A Scaffold Protein JIP-1b Enhances Amyloid Precursor Protein Phosphorylation by JNK and Its Association with Kinesin Light Chain 1*

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Amyloid precursor protein (APP) is the precursor molecule of β-amyloid peptides, the major components of amyloid plaque in patients with Alzheimer’s disease. In this study, we isolated JIP-1b, a JNK signaling scaffold protein, as a binding protein of APP, and analyzed the roles of JIP-1b in APP phosphorylation by JNK and the association of kinesin light chain 1 with APP. APP phosphorylation at threonine 668 by JNK was enhanced on the JIP-1b scaffold in vitro and in cultured cells exogenously expressing APP. APP phosphorylation in nerve growth factor-differentiated PC12 cells was mediated by activation of JNK signaling. JIP-1b also enhanced the association of kinesin light chain 1 with APP. Our results suggest that JIP-1b may function as a protein linking the kinesin-I motor protein to the cargo receptor, APP, and that the JNK signaling pathway may regulate the phosphorylation of this cargo protein through the JIP-1b scaffold.

Received for publication, December 1, 2002, and in revised form, March 5, 2003
Published, JBC Papers in Press, March 28, 2003, DOI 10.1074/jbc.M212160200

The deposition of amyloid plaque is a principal pathological feature in the brain parenchyma and blood vessel walls of patients with Alzheimer’s disease. The major components of amyloid plaque are β-amyloid (Aβ) peptides, including Aβ40 and Aβ42, which are derived by proteolytic cleavage of amyloid precursor protein (APP) (for a review see Ref. 1). APP is a type I membrane spanning protein. Three major isoforms of APP comprising 695, 751, and 770 amino acids are generated by alternative splicing. Enzymes termed β- and γ-secretase cleave APP to form the amino and carboxyl termini of the Aβ peptides. Besides its pathological role in Alzheimer’s disease, APP is thought to be functionally important because mice lacking all APP family genes die in the early postnatal period (2). Although numerous studies have shown various roles of APP, the physiological function of APP is not yet clear.

In neurons, APP is transported within axons by the fast anterograde axonal transport system from cell bodies to nerve terminals (3). When expression of the kinesin heavy chain is inhibited with antisense oligonucleotides, axonal transport of APP is disturbed in cultured hippocampal neurons, suggesting that axonal transport of APP requires the motor protein kinesin-I (4). Recently, it was shown that APP may function as a cargo receptor for kinesin-I. APP forms a complex with kinesin-I by binding directly to the tetratricopeptide repeat (TPR) domain of the kinesin light chain (KLC) subunit (5). Neuronal overexpression or deletion of APP, the Drosophila functional homolog of APP, disrupt axonal transport in Drosophila (6, 7). Furthermore, the fast anterograde axonal transport of β-secretase and presenilin-1 is mediated by APP and kinesin-I, and processing of APP to Aβ by secretases can occur in an axonal membrane compartment transported by kinesin-I (8).

APP is a phosphorylated protein and one known phosphorylation site is threonine 668 (Thr668) (numbering for the APP695 isoform) within the cytoplasmic region of APP (9). Constitutive phosphorylation of APP is observed at Thr668 specifically in neurons. Phosphorylation of APP appears to regulate its function and localization (10). Thus, it is important to elucidate the mechanism of APP phosphorylation. Several kinases were reported to phosphorylate Thr668 of APP. Cdc2 kinase phosphorylates the Thr668 during the G2/M phase of the cell cycle (9). Thr668 of APP is phosphorylated in adult rat brain and in differentiated PC12 cells (10), and this phosphorylation is mediated by Cdk5, a neuronal homolog of Cdc2 kinase (11). Glycogen synthase kinase-3β also phosphorylates Thr668 of APP in vitro (12). Recently, it was shown that APP is more efficiently phosphorylated at Thr668 by e-Jun NH2-terminal kinase-3 (JNK-3) than by Cdk5 or glycogen synthase kinase-3β in vitro (13). However, it is unknown whether JNK-3 phosphorylates APP in neurons.

