Facilitation of Stress-induced Phosphorylation of β-Amyloid Precursor Protein Family Members by X11-like/Mint2 Protein*[^S]

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β-Amyloid precursor protein (APP) is the precursor of β-amyloid (Aβ), which is implicated in Alzheimer’s disease pathogenesis. APP complements amyloid precursor-like protein 2 (APLP2), and together they play essential physiological roles. Phosphorylation at the Thr668 residue of APP (with respect to the numbering conversion for the APP 695 isoform) and the Thr736 residue of APLP2 (with respect to the numbering conversion for the APLP2 763 isoform) in their cytoplasmic domains acts as a molecular switch for their protein-protein interaction and is implicated in neural function(s) and/or Alzheimer’s disease pathogenesis. Here we demonstrate that both APP and APLP2 can be phosphorylated by JNK at the Thr668 and Thr736 residues, respectively, in response to cellular stress. X11-like (X11L, also referred to as X11β and Mint), which is a member of the mammalian LIN-10 protein family and a possible regulator of Aβ production, elevated APP and APLP2 phosphorylation probably by facilitating JNK-mediated phosphorylation, whereas other members of the family, X11 and X11L2, did not. These observations revealed an involvement of X11L in the phosphorylation of APP family proteins in cellular stress and suggest that X11L protein may be important in the physiology of APP family proteins as well as in the regulation of Aβ production.

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[^2]: The abbreviations used are: APP, β-amyloid precursor protein; Aβ, β-amyloid peptide; AD, Alzheimer’s disease; APLP, β-amyloid precursor-like protein; APPct, the cytoplasmic domain of APP; Cdk5, cyclin-dependent kinase 5; DAB1, disabled homolog 1; DLK, dual leucine zipper-bearing kinase; GST, glutathione S-transferase; JBD, JNK-binding domain; JIP, JNK-interacting protein; JNK, c-Jun NH2-terminal kinase; KLC, kinesin light chain; PI domain, phosphoryrosine interaction domain; X11L, X11-like protein; HA, hemagglutinin; HEK, human embryonic kidney; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.

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and JIP-2 are scaffold proteins within the JNK signaling cascade and function in kinesin-mediated axonal transport (33–35). JIP-1, but not JIP-2, was also reported to retard APP metabolism and to suppress Aβ production (36). Among these binding proteins, JIP-1 has been suggested to enhance the phosphorylation of APP by JNK (16, 17). JIP-1 scaffolds JNK and APP to increase the phosphorylation of APP in cell lysates (17). In contrast, however, the phosphorylation level of APP is not dramatically affected by JIP-1 in cells (16). In addition, it remains unclear whether other binding protein(s) could play a prominent role in the regulation of JNK-mediated intracellular phosphorylation.

The cytoplasmic domains of APP family proteins are highly homologous, and the phosphorylation site corresponding to Thr668 of APP is conserved in APLP2 as Thr736 (37) (with respect to the numbering conversion for the APLP2 763 isoform) (4, 37). Indeed, similar to Thr668 of APP, Thr736 of APLP2 is phosphorylated by Cdc2 kinase (37). Moreover, also similar to APP, phosphorylation of APLP2 is suggested to act as a molecular switch for binding to cytosolic proteins such as FE65 (11). However, whether the molecular mechanisms of APLP2 phosphorylation and its regulation are identical to that of APP has not been fully elucidated.

