MINIREVIEW

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Regulation of Amyloid β-Protein Precursor by Phosphorylation and Protein Interactions*

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Amyloid β-protein precursor (APP), a type I membrane protein, is cleaved by primary α- or β-secretase and secondary γ-secretase. Cleavage of APP by β- and γ-secretases generates amyloid β-protein, the main constituent of the cerebrovascular amyloid that accompanies Alzheimer disease. The generation and aggregation of amyloid β-protein in the brain are believed to be a primary cause of Alzheimer disease pathogenesis, and indeed, early onset Alzheimer disease is genetically linked to APP and also to presenilins 1 and 2, which are components of γ-secretase. Proteolytic cleavage of APP has been investigated as a candidate target for Alzheimer disease therapy, but the mechanisms regulating APP metabolism are still unclear. APP is a type I membrane protein with a short cytoplasmic region consisting of 47 amino acids. Recent research has elucidated the significance of the cytoplasmic region in the metabolism, trafficking, and physiological function of APP. The structure and function of the APP cytoplasmic domain can be modified by phosphorylation and through interaction with cytoplasmic proteins. This minireview summarizes a large body of recent information on the regulation of APP by phosphorylation and protein interaction, along with some of the physiological functions of APP. Recent findings regarding the regulation of APP processing contribute to the development of novel drugs and/or therapies for Alzheimer disease.

Characterization of APP and Its Metabolites in Brain and Amino Acid Sequence of the Cytoplasmic Domain of APP

APP[2] has three major isoforms of 695, 751, and 770 amino acids, all of which are derived from alternative splicing of a single gene product (reviewed in Ref. 1). In neurons, APP695 is the predominantly expressed form and is subject to *N*- and *O*-glycosylation within its extracellular/luminal domain. The other two isoforms, APP751 and APP770, are expressed mainly in non-neuronal cells, especially in glial cells in the brain. The *N*-glycosylated form of APP is localized to the endoplasmic reticulum and early Golgi; thus, "core" *N*-glycosylated APP is designated imAPP and is not subject to cleavage by secretases (2). *N*-Glycosylated APP is further trafficked within the Golgi and subjected to *O*-glycosylation, after which it is designated mAPP. Both "complex" *N*- and *O*-glycosylated mAPPs then reach the *trans*-Golgi network and enter into the late secretory pathway. During the late secretory pathway, APP is subjected to consecutive cleavage events in the primary extracellular/luminal juxtamembrane region by α-secretase (ADAM10 and ADAM17) or β-secretase (BACE1) and in the secondary transmembrane region by γ-secretase (reviewed in Ref. 3). As a consequence of this APP trafficking, neurons express two mAPP695 species with different types of *O*-glycosylation and one imAPP695 species (Fig. 1, *upper middle panel*). In contrast, glial cells express two mAPP isoforms (mAPP770 and mAPP751) and two imAPP isoforms (imAPP770 and imAPP751) (Fig. 1, *upper right panel*) (4, 5). Therefore, any metabolic analysis of APP must carefully distinguish mAPP from imAPP. The complement of APP isoforms detected in whole brain does not differ significantly from the neuronal complement (Fig. 1, *upper left panel*), indicating that the majority of APP expressed in the brain is neuronal and also that brain Aβ is secreted largely from neurons, but not from non-neuronal cells.

Aβ is generated from mAPP during the late secretory pathway, especially in the endosomal-lysosomal pathway, in which active β-secretases are highly concentrated (reviewed in Ref. 3). Therefore, it appears that Aβ generation is closely related to APP trafficking in the cell, especially in neurons, because terminally differentiated neurons have developed well organized systems for protein secretion and vesicular transport. The short cytoplasmic region of APP contains a phosphorylation site and functional motifs that play an important role in the regulation of its metabolism, trafficking, and function.

