Alzheimer’s amyloid precursor protein (APP) is a transmembrane protein containing three phosphorylation sites in its cytoplasmic domain. In the present study, we isolated cDNA of APP from electric ray electric lobe (elAPP). This APP (elAPP699) consists of 699 amino acids, contains the β-amyloid domain and has 80.7% similarity with the human APP695 isoform. The cytoplasmic domain, including three phosphorylation sites, was completely conserved. In the nerve terminals of the cholinergic neuron from the electric ray electric organ, we found elAPP699 existed exclusively in the mature form. We found the phosphorylated form of mature elAPP699 in the nerve terminal as well as in cell body. Immature elAPP699 was not subject to phosphorylation. Our findings indicate that, in neurons, the phosphorylation of APP occurs after maturation.

INTRODUCTION

Alzheimer’s amyloid precursor protein (APP) is an integral membrane protein with a receptor-like structure, from which β-amyloid (Aβ) derives [1–4]. APP is a phosphoprotein [5–7] and its cytoplasmic and extracellular domains are phosphorylated in intact cells [8–12]. However, the role of APP phosphorylation in neuronal functions and/or APP metabolism have not yet been well characterized. Among three phosphorylation sites in the APP cytoplasmic domain [5,7,10,11], phosphorylation of APP at threonine-668 (human APP695 isoform) was originally found in cultured cells at the G2/M phase of the cell cycle and it has been well characterized that cdc2 kinase phosphorylates immature APP (imAPP, N-glycosylated form) preferentially [10,11]. The phosphorylation site corresponding to threonine-668 of APP is conserved in the cytoplasmic domain of amyloid precursor-like protein 2 [13] and in the APP-related protein of Caenorhabditis elegans, apl-1 [14]. The conservation analysis suggests the importance of phosphorylation of the site to the physiological function of APP. Our recent study using a phosphorylation-state-specific antibody raised against phosphorythreonine-668 of APP (pAbThr668) has also shown that only the mature (mAPP) N- and O-glycosylated form of APP695, a major APP isoform expressed specifically in neuronal tissues, is phosphorylated in rat brain [11], although cdc2 kinase activity is very low in such neuronal tissues [15]. The previous data indicate that the mechanism of APP phosphorylation at threonine-668 in the brain differs from that in cultured cell lines, although the phosphorylation site is identical. Therefore further analysis of APP phosphorylation and its mechanism are thought to be important to elucidate its role in neuronal tissues.

The electric lobe and the electric organ of electric ray such as Narke japonica are invaluable in studies designed to analyse the function and metabolism of APP. The electric lobe consists only of cell bodies of pure cholinergic neurons and, because the presynaptic nerve terminal is located >2–3 cm distant from the cell body, pure presynaptic nerve terminals of cholinergic neurons can be prepared without contamination by proteins of the cell body [16]. Analysis of APP metabolism and its localization within cholinergic neurons from the electric lobe and electric organ may yield important information concerning the mechanism of neuronal degeneration, and, since the number of cholinergic neurons decrease progressively in the brain of patients with Alzheimer’s disease [17], this may be of importance in the study of the human disease. Using these advantageous factors in the preparation of the electric lobe and the electric organ of Japanese electric ray, Narke japonica, we analysed biochemically the distribution of APP and the localization of phosphorylated forms of APP in the cholinergic neuron. The existence and expression of APP in the electric lobe of the electric ray was confirmed by isolation of cDNA of APP from the electric-lobe cDNA library. The isolated clone, human APP695-like electric ray APP (elAPP), consisted of 699 amino-acid residues and contained an Aβ domain (we designated this elAPP699). The present study demonstrated that only the mature (O- and N-glycosylated form) elAPP699 exists in the nerve terminals of cholinergic neurons and it is phosphorylated in both the cell body and nerve terminal. Immature (N-glycosylated form) elAPP699 is not subject to phosphorylation. Furthermore, elAPP699 represents the first case of cDNA cloning of a human APP homologue containing the Aβ domain from a non-mammalian source.

