples of porcine follicular fluid were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) under reducing or nonreducing conditions and transferred to a polyvinylidene difluoride membrane (Towbin *et al.*, 1979). The blotted membrane was incubated with mouse anti-β-amyloid precursor protein (Zymed Laboratory Inc., South San Francisco, CA) at 1:100 dilution and subsequently with biotinylated anti-mouse IgG antibody. The membrane was further incubated with avidin conjugated with horseradish peroxidase. Immunoreactive signals were detected using an ECL Western Blot Detection Kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RESULTS

Isolation of porcine APP770

We isolated seventeen phage clones by screening 8.0 × 10^5 clones of the porcine ovary cDNA library. We examined the insert sizes of all these clones, and found that the clone T14L contained the longest insert, with a size of 2.8 kb. Nucleotide sequencing analysis revealed that T14L was 2,837 bp long and highly homologous to the nucleotide sequence of human APP770 cDNA (Yoshikai *et al.*, 1990), indicating that it is the porcine homologue of APP770. Because T14L did not contain the whole open reading frame, we conducted an additional 5'RACE to obtain the complete 5'-portion of APP770. We isolated ten amplified clones and sequenced them. It was found that all these clones had the same sequence. Consequently, T14L and the clones generated by 5'RACE spanned 3,051 bp and contained the complete open reading frame of APP770 (Fig. 1). By computer analysis using the program MacVector (Ver. 6.01; Oxford Molecular, Ltd.), the nucleotide sequences of the open reading frame were found to be 92.2%, 88.8%, and 90.1% identical to those of the human (Yoshikai *et al.*, 1990), mouse (Fukuchi *et al.*, 1989; Flood *et al.*, 1997), and guinea pig APP770 (Beck *et al.*, 1997), respectively. When the amino acid sequence of porcine APP770 was compared with those from other species, more striking homologies of 97.8% (for humans), 97.1% (for mice), and 97.4% (for guinea pigs) were observed.

Expression of APP

Northern blot analysis was conducted using total RNAs from various tissues and cells (Fig. 2). A major band was detected at 3.1 kb in all tissues and cells examined, a result consistent with previous reports for other mammalian species