Phosphorylation of APP

APP is a phosphoprotein carrying several phosphorylatable amino acid residues in its cytoplasmic (6, 7) and luminal (8, 9) regions. The physiological phosphorylation state of APP has been investigated in brain, post-mitotic differentiating neuronal cells, and dividing cells (4, 10–13). The phosphorylated forms of APP present in each tissue are mAPP in neurons and imAPP in dividing cells. In either case, Thr^{668} (numbering for the APP695 isoform) in the cytoplasmic region of APP is the phosphorylatable amino acid (Fig. 2). CDK5 (cyclin-dependent kinase-5) and GSK-3β (glycogen synthase kinase-3β) are thought to phosphorylate mAPP at Thr^{668} in neurons, whereas CDK1/CDC2 kinase phosphorylates imAPP at Thr^{668} in dividing cells (4, 10, 11, 14, 15). When cells are subjected to a stress stimulus, JNK also phosphorylates APP at Thr^{668} (13, 16, 17). Thus, Thr^{668} is the sole or at least the major phosphorylation site within the APP molecule, although other amino acids in the APP cytoplasmic domain might be phosphorylated in patho-

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[2] The abbreviations used are: APP, amyloid β-protein precursor; imAPP, immature APP; mAPP, mature APP; Aβ, amyloid β-protein; JNK, c-Jun N-terminal kinase; CTF, C-terminal fragment; AICD, APP intracellular domain; AD, Alzheimer disease; PI, phosphotyrosine interaction; PTB, phosphotyrosine-binding; JIP, JNK-interacting protein; Alc, alcadein.

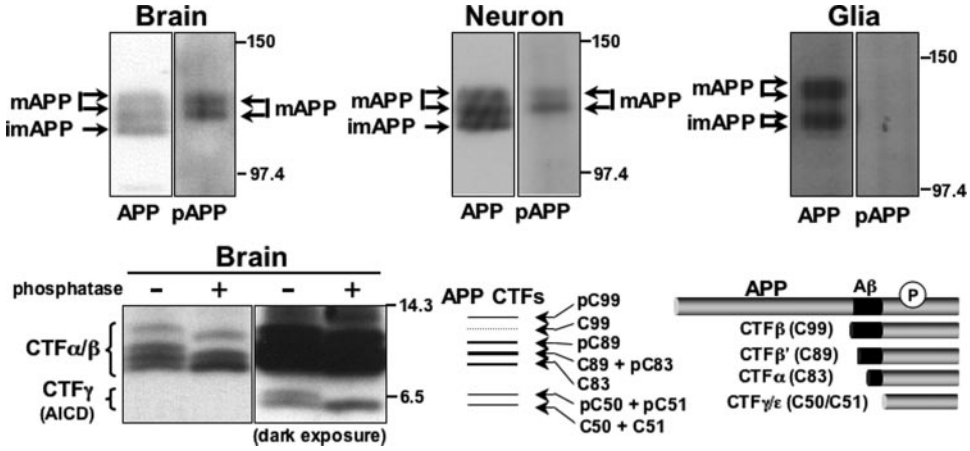

FIGURE 1. **Phosphorylation of APP and APP CTFs in mouse brain.** *Upper panels*, APP phosphorylation state in brain, primary cultured neurons, and glial cells. Lysates of brain (*left*), primary cultured cortical neurons (*middle*), and glial cells (*right*) were subjected to immunoprecipitation with an anti-pan-C-terminal APP antibody. The immunoprecipitates were analyzed by Western blotting using anti-pan-C-terminal APP (*APP*) and anti-phospho-Thr^{668} APP (*pAPP*) antibodies. The APP695 isoform was detected in the samples from brain and primary cultured neurons, whereas APP770 and APP751 were the major isoforms detected in the sample from primary cultured glial cells. *Lower panels*, APP CTFs and their phosphorylation state in brain. Brain lysates were immunoprecipitated with an anti-pan-C-terminal APP antibody and treated with (+) or without (−) λ-phosphatase. The samples were analyzed by Western blotting using an anti-pan-C-terminal APP (*APP*) antibody. C99 and C89 are CTFβ and CTFβ', and C83 is CTFα. C50 and C51 are CTFγ/ϵ (AICD). pC99, pC89, pC83, pC50, and pC51 are CTFs phosphorylated at Thr^{668}. Numbers indicate molecular size markers (in kilodaltons).

logical brain states (18) or in cells overexpressing tyrosine-protein kinases such as Abl and Fyn, which phosphorylate Tyr^{682} (19, 20).