In this study, therefore, we investigated the phosphorylation of APP and APLP2 by JNK and the regulation of these phosphorylation events by their binding proteins. APP and APLP2 were phosphorylated by JNK1, JNK2, and JNK3 in vitro and in cells in response to cellular stress. Furthermore, among several known binding proteins, X11L, but not X11 or X11L2, was identified as a binding protein that could mediate the phosphorylation of APP and APLP2. These findings further elucidate the molecular mechanisms of phosphorylation and physiological function(s) of APP and APLP2 as well as differential functions among X11 family proteins.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal anti-FLAG (M2, Sigma), anti-Myc (Invitrogen), anti-HA (12CA5, Roche Diagnostics), anti-GST (Upstate Biotechnology), and anti-phosphorylated JNK (G-7, Santa Cruz Biotechnology) antibodies were purchased. Polyclonal anti-active JNK and anti-phospho-c-Jun (Ser63) antibodies were purchased from Promega and Cell Signaling Technology, respectively. Polyclonal anti-APP cytoplasmic domain (G369), anti-APLP2 cytoplasmic domain (UT-424), and anti-APLP2 amino-terminal region (G369), anti-APLP2 amino-terminal region (G369) were also inserted into pGEX-4T-1 (Amersham Biosciences) and cloned into pcDNA3-FLAG (36) using EcoRI/XhoI sites. The cDNAs encoding the 47 amino acid residues of the cytoplasmic domain of APLP2 (APLP2cyt, amino acids 717–763) and the amino-terminal region of mouse c-Jun (amino acids 1–79) were also inserted into pGEX-4T-1 (Amersham Biosciences) to generate pGEX-4T-1-APLP2cyt and pGEX-4T-1-c-Jun(1–79). pGEX-4T-1-APLP2cyt, pGEX-4T-1-hX11L, pGEX-4T-1-hX11L-N, and pGEX-4T-1-hX11L-N were used to produce the GST fusion proteins of APPpcyt and human X11L as well as the domain structures of human X11L, respectively, and have been described previously (22).

Expression and Analysis of Proteins in Cultured Cell Lines—Human embryonic kidney 293 (HEK293) cells and HEK293 cells stably expressing the FLAG-tagged APP695 were cultured as described previously (7, 8, 37). For transient protein expression, HEK293 cells were transfected with plasmid expression vectors using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were treated as indicated, collected, lysed in radiomunoprecipitation buffer (25 mM Tris-HCl, 0.1% (w/v) SDS, 0.5% (v/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, 0.15 mM NaCl) containing 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 μM microcin-L, and centrifuged at 12,000 × g for 10 min at 4 °C. Protein kinase inhibitors SP600125 and SB203580 (BIOMOL Research Laboratories Inc.) were added 30 min prior to hypsomotic treatment of cells. The resulting supernatants were subjected to immunoprecipom with G369, UT-424, or M2 anti-FLAG antibody (36). Cell lysates or immunoprecipitated proteins were analyzed by immunoblotting with indicated antibodies using an ECL detection kit (Amersham Biosciences). For quantification, immunoblot analysis was also performed using 125I-protein A (Amersham Biosciences) with radioactivity being quantified using a Fuji BAS 1800C.

Co-immunoprecipitation—Co-immunoprecipitation assay was described previously (36). HEK293 cells were transfected with pcDNA3, pcDNA3-FLAGAPP695, pcDNA3-FLAGAPPA367, or pcDNA3-HA-X11L as indicated, lysed in CHAPS lysis buffer (phosphate-buffered saline containing 10 mM CHAPS, 1 mM Na2VO4, 1 mM NaF, 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 μM microcin-L, and centrifuged at 12,000 × g for 10 min at 4 °C. The resulting supernatants were incubated with anti-FLAG M2 antibody for 4 °C for 1 h. The immunocomplex was recovered with protein G-Sepharose beads (Amersham Biosciences).

Preparation of Proteins—Production and purification of recombinant GST fusion proteins were as described previously (16, 22). Briefly GST fusion proteins were generated in Escherichia coli BL21 containing pGEX-4T-1 cDNA constructs and purified with glutathione-Sepharose 4B (Amersham Biosciences). To prepare FLAG-tagged X11 and X11L proteins, HEK293 cells transiently expressing FLAG-X11 and FLAG-X11L were lysed and subjected to immunoprecipitation with anti-FLAG M2 antibody against X11L (36). Cell lysates or immunoprecipitated proteins were analyzed by immunoblotting with indicated antibodies using an ECL detection kit (Amersham Biosciences). For quantification, immunoblot analysis was also performed using 125I-protein A (Amersham Biosciences) with radioactivity being quantified using a Fuji BAS 1800C.
RESULTS

