Minireview

Regulation of APP by phosphorylation and protein interactions

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Running title: Phosphorylation of APP

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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 presenilin 1 and presenilin 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 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.
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 amyloid β-protein (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-secretase is highly concentrated (reviewed in ref. 3). Therefore, it appears that Aβ generation is closely related to APP trafficking in the cell, especially in neurons, since 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, Thr668 (numbering for APP695 isoform) in the cytoplasmic region of APP is the phosphorylatable amino acid (Fig. 2). Cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase-3β (GSK-3β) are thought to phosphorylate mAPP at Thr668 in neurons, while cyclin-dependent kinase 1 (cdk1)/cdc2 kinase phosphorylates Thr668 of imAPP in dividing cells (4, 10, 11, 14, 15). When cells are subjected to a stress stimulus, c-Jun NH2-terminal kinase (JNK) also phosphorylates APP at Thr668 (13, 16, 17). Thus, Thr668 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 pathological brain states (18) or in cells overexpressing tyrosine protein kinases such as Abl and Fyn which phosphorylate Tyr682 (19, 20).

In mouse and human brain, two mAPP695 species with different types of O-glycosylation are found to be phosphorylated, by Western blot analysis with Thr668 phosphorylation-state specific antibody, while imAPP695 is not phosphorylated (Fig. 1), indicating that a fixed population of mAPP is constitutively phosphorylated in neurons (4).

Three carboxyl-terminal fragments of APP (APP CTFs; 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’), while C83 is a product of cleavage by α-secretase (CTFα) (21). Some APP CTFs are phosphorylated at Thr668 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 intracellular domain fragment, AICD (CTFγ/ε; C50 and C51). The ε-cleavage sites locate several amino acids toward the carboxyl-terminal of γ-cleavage sites (22-25). It is considered that sequential γ-secretase cleavages initiate at ε-site and progressively moves toward 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, while some is translocated into the nucleus. A fixed amount of CTFγ/ε in brain is also phosphorylated at Thr668 to form pCTFγ/ε.
Thr668 is located within the motif 667VTPEER672, which forms a type I β-turn and amino-terminal helix-capping box structure to stabilize its carboxyl-terminal helix structure (28, 29). Phosphorylation of Thr668 induces significant conformational change in APP’s cytoplasmic region, 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 Thr668 phosphorylation is to alter the overall cytoplasmic structure of mAPP, APP CTFα/β and 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 under 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 was suppressed by CTFγ/ε (35). In the nucleus, FE65 induced 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 nucleus in mouse brain (27) and phosphorylation would cause dissociation from FE65 (30). In brain from mutant mice, in which the phosphorylatable threonyl residue at position 668 of APP was replaced with a non-phosphorylatable alanyl residue, Thr668Ala CTFγ/ε was still found to translocate into the nucleus, just as does wild-type CTFγ/ε, although this mutation also suppresses 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 Thr668 renders APP less vulnerable to cytoplasmic cleavage by caspase-3 and caspase-8, which are known to cleave between Asp664 and Ala665 to generate a cytotoxic fragment (36, 37). Thus, phosphorylation of APP may contribute towards preventing cytotoxic cleavage by caspases implicated in Alzheimer disease (AD) pathogenesis.

A potentially pivotal role for the phosphorylation state of Thr668 in the control of brain Aβ levels has been previously proposed (18, 38, 39). However, studies of brains from APP Thr668Ala mutant mice did not reveal any appreciable alterations in the levels and subcellular distributions of APP, nor in its metabolic products including Aβ (5). These observations suggest that the phosphorylation of APP at Thr668 does not play a significant role in governing the physiological generation of Aβ in brain. While the possibility remains that pathological changes in Thr668 phosphorylation of APP in human brain might modulate its metabolism, the phosphorylation of APP at Thr668 is not directly linked to the proteolytic processing that controls Aβ levels in brain. Reliable and quantitative analyses for APP phosphorylation state in the brain 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 667VTPEER672 motif, containing the phosphorylatable amino acid Thr668, the cytoplasmic region of APP contains a 681GYENPTY687 motif containing an NPxY element, a typical internalization signal for membrane proteins (40, 41). In the case of APP, Tyr687 within the 681GYENPTY687 motif is not phosphorylated in brain. However, several cytoplasmic
adaptor proteins bind to this motif through a phosphotyrosine interaction (PI) or binding (PTB) domain (reviewed in ref. 42). The binding of these proteins to APP does not require tyrosine phosphorylation within the 681GYENPTY687 motif. The phosphorylation of APP at Thr668, located 14 amino acids toward the amino-terminal end from 681GYENPTY687 motif, affects the conformation of the 681GYENPTY687 motif (30). This conformational change suppresses the interaction of FE65 with the 681GYENPTY687 motif (30). However, other APP-binding partners such as X11s and JIPs, which also interact with the 681GYENPTY687 motif, are largely unaffected by Thr668 phosphorylation (Fig. 2) (43-46). 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, 47, 48). Expression of X11 and X11L is brain-specific; X11 is largely expressed in inhibitory neurons and X11L is expressed predominantly in excitatory pyramidal neurons, while X11L2 is expressed ubiquitously (49). 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 which also overexpress X11 or X11L, exhibit decreased levels of cerebral Aβ and a reduction of Aβ plaques in the cortex and hippocampus (50, 51). In addition to APP, X11 and X11L are known to associate with several other membrane and cytoplasmic proteins (reviewed by ref. 52).