In mouse and human brains, two mAPP695 species with different types of *O*-glycosylation have been found to be phosphorylated by Western blot analysis with Thr^{668} phosphorylation state-specific antibody, whereas imAPP695 is not phosphorylated (Fig. 1), indicating that a fixed population of mAPP is constitutively phosphorylated in neurons (4).

Three CTFs of APP (C99, C89, and C83; numbers indicate amino acid number) are generated from mAPP in brain. Both C99 and C89 are products of cleavage by β-secretase (and thus are designated CTFβ and CTFβ'), whereas C83 is a product of cleavage by α-secretase (CTFα) (21). Some APP CTFs are phosphorylated at Thr^{668} and detected as phosphopeptides pC99, pC89, and pC83 (22). Using Western blot analysis, typical APP CTFα/β species in brain appear as five CTF bands: pC99, C99, pC89, a mixture of C89 plus pC83, and C83 (5). Treatment of CTFs with phosphatase is effective to identify respective species (Fig. 1) (5, 22). These APP CTFα/β are further cleaved by γ-secretase at the intramembranous γ/ϵ-site to generate the AICD fragment (CTFγ/ϵ; C50 and C51). The ϵ-cleavage sites are located several amino acids toward the C termini of γ-cleavage sites (22–25). It is thought that sequential γ-secretase cleavages initiate at the ϵ-site and move progressively toward the γ-site at every three residues (26). The majority of the CTFγ/ϵ in the brain remains tethered within the membrane fraction by an unknown mechanism, whereas some is translocated into the nucleus. A fixed amount of CTFγ/ϵ in the brain is also phosphorylated at Thr^{668} to form pCTFγ/ϵ (pAICD) (Fig. 1) (27).

Thr^{668} is located within the motif ^{667}VTPEER^{672}, which forms a type I β-turn and N-terminal helix-capping box structure to stabilize its C-terminal helix structure (28, 29). Phosphorylation of Thr^{668} induces significant conformational change in the cytoplasmic region of APP, affecting its interaction with FE65, a neuron-specific adaptor protein (30). APP is phosphorylated not only in mAPP but also in APP CTFs (Fig. 1) (5, 27). Thus, one function of Thr^{668} phosphorylation is to alter the overall cytoplasmic structure of mAPP, APP CTFα/β, and APP CTFγ/ϵ (AICD) and to regulate the interaction with FE65 as a molecular switch (27, 30, 31). The physiological role of FE65 is not yet well understood, but FE65 may function within the nucleus to influence gene expression and/or DNA repair in cooperation with or regulation by CTFγ/ϵ (32, 33). A proportion of FE65 is tethered at the membrane by association with transmembrane proteins such as APP (31, 34). Phosphorylation of APP liberates FE65 into the cytoplasm from the membrane, allowing FE65 to translocate into the nucleus (27, 35). Targeting of FE65 into the nuclear matrix is suppressed by CTFγ/ϵ (35). In the nucleus, FE65 induces the phosphorylation of H2AX, which plays an important role in DNA repair as a cellular response by stress-damaged cells (35). Thus, APP phosphorylation regulates intracellular signaling via FE65. CTFγ/ϵ is unlikely to accompany FE65 during nuclear signaling because the phosphorylated form (pCTFγ/ϵ; pAICD) is also detectable in the nucleus in mouse brain (27), and phosphorylation would cause dissociation from FE65 (30). In brains from mutant mice in which the phosphorylatable threonyl residue at position 668 of APP was replaced with a non-phosphorylatable alanyl residue (T668A), CTFγ/ϵ was still found to translocate into the nucleus, just as does wild-type CTFγ/ϵ, although this mutation also suppressed the interaction with FE65 (5, 30). These *in vivo* observations suggest that FE65 translocates into the nucleus independently of either AICD or the AICD phosphorylation state.