Phosphorylation of APP and APLP2 by JNK Family Proteins—We initially examined the ability of JNK to phosphorylate APP at Thr\(^{668}\) and APLP2 at Thr\(^{736}\) (their phosphorylation sites are schematically represented in Fig. 1A). JNK proteins are expressed from three genes, Jnk1, Jnk2, and Jnk3, as low and high molecular weight splice variants (18). We performed in vitro phosphorylation of purified APPcT protein (Fig. 1B) or APLP2cT protein (Fig. 1C) with activated JNK isoforms including JNK1 (α1 and α2), JNK2 (α1 and α2), and JNK3. Phosphorylation was examined by immunoblotting with antibodies that specifically recognize Thr\(^{668}\)-phosphorylated APP or Thr\(^{736}\)-phosphorylated APLP2. Phosphorylation of APP at Thr\(^{668}\) was induced by all of these JNK isoforms (Fig. 1B, upper panel). In addition, phosphorylation of APLP2 at Thr\(^{736}\) was also mediated by all of the JNK kinases that we examined (Fig. 1C, upper panel). These results indicate that all JNK family proteins are capable of phosphorylating both APP at Thr\(^{668}\) and APLP2 at Thr\(^{736}\).

Stress-induced Phosphorylation of APP and APLP2 by JNK—JNK is a stress-activated protein kinase that is critical for signaling cell death and/or cell survival in response to various cellular stresses (18). We therefore investigated whether phosphorylation of APP could be induced in response to stress (Fig. 2A). HEK293 cells stably expressing APP were treated with various stimuli including a protein synthesis inhibitor (anisomycin), radiation (UV), and hyperosmolarity (high concentration of sorbitol). In cells that had been subjected to these stressful stimuli, activation of endogenous JNK proteins was detected as evidenced by the appearance of dually phosphorylated forms of JNK in immunoblot (Fig. 2A, lower panel). Phosphorylation of APP was detected in response to each of the stressful stimuli and was most pronounced in cells subjected to hyperosmolarity but was hardly observed in non-treated cells (Fig. 2A, upper panel). This indicated that phos-
Treated with or without 0.5 M sorbitol for 30 min. The cell lysates were immunoblotted with anti-phospho-APP Thr 668 (P-APP, upper panel), immunoblotted with UT-33 (stimuli. Such stress-induced phosphorylation of APP at Thr668 (Fig. 2B), as it was in cells stably expressing APP, except that by hyperosmotic stress in cells transiently expressing APP. HEK293 cells were co-transfected with plasmids encoding APP and either FLAG-tagged X11, X11L, or X11L2. The indicated cells were treated with 0.5 M sorbitol for 30 min or simultaneously transfected with FLAG-tagged DLK. APLP2 was immunoprecipitated from cell lysates with G369 and immunoblotted with UT-33 (P-APLP2, upper panel) and G369 (APLP2, middle panel). The cell lysates were also immunoblotted with M2 (X11s, lower panel). C, increase in APLP2 phosphorylation by co-expression of X11L. HEK293 cells were co-transfected with Myc-tagged APLP2 and either FLAG-JNK1 or FLAG-X11L as indicated. The cells were treated with 0.5 M sorbitol for 30 min or simultaneously transfected with FLAG-tagged DLK. APLP2 was immunoprecipitated from cell lysates with UT-424 and immunoblotted with UT-23 (P-APLP2, upper panel) and anti-Myc (APLP2, lower panel).

Phosphorylation of APP at Thr668 was induced by these stress stimuli. Such stress-induced phosphorylation of APP at Thr668 was also observed in other cell lines including mouse neuroblastoma neuro-2a cells (data not shown).