In this study, we isolated human JNK interacting protein-1b (JIP-1b) as a novel molecule interacting with the cytoplasmic region of APP. Although JIP-1b was originally isolated as a cytoplasmic inhibitor of the JNK signal transduction pathway (14), a subsequent study revealed that JIP-1b is a scaffold protein that interacts with specific multiple components of the JNK pathway namely JNK, mitogen-activated protein kinase kinase 7 (MKK7), and mixed lineage kinase 3 (MLK3) (15). JNK is activated in cells treated with inflammatory cytokines or in cells exposed to environmental stress (for a review see Ref. 16). Although the main targets of the JNK pathway include the transcription factor c-Jun, ATF2, and Elk-1, JNK-3 also in-
duces robust phosphorylation of Thr<sup>668</sup> in the cytoplasmic region of APP (13). Thus, JIP-1b could recruit JNK to APP and in turn enhance the phosphorylation of APP by JNK. Moreover, JIP-1b was identified as a binding partner of the TPR domain of KLC, and was suggested to be a scaffold protein linking the kinesin-I motor protein to its membrane cargo (17). Thus, APP could associate with kinesin-I via JIP-1b. In this study, we further examined whether JIP-1b functions as a scaffold protein for the association of APP with JNK and KLC1. Our results demonstrate that APP associates with JNK and KLC1 on the JIP-1b scaffold, and that APP phosphorylation by JNK is enhanced by the association of APP with JIP-1b. These results suggest that JIP-1b may function as a linker protein of the kinesin-I motor protein to the cargo receptor, APP, and that the JNK signaling pathway may regulate the phosphorylation of the cargo protein through the JIP-1b scaffold.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids—**To construct expression vectors (pcDNA/HA and pcDNA/FLAG) with the HA tag or FLAG tag sequence, oligonucleotide fragments encoding HA or FLAG were inserted into the EcoRI and XhoI sites of the expression vector pcDNA3 (Invitrogen). Expression vectors encoding the HA-tagged or FLAG-tagged proteins were constructed by inserting cDNAs into pcDNA/HA or pcDNA/FLAG. Expression vectors encoding the HA-tagged JNK1 and GST-c-Jun were described previously (18). APP cDNA and MLK3 cDNA were obtained by PCR using oligonucleotide primers and human brain Marathon-Ready (<sup>TM</sup>) cDNA (Clontech). MKC7 cDNA and KLC1 cDNA were kindly provided by Dr. J. Yamauchi (Nara Institute of Science and Technology) and Dr. A. Armin (McGill University), respectively. Deletion and point mutants were constructed with a QuickChange site-directed mutagenesis kit (Stratagene).

**Antibodies—**To prepare a polyclonal anti-JIP-1b antibody, the human JIP-1b cDNA (amino acid residues 162–433) was inserted into the pEXK-AT2 vector (Amersham Biosciences). The fusion protein with glutathione S-transferase (GST) was then expressed in *Escherichia coli*, and purified. Rabbits were immunized with the purified protein, and the antiserum (anti-JIP-1b) was raised. Anti-APP antibodies, UT18 and G369, were previously described (19, 20). The anti-FLAG (M2) and anti-β-tubulin antibodies were purchased from Sigma, the anti-HA antibody (3F10) from Roche Diagnostics, the anti-APP antibody (6E10) from Signet, the anti-JNK1 antibody from Pharmingen, and the anti-phospho-c-Jun and anti-phospho-APP antibodies from Cell Signaling Technology.

**Yeast Two-hybrid Screening—**To isolate cDNA clones encoding APP-interacting proteins, yeast two-hybrid screening was performed as previously described (21). A human brain cDNA library inserted downstream of the Ga4 DNA activation domain in the pACT2 vector was obtained from Clontech. The bait plasmid and library were co-transformed into yeast (L40 strain).

**Cell Culture—**HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. PC12 cells were cultured on collagen-coated dishes in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, and 1% non-essential amino acids. Neuronal differentiation of PC12 cells was induced by NGF (50 ng/ml) in RPMI 1640 medium supplemented with 1% horse serum and 1% non-essential amino acids (differentiation medium).

**Transfection—**HEK293 cells were transfected with plasmid expression vectors using FuGENE 6 transfection reagent (Roche Diagnostics). For transfection of the HA-tagged JNK binding domain (JBD) expression vector into PC12 cells, the cells were plated in a 12-well dish at 5 × 10<sup>4</sup> cells per well. At 24 h after plating, the cells were transfected with 1.6 μg of plasmid per well by the LipofectAMINE 2000 (Invitrogen). At 6 h after transfection, the medium was replaced with fresh medium containing NGF, and 24 h later, the cells were subjected to immunofluorescence staining.