EXPERIMENTAL

Antibodies

Anti-APP cytoplasmic domain polyclonal antibody (AbAPP), G-369, was raised against human APP695[315–394] peptide [18] and...
another AbAPP, UT-421, was raised against human APP695-695 (S. Tomita, Y. Kirino and T. Suzuki, unpublished work). Both antibodies were useful in isolating eAPP cDNA by immunoscreening and for detecting eAPP from tissues of *N. japonica* by Western blot and immunoprecipitation. Both AbAPPs react only to APP and not to APP-like proteins ([11] and Y. Satoh, unpublished work). 1 and 2 [19,20]. Phosphorylation-state-specific polyclonal antibodies pAbThr668, G-474 and UT-436, were raised against human APP695-675-[Cys]-[Thr668]. pAbThr668 was purified by affinity chromatography using resin which had been coupled to the antigen peptide before use. The purified antibody was highly specific for APP phosphorylated at threonine-668 and did not cross-react with APP phosphorylated at other sites or with dephosphorylated APP ([11] and Y. Satoh, unpublished work).

**Preparation of synaptosomes from the electric organs of *N. japonica***

Synaptosomes were prepared from fresh electric organs by the method described previously [21]. The purity of samples was confirmed by electron microscopy. The samples were stored at –80 °C until use.

**Immunoblot analysis**

Electric lobe, forebrain and cerebellum dissected from electric ray and synaptosomes prepared from the electric organ were homogenized and sonicated following a procedure for the preparation of rat brain lysate as described previously [11]. Samples were treated with SDS sample buffer [50 mM Tris–HCl (pH 6.8)/12.5 mM EDTA/3.75% (w/v) SDS/10% (v/v) glycerol/4 M urea/2.5% (v/v) 2-mercaptoethanol/0.015% (w/v) Bromophenol Blue], boiled for 5 min, analysed by SDS–PAGE [6% (w/v) acrylamide] and electrophoretically transferred to a nitrocellulose membrane. Membrane was probed with appropriate antibodies and 125I-labelled Protein A (IM144, Amersham). Radioactivity was quantified using a Fuji BAS 2000 Imaging Analyzer (Tokyo, Japan) or by autoradiography.

**Enzymic deglycosylation of eAPP**

Tissue and synaptosome homogenates, prepared as described above, were centrifuged (10000 g for 10 min) and eAPP was immunoprecipitated from the resulting supernatant with AbAPP and Protein A-Sepharose (Pharmacia). Deglycosylation of APP was performed using a procedure described previously [22]. The eAPP–antibody–resin complex was treated for 16 h with a combination of 10 m-units neuraminidase (EC 2.3.1.18; Seika gaku Co., Tokyo, Japan) and 1 m-unit O-glycanase (EC 3.2.1.97; Genzyme Co., Cambridge, MA, U.S.A.) in 40 mM Tris/maleate, pH 6.0, containing 2.25 mM CaCl₂, 200 µg/ml of pepstatin A, 200 µg/ml of chymostatin and 200 µg/ml of leupeptin. After incubation, the antigen–antibody complexes were washed as described previously [10], treated with SDS sample buffer, and separated by SDS/PAGE [6% (w/v) acrylamide]. Samples were electrophoretically transferred to nitrocellulose membranes and analysed by immunoblotting using AbAPP.

**Molecular cloning of eAPP cDNA**

All standard molecular biological techniques were performed as described [23]. cDNA was synthesized with poly(A)+ RNA from the electric lobe of *N. japonica* and was then inserted into AZAP Express vector according to the manufacturer’s instructions (Stratagene). Approx. 10<sup>5</sup> independent plaques were screened with AbAPP and positive plaques were isolated and purified with LambdaSorb Phage Adsorbent (Promega). The cDNA obtained was excised by digestion with *Xho*I/*Nhe*I and subcloned into pMAM2-BSD (Funakoshi, Tokyo, Japan) for sequence analysis by dyeoxy terminator cycle sequencing with Prism 377 DNA Sequencer (Perkin–Elmer/ABI).