To determine whether JNK could be involved in stress-induced phosphorylation of APP, we transiently overexpressed JNK isoforms and APP. Phosphorylation of APP was induced by hyperosmotic stress in cells transiently expressing APP (Fig. 2B), as it was in cells stably expressing APP, except that phosphorylation of the mature form of APP (Fig. 2B, second panel, upper band) was barely detectable. Overexpression of JNK strongly enhanced the hyperosmotic stress-induced phosphorylation of APP (Fig. 2B, first panel). In addition, a small degree of APP phosphorylation was observed in the absence of stressful stimuli, and this appeared to correlate with spontaneous activations of overexpressed JNK in transfected cells (Fig. 2B, first and third panels). This correlation between the amount of phosphorylated APP and that of phosphorylated JNK suggests that JNK may be involved in the phosphorylation of APP.

Therefore, we next examined the involvement of endogenous JNK in stress-induced APP phosphorylation using selective kinase inhibitors in HEK293 cells stably expressing APP. An appropriate concentration of SP600125, a recently described selective chemical inhibitor of JNK (40), significantly inhibited APP phosphorylation induced by hyperosmolality (Fig. 2C). On the contrary, SB203580, a specific inhibitor of another major stress-responsive kinase, p38, did not affect APP phosphorylation. In addition, we observed that co-expression of JNK-binding domain (JBD), which is a short polypeptide within JIP-1 (amino acids 127–281) and inhibits the activity of JNK (41), significantly inhibited APP phosphorylation induced by hyperosmolality (Fig. 2D, left panel). Phosphorylation of APLP2 at Thr736 was also examined in cells. Phosphorylation of APLP2 at Thr736 was observed in sorbitol-treated cells as evidenced by immunoblotting with anti-phospho-APLP2 but was barely detectable in non-treated cells. When JBD was co-expressed, phosphorylation was apparently reduced (Fig. 2D, right panel). Taken together, these observations suggest that APP and APLP2 are phosphorylated at Thr668 and Thr736, respectively, by JNK in response to cellular stress.

Enhanced Phosphorylation of APP and APLP2 by X11L—APP interacts with several cytosolic proteins in its cytoplasmic domain, and these regulate its metabolism including the generation of Aβ (16, 17, 21–26). However, their effects on APP phosphorylation are very unclear. Thus, we examined the effect of APP-binding proteins including FR65, JIP-1, JIP-2, DAB1, KLC1, KLC2, and X11L on APP phosphorylation induced by cellular stress. APP was co-expressed with each of these APP-binding proteins or JNK1 in HEK293 cells, and phosphorylation...
tion was examined following treatment or non-treatment of the cells with sorbitol (Fig. 3A). Phosphorylation of APP at Thr690 was observed in all treated cells. Most of the APP-binding proteins did not significantly affect the level of phosphorylation; however, in the presence of X11L, the level of phosphorylated APP was greatly increased. Consistent with the previous observation that the expression of X11L stabilizes intracellular APP metabolism (30), we also observed an increase in APP in cells expressing X11L (Fig. 3A, left middle panel). However, the increase of the phosphorylated form of APP was too large to be accounted for by accumulation of intracellular APP. It was found that the ratio of phosphorylated to total APP was increased by ~3-fold by co-expression of X11L when an equal amount of APP was analyzed (Fig. 3A, right panels). In the cells co-expressing exogenous JNK, APP phosphorylation was also elevated corresponding to an increase of phosphorylated JNK. On the other hand, no significant increase of phosphorylated JNK was observed in the cells expressing X11L (Fig. 3A, left lower panel). Overexpression of X11L facilitates the activation of neither endogenous nor exogenous JNK induced by hyperosmolarity (Supplemental Fig. 1). Interestingly we also detected phosphorylated APP in cells expressing X11L that were not exposed to stress, although it was much smaller than in cells exposed to hyperosmotic stress (Fig. 3A). This phosphorylation of APP may reflect basal cellular JNK activity because it was suppressed by inhibiting JNK with JBD protein (data not shown). These results suggest that X11L expression could increase both the amount and the ratio of phosphorylated APP in cells without further activation of JNK.