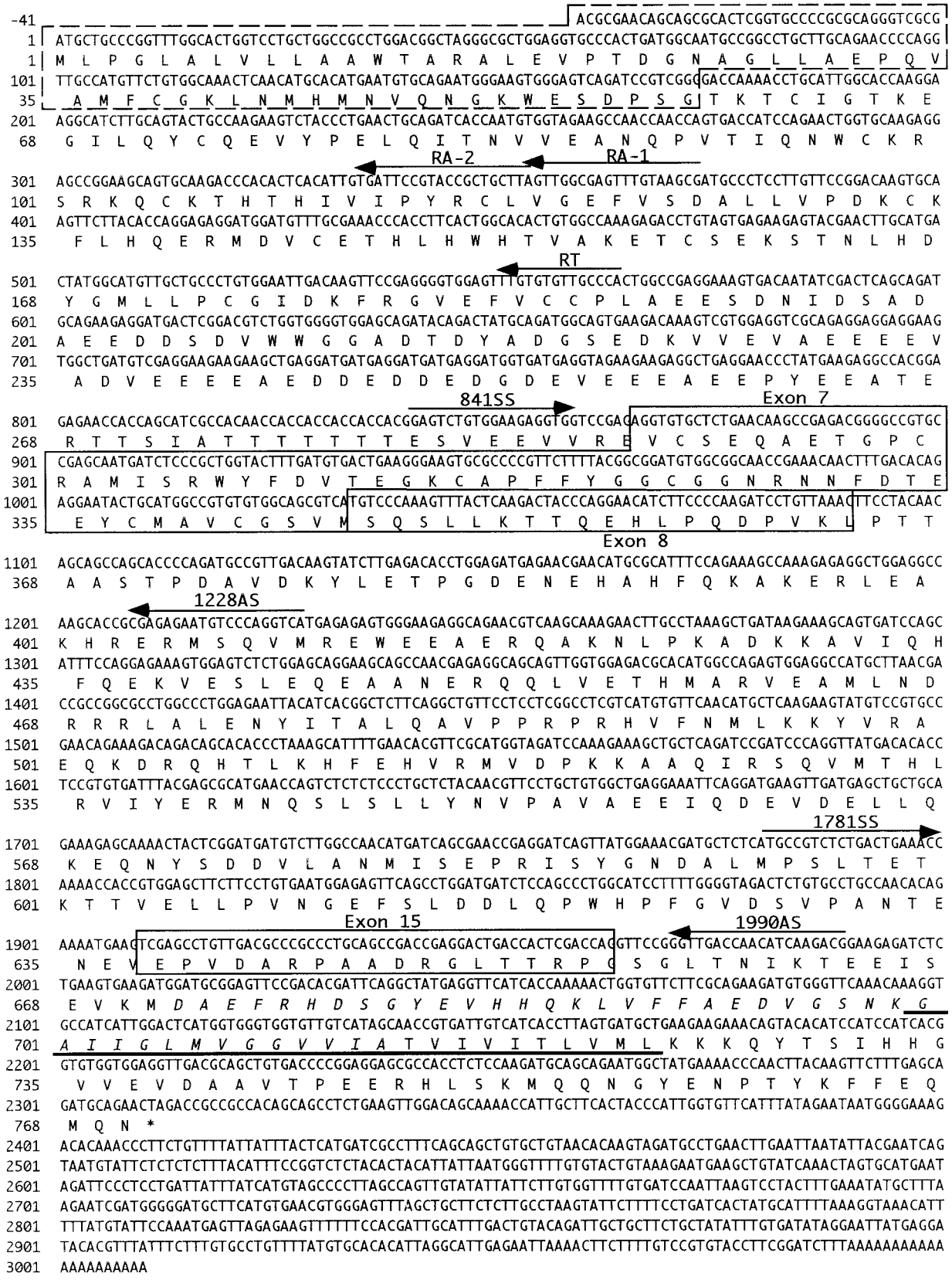

Fig. 1. Nucleotide and deduced amino acid sequences of porcine APP770. The positions of primers are indicated by arrows. Three alternative spliced exons are boxed and the sequence obtained by 5'RACE is boxed with broken lines. The predicted transmembrane sequence is underlined and the amyloid-β peptide is shown in italic type. The sense and antisense primers used for further experiments are shown by arrows (see the text for details). This nucleotide sequence has been submitted to the DDBJ/EMBL/GenBank™ Data Bank with the accession number AB032550.