Interactions of X11 and X11L with other proteins can result in the formation of functional complexes, which can regulate APP metabolism and/or function. Alcadin (Alc), a type I membrane protein, associates with the cytoplasmic domain of APP via X11 or X11L, and metabolism of both APP and Alc are stable by forming the tripartite complex composing of APP-X11s-Alc (53, 54). In contrast, X11L-deficient mice exhibit enhanced amyloidogenic cleavage of APP in the hippocampus, indicating the importance of X11L function in APP metabolism in the brain and a possible role for X11L in AD pathogenesis (55). X11L and X11L2 are shuttled between the cytoplasm and nucleus, and both proteins are detected in nucleus of mouse brain tissues (56), 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 (46). Mediating this may be the association of X11L with the 681GYENPTY687 motif, causing increased exposure of threonyl residues within the 667VTPEER672 motif. Therefore, the two 681GYENPTY687 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 comprised of JIP1a, JIP1b and JIP2 (57), 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, 58). Thus, JIP1b may be the only physiologically-relevant binding partner of APP among the JIP family. In addition to its association with APP through its PI/PTB domain, JIP1b also associates with kinesin light chain (KLC) of the classical kinesin-1 motor (45, 59). Therefore, JIP1b can connect APP to the kinesin-1 motor by molecular bridging, to regulate axonal transport of APP-containing vesicles (60). Phosphorylation of APP at Thr668 does not affect the interaction with JIP1b (46), but phosphorylated APP is observed in neurites and mostly in the growth cones of differentiating neuronal cells (12, 61). Therefore, phosphorylation of APP may have a role in the axonal transport of APP.
within neurons (62). Anterograde axonal transport of APP may occur via several pathways in addition to the one involving JIP1b-bridging between KLC 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 (60, 63). 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 are important for understanding the function and metabolism of APP.

Concluding Remarks

Major phosphorylation site of APP is Thr668 located in the cytoplasmic region. Physiological phosphorylation state of APP at Thr668 is observed in neurons (mAPP alone), dividing cells (imAPP largely), and in stressed cells. Prolin-directed protein kinases such as CDK5, GSK-3β, cdc2 (CDK1) and/or JNK phosphorylate APP at Thr668 in the 667VTPEER672 motif. This phosphorylation induces conformational change in the cytoplasmic region of APP, which functions as a “molecular switch” regulating its interaction of protein(s) such as FE65. Intracellular localization of FE65 is largely controlled 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 postulated role in gene transactivation. Other physiological functions of APP regulated by the phosphorylation may be found out. Finally, there are no obvious evidences that the phosphorylation state of APP at Thr668 plays an significant role in regulating the amyloidogenic processing of APP directly.

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**The abbreviations** used are: APP, amyloid β-protein precursor; Aβ, amiloid β-protein; AD, Alzheimer disease; cdc2, cycline-dependent protein kinase 2; CDK5, cyclin-dependent protein kinase 5; CTF, carboxyl-terminal fragment; GSK-3β, glycogen synthase kinase 3β; JNK, c-Jun NH2-terminal kinase (JNK); JIP; JNK-binding protein.
Figure legends

Figure 1. Phosphorylation of APP and APP CTFs in mouse brain.
A. 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-APP C-terminal antibody. The immunoprecipitates were analyzed by Western blotting using an anti-pan-APP C-terminal (APP) and anti-phosphoThr668-specific (pAPP) antibodies. mAPP, mature APP; imAPP, immature APP. The APP695 isoform is detected in the samples from brain and primary cultured neurons, while APP770 and APP751 are the major isoforms detected in the sample from primary cultured glial cells. B. APP carboxyl-terminal fragments (APP CTFs) and their phosphorylation state in brain. Brain lysates were immunoprecipitated with an anti-pan-APP C-terminal antibody and treated with (+) or without (-) λ phosphatase. The samples were analyzed by Western blotting using an anti-pan-APP C-terminal (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 Thr668. Numbers indicate molecular size marker (kDa).

Figure 2. Amino acid sequence of 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, 667VTPEER672 and 681GYENPTY687, are indicated. Phosphorylation site Thr668 is shown with “P”. B. Relationship between Thr668 phosphorylation in the 667VTPEER672 and 681GYENPTY687 motifs, and interaction of APP-binding partners with the 681GYENPTY687 motif. (i) The APP cytoplasmic region is characterized by the 667VTPEER672 turn as a helix-capping box structure, which stabilizes the helical structure of its carboxyl-terminal, which contains the 681GYENPTY687 motif (28). Major APP-binding partners such as X11L, FE65 and JIP1b interact with the 681GYENPTY687 motif. (ii) Phosphorylation at Thr668 within the 667VTPEER672 motif induces structural change in the cytoplasmic region, releasing FE65 from the 681GYENPTY687 motif (29, 30). (iii) Binding of X11L to the 681GYENPTY687 motif may affect the structure of the 667VTPEER672 motif and expose the Thr668 residue, facilitating its phosphorylation by protein kinases such as JNK (46). Thus, phosphorylation of Thr668 acts as molecular switch to induce conformational change of the cytoplasmic region, and to regulate protein interactions.
Figure 1

Brain

- mAPP ↔ imAPP
- APP pAPP

Neuron

- mAPP ↔ imAPP
- APP pAPP

Glia

- mAPP ↔ imAPP
- APP pAPP

Phosphatase

Brain

phosphatase

CTFα/β

CTFγ

(AICD)

(dark exposure)

14.3

- +

- +

APP CTFs

pC99

C99

C89

C83

C89 + pC83

pC50 + pC51

pC50 + pC51

Aβ (P)

APP

CTFβ (C99)

CTFβ' (C89)

CTFα (C83)

CTFγ/ε (C50/C51)
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