**Assay for in Vitro Binding to Immobilized GST Fusion Proteins (GST Pull-down)—**Cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 20 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 20 mM glycophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). The cell lysate was incubated with purified GST fusion proteins bound to glutathione-Sepharose 4B beads at 4 °C. After the beads were washed three times with washing buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate, and 0.1% Nonidet P-40), the binding proteins were released by boiling in 2× sample buffer (25 mM Tris-Cl, pH 6.5, 1% SDS, 1% mercaptoethanol, and 5% glycerol), and then separated by SDS-PAGE under reducing conditions, before being analyzed by immunoblotting.

**Immunoprecipitation and Immunoblotting—**Cells were lysed in lysis buffer. Immunoprecipitation and immunoblotting were performed as described previously (21).

**In Vitro Phosphorylation of APP—**To prepare purified JIP-1b proteins, HEK293 cells were transfected with expression vectors encoding FLAG-tagged JIP-1b proteins. At 48 h after transfection, the cells were lysed in kinase lysis buffer (20 mM HEPES, pH 7.4, 0.5% Nonidet P-40, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 10 mM NaF, 20 mM glycophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml EGTA). The cell lysate was incubated with an anti-FLAG M2-agarose affinity gel (Sigma) at 4 °C. The binding JIP-1b proteins were eluted with FLAG peptide (Sigma), and concentrated with CENTRICRON (Millipore) in reaction buffer (20 mM HEPES, pH 7.4, 1 mM diethiothreitol, 0.1 mM sodium orthovanadate, 0.1 mM glycophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, and 0.1 μg/ml EGTA). The amount of each JIP-1b protein was measured by Coomassie Brilliant Blue staining. The GST-fused cytoplasmic region of APP (APP<sup>COOH</sup>) proteins was expressed in *E. coli* using the pGEX vector and purified by glutathione 4B beads (Amersham Biosciences). To prepare activated JNK, HEK293 cells were transfected with an expression vector encoding HA-tagged JNK. At 48 h after transfection, the cells were treated with anisomycin (30 μg/ml) for 30 min. The cells were then lysed in kinase lysis buffer. The cell lysate was incubated with an anti-HA antibody at 4 °C, and after the addition of protein G beads the mixture was incubated at 4 °C. The immune complexes were precipitated and then washed twice with kinase lysis buffer. The precipitated complexes were incubated in 30 μl of reaction buffer containing 18 μM ATP, 5 μCi of [γ-<sup>32</sup>PI]ATP (Amersham Biosciences), 5 μg of GST-APP, or its mutants with purified JIP-1b or its mutants for 25 min at 30 °C. The reaction was stopped by adding sample buffer, and the mixture was heated at 95 °C for 5 min. The proteins were then separated by SDS-PAGE. The radioactivity incorporated into APP and JIP-1b was detected by autoradiography.

**Immunofluorescence Staining—**PC12 cells were cultured on collagen-coated glass coverslips and treated with NGF (50 ng/ml). The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline containing 4% sucrose for 10 min at room temperature and then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min. The membranes were then rinsed three times in phosphate-buffered saline with 0.1% Triton X-100 and then washed with the primary antibody for 1 h at room temperature, followed by incubation with Alexa-488- or Alexa-594-conjugated secondary antibody (Molecular Probes). Cells were examined by confocal immunofluorescence microscopy.

**RESULTS**

**Binding of the COOH-terminal Cytoplasmic Region in APP to JIP-1b—**To isolate cDNA clones encoding APP-interacting proteins, we screened a human brain cDNA library using a yeast two-hybrid system. We inserted a cDNA fragment encoding the COOH-terminal cytoplasmic region (amino acid 649–695) of human APP into a pBTM116 plasmid vector, and used it as bait. We isolated 27 clones that reacted positively for β-galactosidase with the bait vector, but not by themselves. Among them, 24 clones encoded proteins that have previously been shown to bind to the cytoplasmic region of APP (22 clones of FE65, a clone of FE65-L2, and a clone of X11). Besides them, three clones, which encoded human JIP-1b, were isolated. Although JIP-1b has recently been shown to bind to APP (20, 23), the significance of the binding has not been characterized. Because the 5'-portion of cDNA was missing, this portion was obtained by PCR amplification of human genomic DNA (Clontech) and then connected to the cDNA clone. The full-length human JIP-1b that we obtained consisted of 711 amino acid residues, and contained a JBD in the NH<sub>2</sub>-terminal region, a
Src homology (SH) 3 domain in the middle region, and a phosphotyrosine interacting domain (PID) and kinesin binding (KB) domain in the COOH-terminal region.