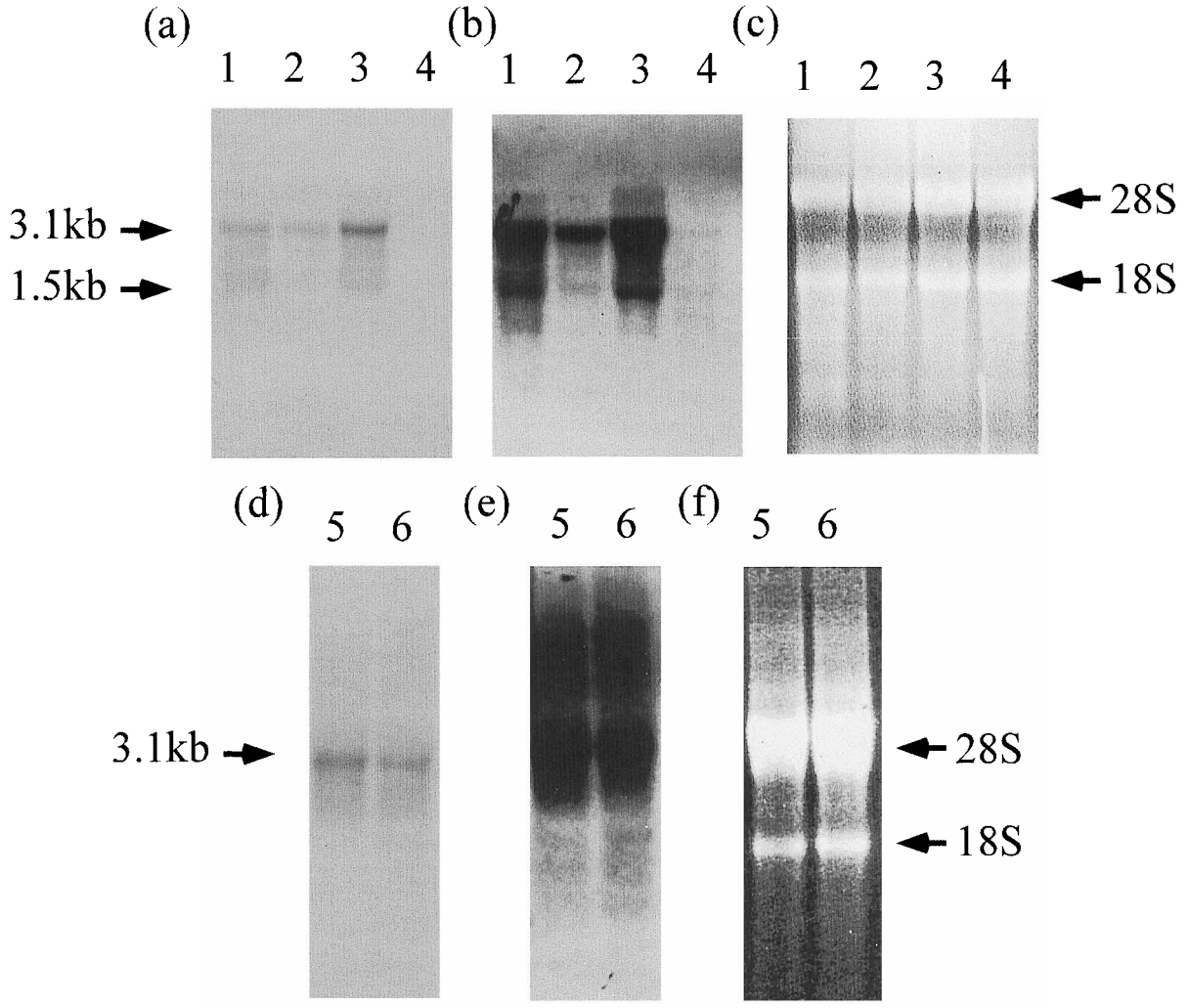

Fig. 2. Northern blot analysis of APP in porcine tissue. Twenty micrograms of total RNA from the ovaries (lane 1), testis (lane 2), adrenal glands (lane 3), liver (lane 4), and granulosa cells from small (lane 5) and large (lane 6) follicles were applied to each lane. (b) and (e) show long exposed images of (a) and (d). The ethidium bromide staining in (c) and (f) confirms that equivalent amounts of RNA were loaded on each lane.

(De Sauvage and Octave, 1989). Strong signals were observed for tissues of the ovaries, testis, and adrenal glands, whereas only a faint signal was detected for the liver even after long exposure of the membrane. Long exposure led to an appearance of a 1.5-kb band in all tissues examined, with the signal being particularly prominent for the ovaries and adrenal glands (Fig. 2b). Several investigators (De Sauvage *et al.*, 1989; Donnelly *et al.*, 1988; Zain *et al.*, 1988) detected APP mRNA signals of 1-2.3 kb in size using human leukemia cells (HL-60 cell) and human brain tissue. We presume that the 1.5-kb transcript in the present study corresponds to those reported previously. As shown in Fig. 2e, the intensity of the 3.1 kb band was similar intensity between the granulosa cells isolated from large- and small-sized follicles, whereas the 1.5 kb band was not observed in either type of cells.