**Rapid amplification of cDNA ends (RACE) procedure for obtaining 5′-cDNA extensions**

The RACE procedure was carried out as described previously [24]. Primers corresponding to nucleotides 282–299, 312–330 and 531–549 of the sequences underlined in Figure 2. The products were subcloned into a PCR II vector by TA cloning (Invitrogen), positive clones were selected and the nucleotide sequences of both strands were determined with Prism 377 DNA Sequencer.

**RESULTS**

**APP in neuronal tissues of *N. japonica***

We probed the existence of APP in neuronal tissues of electric ray with AbAPP. There were no previous reports of APP in electric ray, although APP-like proteins lacking an Aβ domain have been reported from non-mammalian sources [14,25]. Japanese electric ray, *N. japonica*, has three major neuronal organs consisting of forebrain, cerebellum and electric lobe. When protein extracts (400 µg) prepared from these three organs and from rat cerebrum were separated by SDS/PAGE, transferred to nitrocellulose membrane and probed with AbAPP (G-369), immunoreactive proteins from the three neuronal organs were detected, with molecular masses similar to rat APP (Figure 1). These proteins were thought to be electric ray APP because no immunoreactivity was detected when AbAPP was pre-absorbed with human APP645-649 (numbering for human APP695 isoform) antigen peptide (results not shown). Thus the result strongly suggests that the neuronal organs of electric ray express APP as do those of mammals.

**Isolation of cDNA encoding eAPP and structural analysis of eAPP protein**

Immunoblot analysis using AbAPP indicated the existence of APP in electric ray neuronal organs. However, it was still unclear whether the electric ray APP was a ‘true’ APP containing an Aβ domain or was an APP-like protein. To resolve this issue, we isolated a cDNA encoding APP from an electric-organ cDNA library by immunoscreening with AbAPP. Two independent

**Figure 1** APP from electric ray forebrain, cerebellum and electric lobe and rat cerebrum

Protein extracts (400 µg) from the organs indicated was analysed by SDS/PAGE, transferred to nitrocellulose membrane and probed with AbAPP (G-369) as described in the Experimental section. The square bracket indicates APP isoforms.
Alzheimer’s amyloid precursor protein from electric ray neuronal tissues

Figure 2 Nucleotide sequence analysis of elAPP699 cDNA

Nucleotides are numbered on the right. Capital letters indicate sequence encoding protein and the first nucleotide of the first in-frame methionine. The underlined sequences are the primers used for 5'-RACE. cDNA clones were isolated and both clones were found to encode identical genes (results not shown). The size of this cDNA clone was approx. 3.0 kbp and its nucleotide sequence was determined. Approximately 150 nucleotides of the 5'-translating region were missing from this clone. We determined the 5'-nucleotide sequence by RACE using the indicated primers and the entire nucleotide sequence is shown in Figure 2. A single open reading frame was found, extending from nucleotide 1 to a stop codon at position +1998 ~ +2100. The amino-acid sequence deduced from the nucleotide sequence of the cDNA clone is shown in Figure 3. The first methionine residue after the termination codons was chosen and the nucleotide sequence surrounding this first methionine appeared to match consensus sequence described by Kozak [26]. The open reading frame encoded a protein consisting of 699 amino acids (Figure 3). Comparison of the deduced amino-acid sequence of the clone with the database revealed a significantly high similarity with human APP695 (81% identity). The sequence contained an Aβ domain (88% identity; 38 amino acids identical in 43 residues) and the putative cytoplasmic domain of the C-terminal region was completely identical with that of mammalian APP (Figure 3). Furthermore, the clone did not contain a Kunitz-type protease inhibitory domain in its N-terminal domain [27,28], which indicates that the clone encodes an APP695-like protein. The results indicated that the clone encodes a homologue of human APP695, a neuron-specific isoform of APP.