Several researchers have reported that all three mammalian LIN-10 family proteins, X11, X11L, and X11L2, commonly interact with the cytoplasmic domain of APP and modulate APP metabolism (21–23, 27–30). Thus, we examined whether X11 and X11L2 could elevate the phosphorylation level of APP in a manner similar to X11L (Fig. 3B). Similar to X11L, co-expression of either X11 or X11L2 with APP in HEK293 cells increased cellular APP, suggesting that they may play a role in stabilizing cellular APP (27, 28, 30). When APP phosphorylation was induced by high osmotic stress, the level of phosphorylated APP was increased slightly in cells expressing X11 or X11L2 compared with cells expressing APP alone, probably as a consequence of increasing total cellular APP. However, these increases in the cells expressing X11 and X11L2 were much lower than that observed in cells expressing X11L, and the ratio of phosphorylated APP to cellular APP was not significantly elevated. We also investigated the effect of X11L and its family proteins on APP phosphorylation induced by DLK over-expression (16) as this protein activates JNK kinase in cells. In cells not expressing any X11 family proteins, DLK-induced phosphorylation was weak, and this was slightly enhanced by expression of X11 or X11L2, probably reflecting increased cellular APP. On the contrary, in cells expressing X11L a high level of phosphorylated APP was detected. Taken together, these results suggest that the ability to elevate APP phosphorylation is a unique function of X11L among the X11 family and is probably independent of the effects of this family on cellular APP accumulation.

Unlike APP, the interaction of APLP2 with intracellular proteins has not been well characterized. However, similar to APP, APLP2 is known to bind X11L in its cytoplasmic domain (22). Therefore, it is likely that the phosphorylation of APLP2 at Thr736 is elevated in the presence of X11L. Thus, APLP2 was co-expressed with JNK1 or X11L, and the level of phosphorylated APLP2 was examined after hyperosmotic stress and DLK overexpression (Fig. 3C). Both hyperosmotic stress and DLK overexpression induced the phosphorylation of APLP2 at Thr736, and co-expression of JNK1, which could act as the responsible kinase, increased the amount of phosphorylated APLP2. Co-expression of X11L apparently increased the level of phosphorylated APLP2 as did co-expression of JNK1. These results suggest that, similar to APP, phosphorylation of APLP2 at Thr736 is likely to be enhanced in the presence of X11L.

Interaction of X11L with APP Is Required to Elevate the Phosphorylation Level of APP—To address the detailed mechanism of X11L on APP phosphorylation, we examined whether a direct interaction between X11L and APP was necessary to increase phosphorylated APP. The interaction involves the PI domain of X11L and the 681-GYENPTY687 motif of APP. APP lacking amino acid residues 681–690 (APPΔ681–690) failed to bind X11L in yeast two-hybrid binding analysis (22). Therefore, we first examined the binding abilities of APP and APPΔ681–690 to X11L in cells by co-immunoprecipitation analysis (Fig. 4A). X11L was co-precipitated with APP but not with APPΔ681–690, indicating that APPΔ681–690 is indeed unable to interact with X11L in cells. Then we examined the effect of X11L on phosphorylation of APPΔ681–690 by JNK (Fig. 4B). In the absence of X11L, APPΔ681–690 was phosphorylated as efficiently as wild-type APP after high osmotic stimulation. This indicates that the deletion does not by itself affect the...
phosphorylation at Thr^688 by JNK. However, when X11L was co-expressed, no elevation of phosphorylated APP^3681–690 could be observed, while the level of phosphorylated wild-type APP was markedly increased. Similarly X11L failed to increase the phosphorylated form of APP^3681–690 when its phosphorylation was induced by overexpression of DLK. This suggests that the interaction of X11L with APP is necessary for its increase in APP phosphorylation.