Identification of alternatively spliced products in the porcine ovary

It is well known that several distinct forms of APP are generated through alternative splicing of the mRNA. Previous studies on the human and rat APP genes have clearly documented that splicing events involving the exons 7, 8, and 15 are responsible for the production of APP isoforms. We therefore examined whether such an alternative splicing mechanism also operates in the porcine ovary, although the APP gene structure, including its exon/intron organization, is not yet known in this species. To this end, RT-PCR analyses were performed using two primer sets, the first being a combination of the primers 841SS and 1228AS, and the second of the primers 1781SS and 1990AS (Fig. 1). Using the former set, three differently sized bands were amplified (Fig. 3a). Nucleotide sequencing analysis of the products revealed that the 388-bp band included putative exons 7 and 8, the 331-bp band only exon 7, and the 163-bp band neither exons 7 nor 8. Using the latter primer set, two amplified products were obtained (Fig. 3b). The nucleotide sequencing showed that the 210-bp band included putative exon 15 while the 156-bp band did not. These results clearly demonstrate that the porcine ovary

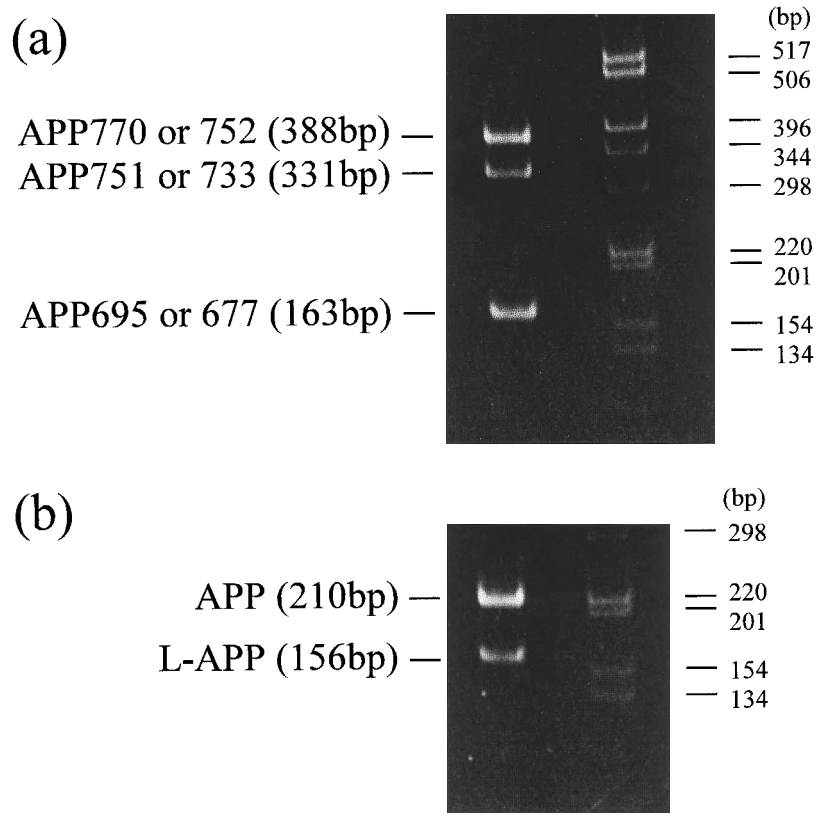

Fig. 3. Identification of alternative spliced products of APP in the porcine ovary by RT-PCR. (a) A primer pair of 841SS and 1228AS was used to detect the alternative splicing of exons 7 and/or 8. Three bands were amplified and their sizes are indicated to the left along with the predicted isoform names. (b) A primer pair of 1781SS and 1990AS was used to detect L-APP, an isoform lacking exon 15. Two bands were amplified and their sizes are indicated to the left. The molecular size markers (1 kb DNA ladder, Life Technologies, Inc.) were applied and their sizes are shown to the right.

