cDNA isolation of Alzheimer's amyloid precursor protein from cholinergic nerve terminals of the electric organ of the electric ray

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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 (*elAPP*699) 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 *elAPP*699 existed exclusively in the mature form. We found the phosphorylated form of mature *elAPP*699 in the nerve terminal as well as in cell body. Immature *elAPP*699 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 ($A\beta$) 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 phosphothreonine-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, *N. 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 $A\beta$ domain (we designated this *elAPP*699). The present study demonstrated that only the mature (*O*- and *N*-glycosylated form) *elAPP*699 exists in the nerve terminals of cholinergic neurons and it is phosphorylated in both the cell body and nerve terminal. Immature (*N*-glycosylated form) *elAPP*699 is not subject to phosphorylation. Furthermore, *elAPP*699 represents the first case of cDNA cloning of a human APP homologue containing the $A\beta$ domain from a non-mammalian source.

EXPERIMENTAL

Antibodies

Anti-APP cytoplasmic domain polyclonal antibody (AbAPP), G-369, was raised against human APP695^{645–694} peptide [18] and

Abbreviations used: APP, amyloid precursor protein; AbAPP, anti-APP cytoplasmic-domain antibody; $A\beta$, β -amyloid; *elAPP*, electric ray amyloid precursor protein; imAPP, immature form APP; mAPP, mature form APP; pAbThr668, phosphorylation-state-specific antibody to threonine-668 of APP695; RACE, rapid amplification of cDNA ends.

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another AbAPP, UT-421, was raised against human APP695⁶⁷⁶⁻⁶⁹⁵ (S. Tomita, Y. Kirino and T. Suzuki, unpublished work). Both antibodies were useful in isolating *e*/APP cDNA by immunoscreening and for detecting *e*/APP 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 APP695⁶⁶⁵⁻⁶⁷³[Cys]-[P_iThr668]. 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 *e*/APP

Tissue and synaptosome homogenates, prepared as described above, were centrifuged (10000 *g* for 10 min) and *e*/APP 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 *e*/APP-antibody-resin complex was treated for 16 h with a combination of 10 m-units neuraminidase (EC 3.2.1.18; 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 $\mu\text{g}/\text{ml}$ of pepstatin A, 200 $\mu\text{g}/\text{ml}$ of chymostatin and 200 $\mu\text{g}/\text{ml}$ 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 *e*/APP 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 λ ZAP Express vector according to the manufacturer's instructions (Stratagene). Approx. 10⁶ 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 dideoxy 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 pCRII 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 A β 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 APP⁶⁴⁵⁻⁶⁹⁴ (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 *e*/APP and structural analysis of *e*/APP 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 A β domain or was an APP-like protein. To resolve this issue, we isolated a cDNA encoding APP from an electric-lobe 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.

[illegible]

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 A β 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-like APP, and we have confirmed that the neuronal organs of the electric ray, or at least the electric lobe, express APP, as do mammalian neuronal tissues, and that the immunoreactive proteins detected with AbAPP, shown in Figure 1, is indeed APP and not APP-like protein. We tentatively assigned this APP as electric lobe APP699, or *el*/APP699, to distinguish it from the human APP species. When hydropathy of *el*/APP699 and human APP695 was compared, they showed similar profiles (results not shown), which also confirms that *el*/APP699 is a

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 are a source of pure cholinergic nerve terminals which are not contaminated with the plasma membrane fraction derived from the electric lobe cell body. Protein samples (400 μ g) of electric lobe and 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 *e*lAPP bands. The two major *e*lAPP bands (Figure 4a; arrows 2 and 3) could be the protein products of *e*lAPP699, the cDNA of which was isolated in this study, as the size of *e*lAPP is almost identical with the rat

Figure 4 Characterization and distribution of *e*APP and the phosphorylated form of *e*APP at threonine-672

(a) *e*APP was recovered from synaptosomes (left) or electric lobe (right) samples (1 mg protein) by immunoprecipitation with AbAPP (G-369), analysed on SDS/PAGE, transferred to membrane and probed with the same antibody. The arrows on the right indicate *e*APP isoforms. The protein molecular mass marker is shown on the left. (b) *e*APP recovered with AbAPP (G-369) and Protein A–Sepharose from synaptosomes (left) and electric lobe (right) samples (1 mg protein) was treated with a combination of neuraminidase and *O*-glycanase as described in the Experimental section. Treated samples were analysed by immunoblotting with AbAPP (G-369). The arrows on the right indicate *e*APP isoforms. (c) *e*APP recovered from synaptosomes (left) and electric lobe (right) samples by immunoprecipitation with AbAPP (G-369) was probed with pAbThr668 (UT-436). The arrows indicate *e*APP phosphorylated at threonine-672 (numbering for *e*APP699, a corresponding site for threonine-668 of human APP695).

APP695 isoform (Figure 1). The minor protein (Figure 4a, arrow 1) could be another isoform of *e*APP or a differently modified form of *e*APP699. 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, mAPP (*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 *e*APP detected in nerve terminal was mAPP, *e*APP isolated from the electric lobe and synaptosomes were treated with a combination of neuraminidase and *O*-glycanase (Figure 4b). The quantity of the major *e*APP 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 *e*APP 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 *e*APP699. A relatively large amount of the electric lobe band (arrow 2) was observed even after enzyme treatment of *e*APP (Figure 4b, right lane), although a minor *e*APP 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 *e*APP699 and immature minor *e*APP isoforms. Thus a possible explanation is as follows. The bands indicated by arrow 1 is a mature unidentified minor *e*APP isoform; those indicated by arrow 2 are a mixture of mature *e*APP699 and an immature, unidentified minor *e*APP isoform; and those indicated by arrow 3 are immature *e*APP699. The results of this study, using enzymes, demonstrated that only mature *e*APP 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 *e*APP699 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 *e*APP in a cholinergic neuron. The *e*APP recovered from synaptosomes and electric lobe was probed with pAbThr668 (Figure 4c). Immature *e*APP from the electric lobe (Figure 4a, arrow 3) was not detected in the phosphorylated form (Figure 4c). Only mature *e*APP699 from synaptosomes and electric lobe was detected in phosphorylated form (Figure 4c, arrow 2). The mature minor *e*APP species was also phosphorylated in the electric lobe (Figure 4c, arrow 1). The results demonstrate that only the mature *e*APP species is distributed in nerve terminals and it is subject to phosphorylation at threonine-672 (numbering for *e*APP699).

DISCUSSION

*e*APP699 is the first example of APP isolated from non-mammalian origins. Fruit fly, *Drosophila melanogaster* [25], and nematoda, *C. elegans* [14], possess APP-like proteins but lack the A β domain. *e*APP699 contains an A β domain and the amino-acid sequence surrounding the α -secretase site within the A β domain has been conserved. It is possible that *e*APP699 is cleaved by a putative α -secretase as is mammalian APP, although we failed to detect the truncated C-terminal fragment from *e*APP produced by cleavage of α -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 A β domain [14,25], the A β domain itself may be a characteristic sequence of vertebrate animals. The *e*APP699 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 *e*APP699 may be a neuron-specific isoform, although a minor *e*APP species (Figure 4a, arrow 1) may be a ubiquitous isoform containing a Kunitz-type protease inhibitory domain.

We found the existence of mature *e*APP 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 mAPP 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 *e*APP 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 *e*APP is localized in the nerve terminals. The phosphorylated form of mature *e*APP 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 *e*APP at threonine-672 (numbering for *e*APP699) 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 *e*APP at threonine-672 is still unclear, the present biochemical analyses for *e*APP 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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