Next we further characterized the effect of X11L using several truncated X11L proteins. X11L is composed of a unique amino-terminal region, a central PI domain, and a carboxy-terminal region containing two PDZ domains. We utilized truncated X11L including N+PI (containing the amino-terminal region and attached PI domain), PI+C (containing the carboxy-terminal half of X11L including PI and PDZ domains), and N (the amino-terminal region alone) (Fig. 5A). APP lacking a part of the extracellular region (APP^124–303), which behaves in the same manner as full-length APP in cells, was also utilized instead of full-length APP for precise determination of APP phosphorylation since phosphorylated full-length APP could overlap with N+PI, which was nonspecifically recognized with anti-phospho-APP antibody in immunoblots. APP phosphorylation induced by hyperosmotic stress was examined in cells co-expressing full-length X11L or these truncated proteins, and the ratio of the phosphorylated form of APP to total APP was compared. Expression of N+PI markedly elevated the ratio of APP phosphorylation as did full-length X11L, whereas expression of PI+C and N was without significant effect (Fig. 5B).

When we induced APP phosphorylation by DLK overexpression instead of high osmotic stress, we obtained similar results. The ratio of APP phosphorylation was increased by co-expression of full-length X11L and N+PI by ~8-fold but was not influenced by PI+C and N (Fig. 5C). Expression of the PI domain alone failed to elevate phosphorylated APP, although it is not possible to conclude that this domain alone does not influence phosphorylation because its cellular expression level was much lower than the other domains (data not shown). These results suggest that the interaction of X11L with APP via its PI domain is responsible, and the amino-terminal region of X11L is also important for its effect on APP phosphorylation. The ability of X11L to stabilize cellular APP is likely insufficient to elevate APP phosphorylation, and the effects of X11L on the stability and phosphorylation of APP might be constituted of independent mechanisms since PI+C could stabilize APP but could not enhance the phosphorylation. This conclusion seems to be consistent with the observations that X11 and X11L2, which resemble X11L in their PI and carboxy-terminal regions and stabilize cellular APP (21–23), did not elevate APP phosphorylation as X11L did (Fig. 3B).

**X11L Facilitates the Phosphorylation of APP by JNK in Vitro—**Association of X11L with APP required to elevate the phosphorylation level (Fig. 4). However, expression of X11L did not affect JNK activation (Fig. 3 and Supplemental Fig. 1). In addition, insofar as we examined, there was no interaction of X11L with JNK protein, whereas another APP-binding protein, JIP-1, can bind JNK (Supplemental Fig. 2). Therefore, a more likely mechanism of increased APP phosphorylation by X11L is that X11L renders APP more susceptible to phosphorylation by JNK through its direct interaction rather than facilitating the activation of JNK or scaffolds between JNK and APP as is observed for JIP-1 (16, 17, 33). Thus, we tested whether X11L could enhance the phosphorylation of APP by JNK in vitro using purified proteins. APP^Cyt protein was subjected to phosphorylation with activated JNK in the presence or absence of either purified X11L or X11 protein, and the phosphorylation at Thr^688 was examined (Fig. 6A, left panels). APP was similarly phosphorylated in the absence and presence of X11. However, in the presence of X11L, the level of APP phosphorylation was higher than in the absence of X11L. This is consistent with the observation in cultured cells that X11L, but not X11, increased APP phosphorylation (Fig. 3B). On the other hand, X11L did not affect JNK-mediated phosphorylation of GST-e-Jun (Fig. 6A, right panels), which is a widely used substrate to analyze catalytic activity of JNK (42), suggesting that X11L selectively facilitates the phosphorylation of APP rather than directly elevates catalytic activity of JNK. We also examined the effect of truncated X11L proteins purified as GST fusion proteins on APP phosphorylation (Fig. 6B). Addition of GST-X11L (full length) and GST-N+PI to the reaction increased APP phosphorylation, while GST protein (Fig. 6B, ctrl) and GST-N were