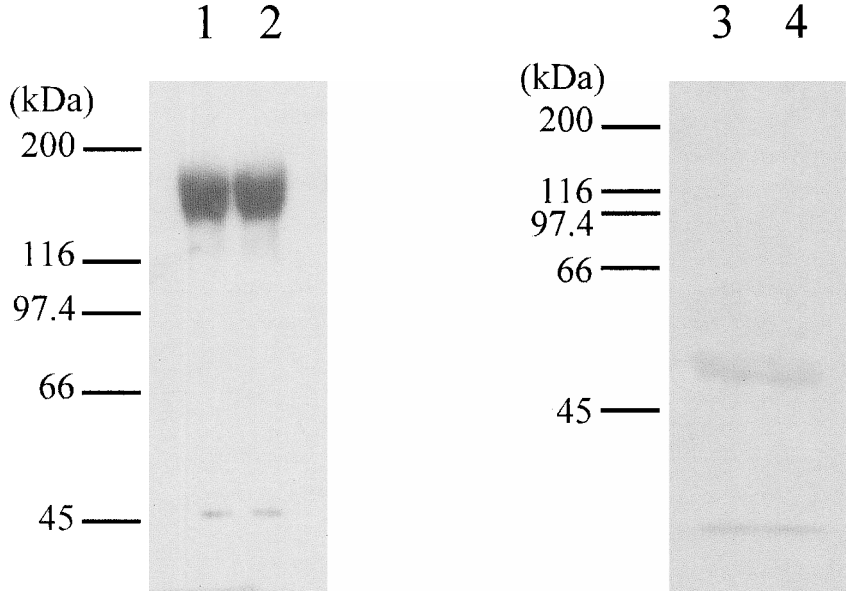

Fig. 4. Immunological detection of APP in follicular fluid. Sixty micrograms of follicular fluids from small (1 and 3) or large (2 and 4) follicles of porcine ovary were applied to each lane. SDS-PAGE analysis under reducing (3 and 4) and nonreducing (1 and 2) conditions was conducted on 6% and 8% polyacrylamide gels, respectively. The signals were detected with mouse anti-β-amyloid precursor protein antibody. The positions of the molecular size markers are shown to the left.

mRNA fraction contained 4-6 species of APP transcripts generated through alternative splicing involving the exons 7, 8 and 15.

Immunological detection of APP in the porcine ovary

To detect APP expressed in the porcine ovary, we conducted Western blot analysis using the extract of granulosa cells and the fluid of ovarian follicles. Since the primary structure of porcine APP is highly homologous to that of its human counterpart (97.8% identity), we initially thought that any commercially available anti-human APP antibody would cross-react with the porcine protein. Of three antibody products tested, only one was found to be suitable. The antibody used here was raised against human APP, and specifically recognizes the amino-terminal 200 amino acids of APP. As shown in Fig. 4, the follicular fluid from small (1–2 mm in diameter) and large (5-8 mm in diameter) follicles gave signals at 47 kDa and 134–178 kDa under nonreducing conditions (Fig. 4, lanes 1 and 2), and at 48 kDa and 32 kDa under reducing conditions (Fig. 4, lanes 3 and 4). The separation pattern and intensity of the respective bands were essentially the same between the two sizes of follicles. Soluble and membrane fractions were also prepared from the granulosa cells of small- and large-sized follicles, and analyzed for APP by Western blotting under the same conditions. However, no specific, immunoreactive band was visualized even when as much as 100 μg of the protein was applied (data not shown). These results indicate that APP detectable with the antibody used in this experiment is exclusively present in the follicular fluid of the porcine ovary.

DISCUSSION

In this study, we determined the complete nucleotide sequence of an APP cDNA clone isolated from a porcine ovary cDNA library. Since Johnstone *et al.* (1991) reported a 175-bp nucleotide sequence for APP cDNA from the same species, we compared our clone with their sequence. The reported 175-bp sequence corresponded to the nucleotides 1997–2171 of the present sequence (Fig. 1). The two sequences found to be perfectly matched with the exception of a single base substitution of guanine for adenine at 2037 in the present sequence. However, this substitution does not give rise to any change in the coding of amino acids. At present, we presume that this substitution may be due to the polymorphism of the gene in this species.

The porcine liver was found to express APP mRNA at a drastically lower level than the ovaries and adrenal glands. Consistent with our present findings, Tanzi *et al.* (1988) detected no APP expression in the human fetal liver. These findings may indicate that a dearth of APP expression in the liver is common among mammals. This is of particular interest because the APP gene is thought to be a housekeeping gene and indispensable for maintaining cellular activity in all types of cells. The present observation suggests the lack of a factor(s) necessary for hepatic expression of this gene. The extent of ovarian expression of APP mRNA relative to brain was not examined in this study. Previous data indicate that in human the gene is expressed in all tissues, especially in brain, heart, spleen and muscle (Tanzi *et al.* 1989). No result is available to date for its expression in the ovary. However, a moderate expression of APP mRNA was previously observed in the human adrenal gland (Tanzi *et al.* 1989). Since APP mRNA was detected with the adrenal gland and ovary at a similar level in this study, we presume that ovarian expression of this gene is not strong but moderate.