Phosphorylation at Thr^{668} renders APP less vulnerable to cytoplasmic cleavage by caspase-3 and caspase-8, which are known to cleave between Asp^{664} and Ala^{665} to generate a cytotoxic fragment (36, 37). Thus, phosphorylation of APP may contribute toward preventing cytotoxic cleavage by caspases implicated in AD pathogenesis.

A potentially pivotal role for the phosphorylation state of Thr^{668} in the control of brain Aβ levels has been proposed previously (18, 38, 39). However, studies of brains from APP T668A mutant mice did not reveal any appreciable alterations in the levels and subcellular distributions of APP or in its metabolic products, including Aβ (5). These observations suggest that the phosphorylation of APP at Thr^{668} does not play a significant role in governing the physiological generation of Aβ in brain. Although the possibility remains that pathological changes in Thr^{668} phosphorylation of APP in human brain might modulate its metabolism, the phosphorylation of APP at Thr^{668} is not directly linked to the proteolytic processing that

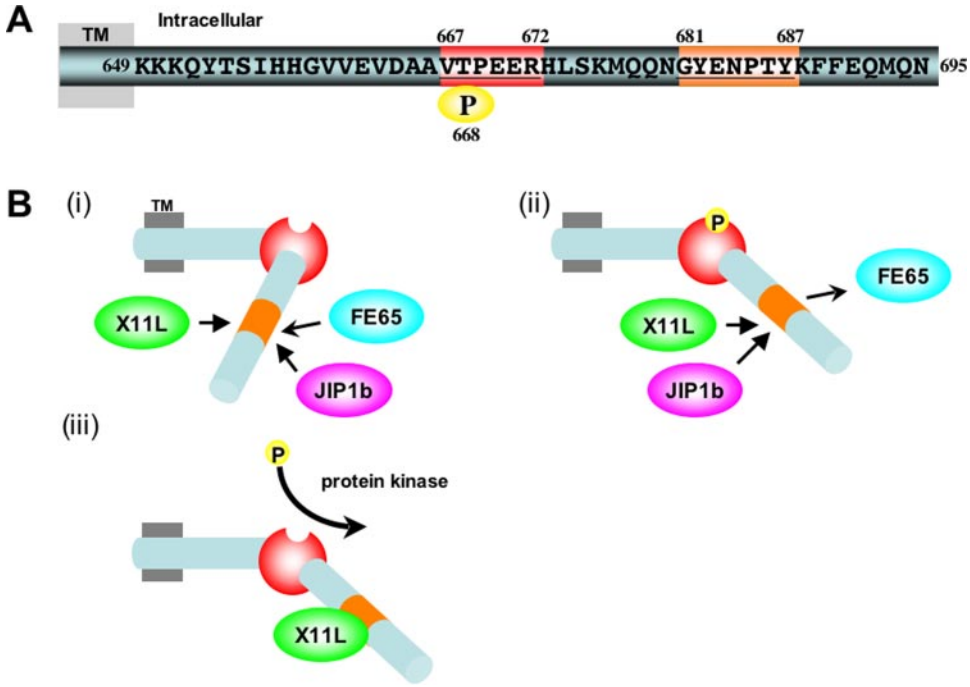

FIGURE 2. **Amino acid sequence of the APP cytoplasmic region, functional motifs, and APP-binding partners.** *A*, amino acid sequence of the APP cytoplasmic region. Numbers indicate amino acid positions of the APP695 isoform. Two functional motifs, ^{667}VTPEER^{672} and ^{681}GYENPTY^{687}, are indicated. The Thr^{668} phosphorylation site is indicated (*P*). *B*, relationship between Thr^{668} phosphorylation in the ^{667}VTPEER^{672} and ^{681}GYENPTY^{687} motifs and interaction of APP-binding partners with the ^{681}GYENPTY^{687} motif. *Panel (i)*, the APP cytoplasmic region is characterized by the ^{667}VTPEER^{672} turn as a helix-capping box structure, which stabilizes the helical structure of its C terminus, which contains the ^{681}GYENPTY^{687} motif (28). Major APP-binding partners such as X11L, FE65, and JIP1b interact with the ^{681}GYENPTY^{687} motif. *Panel (ii)*, phosphorylation at Thr^{668} within the ^{667}VTPEER^{672} motif induces structural change in the cytoplasmic region, releasing FE65 from the ^{681}GYENPTY^{687} motif (29, 30). *Panel (iii)*, binding of X11L to the ^{681}GYENPTY^{687} motif may affect the structure of the ^{667}VTPEER^{672} motif and expose Thr^{668}, facilitating its phosphorylation by protein kinases such as JNK (45). Thus, phosphorylation of Thr^{668} acts as molecular switch to induce conformational change in the cytoplasmic region and to regulate protein interactions. *TM*, transmembrane domain.