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3 K. Iijima and T. Suzuki, unpublished observation.
rylate the Thr668 residue to a comparable extent on APP, we found that JNK1, JNK2, and JNK3 can all phosphorylate APP? JNK protein kinases are encoded by three genes, Jnk1, Jnk2, and Jnk3, which are highly homologous and are involved in signaling apoptosis, stress responses, and proliferation (18). APP is a possible cytosolic substrate of JNK. During the preparation of this report, Inomata et al. (17) and Scheinfeld et al. (43) also reported APP phosphorylation to be induced by stress. However, our analysis extended these findings, particularly with regard to answering two previously unresolved questions. First, what kind of JNK protein kinases can phosphorylate APP? JNK protein kinases are encoded by three genes, Jnk1, Jnk2, and Jnk3, which are highly homologous and are involved in signaling apoptosis, stress responses, and proliferation (18). However, physiological differences among the three JNK protein kinases have also been reported. JNK1 and JNK2 are expressed ubiquitously, whereas JNK3 is expressed most abundantly in brain (18). JNK1 and JNK2 also exhibit differences in substrate specificities, in signal-specific induction of apoptosis in fibroblast, and in response to withdrawal of trophic support in cerebellar neurons (18, 44, 45). In the case of APP, we found that JNK1, JNK2, and JNK3 can all phosphorylate the Thr668 residue to a comparable extent in vitro (Fig. 1B) and that JNK1 and JNK2 isoforms can commensurately contribute to stress-induced APP phosphorylation in cells (Fig. 2B). The ability of all JNK protein kinases to phosphorylate APP is consistent with the observation that APP phosphorylation was induced not only in neuronal cells but also in non-neuronal cells. The second question was whether another APP family protein, APLP2, was phosphorylated by JNK in response to cellular stress. We demonstrated that APLP2 was phosphorylated at Thr726, which corresponds to Thr668 of APP, by JNK1, JNK2, and JNK3 in vitro (Fig. 1C) and that this phosphorylation event was induced by cellular stress (Fig. 2D). Therefore, we conclude that APP and APLP2 can be phosphorylated by JNK protein kinases JNK1, JNK2, and JNK3 in a similar manner.

DISCUSSION
Phosphorylation of APP at Thr668 is proposed to act as a conformational switch for protein-protein interactions and is involved in the regulation of its metabolism and putative function(s) (6–11). In this study, we demonstrated that phosphorylation of APP and APLP2 is induced by JNK family proteins in response to cellular stress and is regulated by one of their binding proteins, X11L. JNK is implicated in the phosphorylation of APP at Thr668 by our group and others (15, 16). Here we found that JNK-medi- ated phosphorylation of APP at Thr668 was induced in response to cellular stress (Fig. 2). Well known targets of activated JNK include transcription factors such as c-Jun and ATF2; however, it is also known that activated JNK localizes to the cytoplasm and contributes to the phosphorylation of several cytosolic proteins (18). APP is a possible cytosolic substrate of JNK. During the preparation of this report, Inomata et al. (17) and Scheinfeld et al. (43) also reported APP phosphorylation to be induced by stress. However, our analysis extended these findings, particularly with regard to answering two previously unresolved questions. First, what kind of JNK protein kinases can phosphorylate APP? JNK protein kinases are encoded by three genes, Jnk1, Jnk2, and Jnk3, which are highly homologous and are involved in signaling apoptosis, stress responses, and proliferation (18). However, physiological differences among the three JNK protein kinases have also been reported. JNK1 and JNK2 are expressed ubiquitously, whereas JNK3 is expressed most abundantly in brain (18). JNK1 and JNK2 also exhibit differences in substrate specificities, in signal-specific induction of apoptosis in fibroblast, and in response to withdrawal of trophic support in cerebellar neurons (18, 44, 45). In the case of APP, we found that JNK1, JNK2, and JNK3 can all phosphorylate the Thr668 residue to a comparable extent in vitro (Fig. 1B) and that JNK1 and JNK2 isoforms can commensurately contribute to stress-induced APP phosphorylation in cells (Fig. 2B). The ability of all JNK protein kinases to phosphorylate APP is consistent with the observation that APP phosphorylation was induced not only in neuronal cells but also in non-neuronal cells. The second question was whether another APP family protein, APLP2, was phosphorylated by JNK in response to cellular stress. We demonstrated that APLP2 was phosphorylated at Thr726, which corresponds to Thr668 of APP, by JNK1, JNK2, and JNK3 in vitro (Fig. 1C) and that this phosphorylation event was induced by cellular stress (Fig. 2D). Therefore, we conclude that APP and APLP2 can be phosphorylated by JNK protein kinases JNK1, JNK2, and JNK3 in a similar manner.