The ovaries expressed not only the APP mRNA but also the 1.5 kb mRNA. The nature of this band is not known at present, but it might be a transcript coding for an APP-related protein. Recently, cDNA clones for amyloid precursor-like protein (APLPs), which display remarkable homology to APPs, were isolated from mammalian brains. Two distinct APLPs, APLP1 (Wasco *et al.*, 1992) and APLP2 (Wasco *et al.*, 1993), have been identified. The sizes of APLP1 mRNA have been reported to be 2.4 and 1.6 kb in the mouse brain (Wasco *et al.*, 1992), while that of APLP2 mRNA is about 4 kb in the human brain (Wasco *et al.*, 1993). Since the 1.5 kb band detected in the present study is close in size to one of the mouse APLP1 mRNAs, we tentatively presume that this signal represents a smaller porcine APLP1 transcript. Interestingly, the 1.5 kb mRNA was not expressed in the granulosa cells of small- or large-sized follicles, indicating that the signal originated from ovarian cells other than granulosa cells.

RT-PCR experiments using porcine ovary mRNA demonstrated that, as in the case of rat and human APP genes, alternative splicing products are generated from the splicing of porcine APP mRNA precursor. Further, exons 7, 8, and 15 have been clearly demonstrated to be involved in the splicing events. We observed three PCR bands when the primers 841SS and 1228AS were used. On the other hand, two PCR products were amplified with the primers 1781SS and 1990AS. These results indicate that porcine ovary mRNA possibly contains six different types of APP transcripts (Fig. 5). Although all three exons are involved in the production of such APP mRNA isoforms, splicing products containing the exon 8 but not the exon 7 were not detected in the present study. These results are compatible with the observation of Sandbrink *et al.* (1994) that, in the rat, the corresponding APP mRNA isoforms (namely, APP714 and APP696) comprise less than 5% of the total.

We found that the porcine ovarian follicular fluid contains APP in soluble state. As indicated by the results of Western blot analysis, APP is present in the fluid in several isoforms. At present we know little about the nature of these individual APPs, but certainly these isoproteins must be generated as a result of proteolytic processing of APP after APP is embedded in the cell membrane. It is generally believed that the major pathway for producing soluble APP involves a peptide bond cleavage at the α secretory cleavage site (Lys^{687}-Leu^{688} in the human APP sequence) within the extracellular portion (Hendriks and van Broeckhoven, 1996). A putative enzyme involved in this proteolytic cleavage is called α secretase, but its entity has not yet been clarified. So far, cathepsin B