controls Aβ levels in brain. Reliable and quantitative analyses for APP phosphorylation state in the brains of AD patients, if possible using biopsy samples, would facilitate our understanding of the degree to which APP phosphorylation is related to the pathogenic state of APP in AD.

Cytoplasmic Regulators of APP

In addition to the ^{667}VTPEER^{672} motif, which contains the phosphorylatable amino acid Thr^{668}, the cytoplasmic region of APP contains a ^{681}GYENPTY^{687} motif containing an NP*X*pY element, a typical internalization signal for membrane proteins (40, 41). In the case of APP, Tyr^{687} within the ^{681}GYENPTY^{687} motif is not phosphorylated in brain. However, several cytoplasmic adaptor proteins bind to this motif through a PI or PTB domain (reviewed in Ref. 42). The binding of these proteins to APP does not require tyrosine phosphorylation within the ^{681}GYENPTY^{687} motif. The phosphorylation of APP at Thr^{668}, located 14 amino acids toward the N-terminal end from the ^{681}GYENPTY^{687} motif, affects the conformation of the ^{681}GYENPTY^{687} motif (30). This conformational change suppresses the interaction of FE65 with the ^{681}GYENPTY^{687} motif (30). However, other APP-binding partners such as X11s and JIPs, which also interact with the ^{681}GYENPTY^{687} motif, are largely unaffected by Thr^{668} phosphorylation (Fig. 2) (17, 43–45). Both X11s and JIPs play an important role in the regulation of the metabolism and trafficking of APP.

Proteins of the X11 family, such as X11 (X11α), X11-like (X11L/X11β), and X11-like 2 (X11L2/X11γ), stabilize intracellular APP metabolism and suppress Aβ production (43, 44, 46, 47). Expression of X11 and X11L is brain-specific; X11 is expressed largely in inhibitory neurons, and X11L is expressed predominantly in excitatory pyramidal neurons, whereas X11L2 is expressed ubiquitously (48). Thus, the interaction of APP with both X11 and X11L has significant effects upon the regulation of APP metabolism. Indeed, transgenic Tg2576 mice carrying the APP Swedish mutation and also overexpressing X11 or X11L exhibit decreased levels of cerebral Aβ and a reduction of Aβ plaques in the cortex and hippocampus (49, 50). In addition to APP, X11 and X11L are known to associate with several other membrane and cytoplasmic proteins (reviewed in Ref. 51).