Characterization and intracellular localization of elAPP

The electric lobe consists of cell bodies of pure cholinergic neurons with nerve terminals to the electric organ, which is located distantly (~2 cm) from the electric lobe cell body. Synaptosome preparations from the electric organ were separated by SDS/PAGE, transferred to a nitrocellulose membrane and probed with AbAPP (Figure 4a). The electric-lobe preparation had two major and one minor elAPP bands. The two major elAPP bands (Figure 4a; arrows 2 and 3) could be the protein products of elAPP699, the cDNA of which was isolated in this study, as the size of elAPP699 is a homologue of human APP695, a neuron-specific isoform of APP.

An alignment of the human APP695 and elAPP699 amino-acid sequences was compared. Gaps produced by the alignment are indicated by a hyphen in the sequence. Predicted transmembrane regions are shown in italics and β-amyloid domains are boxed. The cytoplasmic domain is indicated by bold underlining. Phosphorylation sites are indicated by an arrowhead; * and + indicate the positions of identical or conservatively substituted amino acid residues respectively. The bold dotted underlining indicates a possible signal sequence.
APP695 isoform (Figure 1). The minor protein (Figure 4a, arrow 1) could be another isoform of eAPP or a differently modified form of eAPP699. The synaptosome preparation contained only two immunoreactive proteins (Figure 4a, arrows 1 and 2).

It has been well documented that mammalian APP is modified with N- and O-glycans on its extracellular domain [29,30]. In cells, mAAPP (N- and O-glycosylated form) is localized in compartments following trans-Golgi and on the plasma membrane, but imAPP (N-glycosylated form) localizes in the endoplasmic reticulum and cis-Golgi. Furthermore, in neurons, it has been documented that APP is delivered first to the nerve terminal by axonal transport after maturation and then secondarily is transported to the somato plasma membrane and to dendrites by transcytosis [31]. To confirm that the elAPP detected in nerve terminal was mAAPP, elAPP isolated from the electric lobe and synaptosomes were treated with a combination of neuraminidase and O-glycosidase (Figure 4b). The quantity of the major elAPP species (Figure 4a, arrow 2) extracted from synaptosomes and the electric lobe was decreased remarkably (Figure 4b, left lane) and the quantity of another major elAPP species (Figure 4a, arrow 3) was found in increased quantity after treatment of the samples with the enzymes (compare Figures 4b and 4a). The results show that the band indicated by arrow 2 contains the mature and that indicated by arrow 3 the immature isoform of elAPP699. A relatively large amount of the electric lobe band (arrow 2) was observed even after enzyme treatment of elAPP (Figure 4b, right lane), although a minor elAPP species (Figure 4a, arrow 1) was also decreased after the treatment. This suggested that the band indicated by arrow 2 must be a mixture of mature major elAPP699 and immature minor elAPP isoforms. Thus a possible explanation is as follows, The bands indicated by arrow 1 is a mature unidentified minor elAPP isoform; those indicated by arrow 2 are a mixture of mature elAPP699 and an immature, unidentified minor elAPP isoform; and those indicated by arrow 3 are immature elAPP699. The results of this study, using enzymes, demonstrated that only mature elAPP is distributed in the nerve terminals of cholinergic neurons.

APP is a phosphoprotein [5–13] and phosphorylation of the cytoplasmic domain at threonine-668 (numbering for the human APP695 isoform) has been well characterized by studies using cultured cells and rat brain tissues [10,11]. Amino-acid sequence of the elAPP699 cytoplasmic domain was completely identical with that of mammalian APP (Figure 3). Therefore it was feasible to use pAbThr668, raised against a peptide from human APP phosphorylated at threonine-668, to analyse the distribution of the phosphorylated form of elAPP in a cholinergic neuron. The elAPP recovered from synaptosomes and electric lobe was probed with pAbThr668 (Figure 4c). Immature elAPP from the electric lobe (Figure 4a, arrow 3) was not detected in the phosphorylated form (Figure 4c). Only mature elAPP699 from synaptosomes and electric lobe was detected in phosphorylated form (Figure 4c, arrow 2). The mature minor elAPP species was also phosphorylated in the electric lobe (Figure 4c, arrow 1). The results demonstrate that only the mature elAPP species is distributed in nerve terminals and it is subject to phosphorylation at threonine-672 (numbering for eAPP699).