Structural analyses using NMR and CD spectroscopy indicate that phosphorylation causes prolyl cis/trans isomerization of the Thr668-Pro669 peptide bond of the VTPEER motif and alters the overall structure of the cytoplasmic domain of APP (10, 11). The conformational changes induced by phosphorylation affect the interaction of APP with binding proteins such as FE65 (11). The VTPEER motif of APP is highly conserved in APLP2 as LTPPEER, and a mutation at Thr736 of APLP2 affects its interaction with FE65 protein as does mutation of Thr668 of APP (5, 11). Thus, phosphorylation of APP and APLP2 probably acts as a conformational switch for the complementary physiological functions of these proteins.

Recently, Lee et al. (46) reported that Thr668-phosphorylated APP, especially phosphorylated carboxyl-terminal fragments, was elevated and accumulated in human AD brain and raised the possibility that the phosphorylation may increase Aβ generation. In the pathogenesis of AD, activated JNK is significantly increased and is localized in the cytoplasm of neurons in a manner that correlates with the progression of the disease (19, 20). Activation of JNK is also observed in a mouse model of AD (47). Therefore, it is possible that APP and APLP2 could serve as substrates of cytoplasmic JNK in AD and that this results in neural dysfunction. Detailed immunohistochemical analysis with a highly specific antibody aimed at examining whether abnormal phosphorylation of APP and APLP2 correlates with JNK activation may contribute to elucidating the neuronal degeneration process of AD. However, the antibodies we used here are not suitable for immunohistochemical analysis on fixed brain sections.

The influence of interactions between APP and cytosolic proteins on the phosphorylation at Thr668 has not been well understood. Among major APP-binding proteins, we found that only X11L significantly increases the phosphorylation of APP at Thr668 by JNK. In our study, much higher levels of phosphorylated APP were observed in cells expressing X11L with APP than in the cells expressing APP alone or APP with other APP-binding proteins (Fig. 3). In the in vitro phosphorylation assay in which dephosphorylation of APP was inhibited, X11L
X11L Regulates the Phosphorylation of APP Family by JNK

![Diagram: APP/PLP2](image)

**Fig. 7.** Putative models for the effect of APP-binding proteins on the phosphorylation of APP and PLP2 by JNK. APP and PLP2 are phosphorylated at Thr<sup>560</sup> and Thr<sup>576</sup> in their cytoplasmic domain, respectively, by JNK protein kinases, which are activated in response to cellular stress (left). JIP-1 interacts with both JNK and APP to regulate the phosphorylation of APP. JIP-1 also regulates the JNK signaling cascade by scaffolding the signaling components (middle). X11L interacts with APP and facilitates the phosphorylation of APP by activated JNK (right). Open arrows indicate direction of the phosphorylation.

had no significant effects on APP phosphorylation (Fig. 3B), suggesting that X11 family proteins may have differential roles in APP metabolism. X11L is possibly involved in the metabolism and functions of APP in multiple ways: not only by stabilizing cellular APP and suppressing Aβ generation directly but also by regulating the interactions of APP with other proteins by enhancing APP phosphorylation. The observations that the interaction of X11L with APP can be positively and negatively regulated by Alcadin and XB51 protein, respectively, suggest the presence of a cellular regulatory system for APP that involves X11L (30, 48, 49).

X11L interacts with both APP and PLP2 (22), whereas JIP-1 prefera APP over PLP2 as a binding partner (50). In the presence of X11L, phosphorylation of PLP2 at Thr<sup>576</sup> was enhanced as efficiently as that of APP (Fig. 3C). Therefore, X11L is a common regulator for APP family proteins, which may be important not only in AD but also in physiological conditions.

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