Interactions of X11 and X11L with other proteins can result in the formation of functional complexes, which can regulate APP metabolism and/or function. Alcadein (Alc), a type I membrane protein, associates with the cytoplasmic domain of APP via X11 or X11L, and the metabolism of both APP and Alc is stable upon formation of a tripartite complex composed of APP, X11, and Alc (52, 53). In contrast, X11L-deficient mice exhibit enhanced amyloidogenic cleavage of APP in the hippocampus, indicating the importance of X11L function in APP metabolism in brain and a possible role for X11L in AD pathogenesis (54). X11L and X11L2 are shuttled between the cytoplasm and nucleus, and both proteins are detected in the nucleus of mouse brain tissues (55), suggesting that the function of X11L and X11L2 in the nucleus might be similar to that of FE65.

The binding of X11L to APP elevates the JNK-mediated phosphorylation of APP (45). This may be mediated by the association of X11L with the ^{681}GYENPTY^{687} motif, causing increased exposure of threonyl residues within the ^{667}VTPEER^{672} motif. Therefore, the two ^{667}VTPEER^{672} and ^{681}GYENPTY^{687} motifs might have closely related roles in phosphorylation and protein interaction in the context of APP regulation (Fig. 2).

JIPs are JNK-interacting proteins containing Src homology 3 and PI/PTB domains. Mammalian JIPs are composed of JIP1a, JIP1b, and JIP2 (56), all of which display scaffold functions in the JNK signaling pathway. JIP1a and JIP2 bind weakly to APP,

but JIP1a lacks part of the PI domain, which further reduces its capacity to bind to APP (17, 57). Thus, JIP1b may be the only physiologically relevant binding partner of APP in the JIP family. In addition to its association with APP through its PI/PTB domain, JIP1b also associates with the kinesin light chain of the classical kinesin-1 motor (17, 58). Therefore, JIP1b can connect APP to the kinesin-1 motor by molecular bridging to regulate axonal transport of APP-containing vesicles (59). Phosphorylation of APP at Thr^{668} does not affect the interaction with JIP1b (45), but phosphorylated APP is observed in neurites and mostly in the growth cones of differentiating neuronal cells (12, 60). Therefore, phosphorylation of APP may have a role in the axonal transport of APP within neurons (61). Anterograde axonal transport of APP may occur via several pathways in addition to the one involving JIP1b bridging between the kinesin light chain and APP. Appropriate regulation of the axonal transport of APP-containing vesicles is important for the maintenance of non-amyloidogenic APP metabolism (reviewed in Ref. 42). In fact, disruption of the axonal transport of APP-containing vesicles through kinesin-1 motor dysfunction or through imbalances in APP and Alc (which are transported by the same kinesin-1 motor) can enhance the generation of Aβ (59, 62). In this process, untransported APP-containing vesicles accumulate in both the soma and the axon and may enter into the endosomal-lysosomal pathway in which amyloidogenic processing of APP is active (reviewed in Ref. 3). Therefore, regulation of the connections between JIP1b and the kinesin-1 motor and/or between JIP1b and APP is important for understanding the function and metabolism of APP.

Concluding Remarks

The major phosphorylation site of APP is Thr^{668}, located in the cytoplasmic region. The physiological phosphorylation state of APP at Thr^{668} is observed in neurons (mAPP alone), dividing cells (imAPP largely), and stressed cells. Proline-directed protein kinases such as CDK5, GSK-3β, CDC2 (CDK1), and JNK phosphorylate APP at Thr^{668} in the ^{667}VTPEER^{672} motif. This phosphorylation induces conformational change in the cytoplasmic region of APP, which functions as a "molecular switch" that regulates its interaction of protein(s) such as FE65. Intracellular localization of FE65 is controlled largely by the phosphorylation of APP, and APP-regulated FE65 is likely to play an important role in signal transduction at least during cellular stress, rather than the postulated role in gene transactivation. Other physiological functions of APP regulated by phosphorylation may be discovered. Finally, there is no obvious evidence that the phosphorylation state of APP at Thr^{668} plays an significant role in regulating the amyloidogenic processing of APP directly.

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