Binding sites between APP and JIP-1b were first determined by the yeast two-hybrid system. A series of deletion mutants of JIP-1b were inserted into a pACT2 vector, and assayed for their binding to the cytoplasmic region of APP. The NH₂-terminal truncated mutant including the PID (Del 4) bound to the cytoplasmic region of APP, whereas the mutant with a further 47-amino acid deletion (Del 6) did not (Fig. 1A). The PID deletion mutant lacking the COOH-terminal 25 amino acids (Del 8) did not bind to the cytoplasmic region of APP. These results suggest that the PID of JIP-1b is the domain that binds to the cytoplasmic region of APP, but did not exclude the possibility that the KB domain is also required for the binding. It is known that the PID of FE65 and X11 recognizes the NPTY motif within the cytoplasmic region of APP and binds to it (24). To examine whether this motif is also required for JIP-1b to bind to the cytoplasmic region of APP, the cytoplasmic region of APP and its deletion mutant lacking the NPTY motif were inserted into a pBTM116 vector, and assayed for their binding to JIP-1b. The mutant did not bind to JIP-1b (Fig. 1A), indicating that JIP-1b recognizes the NPTY motif within the cytoplasmic region of APP.

Next, the binding of APP to JIP-1b was examined by the GST pull-down method. Recently, it was shown that the COOH-terminal tail region (11 amino acids) of JIP-1b interacts with the TPR domain of kinesin light chain (17). Thus, we also examined whether this KB domain affects the binding of APP to JIP-1b. The cytoplasmic region of APP (APPC) and its mutant lacking the NPTY motif (ΔNPTY) were expressed as GST fusion proteins in E. coli. The FLAG-tagged full-length JIP-1b and its truncated mutants lacking the KB domain (ΔKB) or lacking the PID and KB domain (ΔPID) (Fig. 1C) were expressed in HEK293 cells, and assayed for binding to the GST-fused cytoplasmic region of APP (GST-APPC) and its deletion mutant (GST-ΔNPTY). The full-length JIP-1b bound to the GST-APPC, but not to the GST-ΔNPTY (Fig. 1D). ΔKB that retained the PID was still able to bind to the GST-APPC, suggesting that the KB domain is not required for the binding of APP to JIP-1b. The truncated mutant lacking the whole PID (ΔPID) did not bind to the GST-APPC. These results confirmed that the NPTY motif of APP and the PID of JIP-1b are responsible for the binding between APP and JIP-1b.

The binding of APP and JIP-1b in mammalian cells was examined by coimmunoprecipitation. The FLAG-tagged JIP-1b with or without APP was expressed in HEK293 cells. Lysates of the cells were immunoprecipitated with an anti-FLAG or anti-APP (6E10) antibody, and the immunoprecipitates were immunoblotted with an anti-APP (UT18) or anti-FLAG antibody.
APP was detected in the immunoprecipitates with the anti-FLAG antibody from cells expressing JIP-1b and APP (Fig. 1E). The coimmunoprecipitated APP corresponded to the immature form of APP. Thus, the immature form appeared to be more efficiently coimmunoprecipitated with JIP-1b than the mature forms, although a long exposure of the immunoblot showed that the mature forms of APP were also coimmunoprecipitated with JIP-1b (data not shown). Although a small amount of JIP-1b was immunoprecipitated by the anti-APP antibody (6E10), the increased amount of JIP-1b was detected in the immunoprecipitates with the anti-APP antibody from cells expressing JIP-1b and APP. Because JIP-1b was shown to selectively bind JNK, it is possible that JNK may affect the binding of APP to JIP-1b. To examine the effect of JNK on the binding, the FLAG-tagged JIP-1b was expressed together with APP and the FLAG-tagged JIP-1b was immunoprecipitated by the anti-APP antibody (Fig. 1F). JNK activation induced the phosphorylation of APP, as shown by immunoblotting (Fig. 1A), and their amounts were measured by Coomassie Brilliant Blue staining. In addition the vector (pcDNA3) used for the expression of JIP-1b was also transfected into HEK293 cells, and the cell lysate was treated as was done for the purification of the FLAG-tagged JIP-1b, for use as the control (pcDNA3). The presence of the purified JIP-1b (1.25 µg) enhanced the phosphorylation of APP by the activated JNK, whereas the level of phosphorylation detected was much less in the presence of the control (pcDNA3) (Fig. 3B). JIP-1b was also phosphorylated by JNK. When the phosphorylation site (Thr668) of APPC was mutated to an alanine (T668A), no phosphorylation was observed (Fig. 3C), confirming that the site phosphorylated by JNK is Thr668. The deletion mutant of APP (ΔNPTY) was phosphorylated to a lower extent. The presence of more JIP-1b (3.75 µg) further enhanced the phosphorylation of APP by JNK. A mutant JIP-1b with a deletion in a part of the PID (Δdel) or a deletion of the PID and KB domain (ΔPID) did not enhance the phosphorylation of either APPC or JIP-1b, whereas a deletion of the kinesin-binding domain (ΔKB) did not affect the enhancement of phosphorylation (Fig. 3D). In addition mutant JIP-1b with a deletion in a part of the PID (Δdel) or a deletion of the PID and KB domain (ΔPID) did not enhance APP phosphorylation, although their own phosphorylation was not affected (Fig. 3D). Note that APPC phosphorylation by JNK occurred in the absence of JIP-1b (compare lanes a and d in Fig. 3D). Thus, these results suggest that JIP-1b linking APP to JNK enhances the in vitro phosphorylation of APP by JNK.