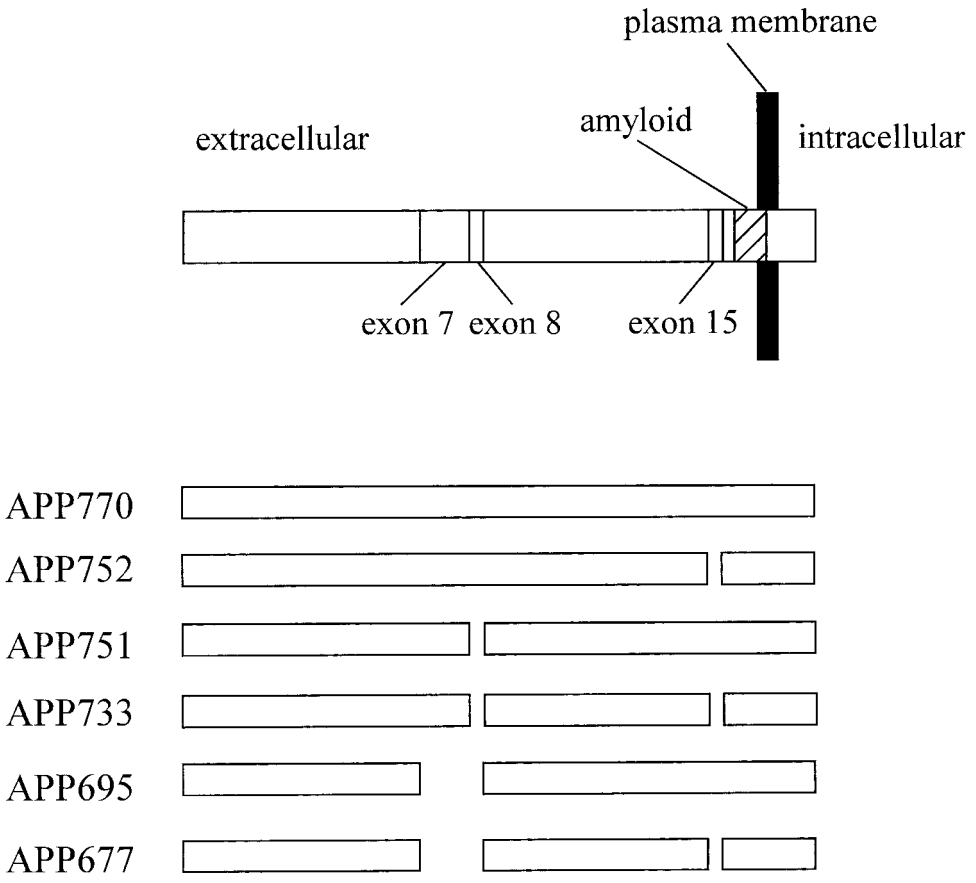

Fig. 5. Schematic drawing of possible APP mRNA splice isoforms present in the porcine ovary. The structure of the full-length APP transcript is indicated at the top. Below this, six isoforms generated by alternative splicing of exons 7, 8, and 15 are illustrated with their names.

(Tagawa *et al.*, 1991), tumor necrosis factor α converting enzyme (Buxbaum *et al.*, 1998), and membrane-anchored metalloprotease MDC9 (Koike *et al.*, 1999) have been proposed as candidate enzymes for α-secretase. As demonstrated in this study, the primary structure of porcine APP is strikingly homologous to that of human APP, and the amino acid sequences around the α secretory cleavage site are perfectly conserved in the two species. Therefore, we may be able to assume that the same molecular mechanism, including an involvement of α-secretase, underlies the production of soluble APP in the follicles of the porcine ovary. Our present data strongly suggest that porcine ovarian follicular fluid certainly contains α-secretase or an enzyme(s) having α-secretase-like activity. It would be interesting to determine whether this putative fluid enzyme would be one of the reported candidates described above. However, it must be pointed out that the molecular weights of soluble APP found in the fluid are much smaller than expected when we assume a sole action of α-secretase or α-secretase-like enzyme. This finding clearly indicates that an additional proteinase(s) responsible for further metabolism of soluble APP is present in the follicular fluid of the porcine ovary.

This study was initiated in the hope of determining the biological role(s) of APP in mammalian ovaries. One of the most intriguing issues in reproductive biology of the ovary concerns the mechanisms by which a cohort of primodial follicles is either selected to become dominant or destined for programmed cell death by atresia. We are particularly interested in the relation between APP metabolism and atresia. Since formation of Aβ peptides (Loo *et al.*, 1993; Moechars *et al.*, 1996; Yamatsuji *et al.*, 1996; Zhao *et al.*, 1997) and intracellular accumulation of APP (Yoshikawa *et al.*, 1992; Nishimura *et al.*, 1998; Bursztajin *et al.*, 1998; Uetsuki *et al.*, 1999) are both known to induce neurodegeneration characteristic of apoptosis, one may speculate that an alteration in the metabolic pathway of APP in ovarian follicles could be closely associated with atresia. To our knowledge, no such studies have been reported. For this purpose, porcine ovaries probably serve as a good experimental system in that biochemical studies are also feasible because large amounts of materials are easily available. As a first step, we needed to establish a basis for addressing the above problem. The current information concerning the expression of APP mRNA and protein in ovarian follicles is indispensable for our future studies.

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