**DISCUSSION**

elAPP699 is the first example of APP isolated from non-mammalian organs. Fruit fly, Drosophila melanogaster [25], and nematode, C. elegans [14], possess APP-like proteins but lack the \( \alpha \)-domain. elAPP699 contains an \( \alpha \)-domain and the amino-acid sequence surrounding the \( \alpha \)-secretase site within the \( \alpha \)-domain has been conserved. It is possible that elAPP699 is cleaved by a putative \( \alpha \)-secretase as is mammalian APP, although we failed to detect the truncated C-terminal fragment from elAPP produced by cleavage of \( \alpha \)-secretase. This may be because of the low concentration of the fragment in the electric lobe and nerve terminal. Furthermore, since APP-like proteins from non-vertebrates do not contain the \( \alpha \)-domain [14,25], the \( \alpha \)-domain itself may be a characteristic sequence of vertebrate animals. The elAPP699 cloned in this study does not contain a Kunitz-type protease inhibitory domain. APP isoforms including the Kunitz-type protease inhibitory domain have been detected in mammalian APP770 and APP751, which are ubiquitous isoforms found in all tissues [27,28,32]. Thus elAPP699 may be a neuron-specific isoform, although a minor elAPP species (Figure 4a, arrow 1) may be a ubiquitously isoform containing a Kunitz-type protease inhibitory domain.

We found the existence of mature elAPP in synaptosomes. This suggests that APP may play an important role in nerve-terminal functions such as neurotransmitter release or synapse formation. Immunohistochemical staining of rat brain tissues with AbAPP has demonstrated the distribution of APP in cytoplasm and dendrites of neurons [33]. In this type of study, however, it was impossible to differentiate the distribution of mAAPP from that of imAPP. When preparing synaptosomes from rat brain, it is very difficult to prepare extremely pure synaptosomes that do not contain post-synaptic membrane. Thus a biochemical study using rat synaptosome cannot show the exact distribution of APP in the presynaptic terminals. Synaptosomes prepared from the electric organ do not contain post-synaptic membrane or plasma membrane from the cell body [16,21]. Therefore the results presented in the present study show exactly the localization of mature elAPP in nerve terminals.

Recently, we have shown that APP is phosphorylated at threonine-668 in rat brain tissues [11], and the results of the present study also clearly demonstrate that the phosphorylated form of mature elAPP is localized in the nerve terminals. The phosphorylated form of mature elAPP is also detected in samples from the electric lobe. The molecular mechanism of APP phosphorylation in neurons is still unknown. It is thought that APP is delivered to the nerve terminal by axonal transport following protein synthesis [31], and it is possible that APP is phosphorylated before axonal transport and that the phosphorylated form is delivered to the nerve terminal. Another possibility is that APP may be phosphorylated at the nerve...
terminal after axonal transport, and that the phosphorylated form is then subject to transcytosis into the plasma membrane of the cell body and dendrites.

A protein kinase responsible for the phosphorylation of APP at threonine-668 (numbering for human APP695) and/or of elAPP at threonine-672 (numbering for elAPP699) has yet to be identified. Possible candidates for proline-directed serine/threonine kinase are cyclin-dependent kinase 5, the regulatory subunit of which is expressed at high levels in brain [34,35], and glycogen synthase kinase-3, a kinase that is abundant in neurons [36]. We have detected a protein that shows reactivity to rat cyclin-dependent kinase 5 antibody in synaptosomes prepared from the electric organ (results not shown). If APP is phosphorylated at the nerve terminal, the synaptosome preparation from the electric organ may be useful material for the identification of APP kinase.

Although the role of phosphorylation of human APP at threonine-668 and/or elAPP at threonine-672 is still unclear, the present biochemical analyses for elAPP using preparations from cholinergic neurons may aid the understanding of the biological function of APP and APP phosphorylation, and the pathogenesis of Alzheimer’s disease.

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