The phosphorylation of APP in mammalian cells was examined in HEK293 cells exogenously expressing APP, JNK, and JIP-1b. The cells were treated with anisomycin to activate JNK, and the JNK activity was measured by in vitro kinase assay using GST-e-Jun as a substrate. The phosphorylation of APP was detected by immunoblotting using an anti-phospho-APP antibody. JNK activation induced the phosphorylation of APP (Fig. 4A). As previously reported (14), overexpression of JIP-1b suppressed the activity of JNK. In proportion to the suppression, APP phosphorylation was decreased, suggesting that APP phosphorylation depends on the JNK activity. Overexpression of the ΔPID mutant of JIP-1b, which lacked the APP binding site, further decreased APP phosphorylation, although the level of JNK activity was similar to that in the cells overexpressing the wild-type JIP-1b. These results suggest that JIP-1b functions as a scaffold protein linking JNK to APP in mammalian cells. Overexpression of the ΔJBD mutant of JIP-1b that lacked the JNK binding domain did not affect the activity of JNK, but reduced APP phosphorylation, suggesting that association of ΔJBD with APP inhibited the phosphorylation of APP by JNK. When the Thr668 phosphorylation site of APP was mutated to an alanine, no phosphorylation by activated JNK was observed, indicating that the site phosphorylated by JNK in mammalian cells is Thr668.

JIP-1b was shown to be a scaffold protein that interacts with specific multiple components of the JNK pathway namely JNK, MKK7, and MLK3. It was shown that overexpression of JIP-1b enhances the activity of JNK induced by MKK7 or MLK3 (15). Thus, the upstream components of JNK may affect the APP phosphorylation by JNK through JIP-1b. To examine the effect...
of upstream components, MKK7 or MLK3 were expressed together with APP, JNK, and JIP-1b in HEK293 cells, and the JNK activity as well as APP phosphorylation were evaluated. Expression of MKK7 alone slightly enhanced the JNK activity, but did not enhance APP phosphorylation (Fig. 4B). Coexpression of MKK7 with JIP-1b enhanced the JNK activity and APP phosphorylation. Coexpression of MKK7 with the ∆PID of JIP-1b, which can interact with MKK7 (15) but not with APP, enhanced the JNK activity, but not the APP phosphorylation. Expression of MLK3 alone enhanced the JNK activity and APP phosphorylation (Fig. 4C). Coexpression of MLK3 with JIP-1b further enhanced both the JNK activity and APP phosphorylation. Coexpression of MLK3 with the ∆del mutant of JIP-1b, which can interact with MLK3 (15) but not with APP, enhanced the JNK activity but not the APP phosphorylation. These results further support the suggestion that JIP-1b functions as a scaffold protein linking JNK to APP in mammalian cells.

**The Phosphorylation of Endogenous APP by JNK**—As the JNK signaling pathway plays a crucial role in cellular responses stimulated by a variety of stress-inducing agents, it is assumed that the activated JNK in the cells stimulated by these agents enhances the phosphorylation of endogenous APP. To examine the phosphorylation of endogenous APP by JNK, PC12 cells were differentiated by treatment with 50 ng/ml NGF for 3 days, and then stimulated by various stress-inducing agents. The JNK activity was measured by *in vitro* kinase assay.
assay using GST-c-Jun as a substrate, and APP phosphorylation was detected by immunoblotting using the anti-phospho-APP antibody. Treatment by anisomycin or staurosporin slightly enhanced the JNK activity in the differentiated PC12 cells (data not shown). UV radiation enhanced the JNK activity and slightly induced the APP phosphorylation (upper panel) and APP expression (middle panel) were detected by immunoblotting of the cell lysates. To measure JNK activity, cell lysates were prepared by the standard method and were immunoprecipitated with an anti-JNK antibody. In vitro kinase assays were performed with the immunoprecipitated JNK using c-Jun as a substrate (bottom panel). B, NGF-differentiated PC12 cells were unstimulated (−) or stimulated with 0.4 M sorbitol (SRB) for 30 min. The cells were fixed, permeabilized, and incubated with anti-APP (G369) (a, b, i, and j), anti-JIP-1b (c, d, k, and l), or anti-phospho-APP (e, f, g, h, m, n, and o) antibodies and an anti-tubulin (TU) antibody followed by Alexa-488 (for APP, JIP-1b, and phospho-APP) or Alexa-594 (for tubulin) conjugated secondary antibodies. Nuclei were stained with Sytox Green (Molecular Probes) (h). o shows an enhancement of the signal in m.

Fig. 5. Phosphorylation of endogenous APP in NGF-differentiated PC12 cells stimulated with sorbitol. A, PC12 cells were differentiated by treatment with 50 ng/ml NGF for 3 days, and then stimulated with 0.4 M sorbitol (SRB). Cell lysates were prepared at the indicated times according to the manufacturer’s instruction for immunoblotting using the anti-phospho-APP antibody. APP phosphorylation (upper panel) and APP expression (middle panel) were detected by immunoblotting of the cell lysates. To measure JNK activity, cell lysates were prepared by the standard method and were immunoprecipitated with an anti-JNK antibody. In vitro kinase assays were performed with the immunoprecipitated JNK using c-Jun as a substrate (bottom panel). B, NGF-differentiated PC12 cells were unstimulated (−) or stimulated with 0.4 M sorbitol (SRB) for 30 min. The cells were fixed, permeabilized, and incubated with anti-APP (G369) (a, b, i, and j), anti-JIP-1b (c, d, k, and l), or anti-phospho-APP (e, f, g, h, m, n, and o) antibodies and an anti-tubulin (TU) antibody followed by Alexa-488 (for APP, JIP-1b, and phospho-APP) or Alexa-594 (for tubulin) conjugated secondary antibodies. Nuclei were stained with Sytox Green (Molecular Probes) (h). o shows an enhancement of the signal in m.

It was shown that overexpression of the JBD region in JIP-1b suppresses the JNK activity in the cells (14, 25). Thus, to prove that the phosphorylation of endogenous APP is catalyzed by JNK, the HA-tagged JBD (Fig. 1C) was expressed in PC12 cells, and the APP phosphorylation examined by immunofluorescence staining. Overexpression of JBD did not alter the subcellular localization of APP in the untreated and sorbitol-treated cells (Fig. 6). Overexpression of JBD inhibited APP phosphorylation in the cells treated with sorbitol. These results indicate that the phosphorylation of endogenous APP is catalyzed by the activated JNK.

It was recently shown that overexpression of MLK3 induced the activation of JNK in PC12 cells and effectively induced apoptotic cell death (22, 26). Thus, to examine whether the JNK activity that leads to apoptotic cell death also induces APP phosphorylation, the HA-tagged MLK3 was transiently expressed in neurally differentiated PC12 cells, and APP phosphorylation was detected by immunofluorescence staining. Overexpression of MLK3 increased the levels of nuclear-phosphorylated c-Jun, but did not alter the subcellular localization of endogenous APP (Fig. 7). APP phosphorylation was induced...
in cells overexpressing MLK3, suggesting that JNK activity that leads to the apoptotic death of neuronal differentiated PC12 cells also induces APP phosphorylation.

**APP Associates with KLC1 on the JIP-1b Scaffold**—Recently, it was shown that the cytoplasmic domain of APP forms a complex with the TPR domain of the KLC (5). It was also shown that JIP-1b interacts with the TPR domain of KLC (17). These findings raised the question as to whether the interaction of APP with KLC is enhanced or inhibited by JIP-1b. To examine this question, the FLAG-tagged TPR domain (amino acid 185–500) of KLC1 and/or the HA-tagged JIP-1b lacking the NH2-terminal region (ΔN) (Fig. 1C) were expressed in HEK293 cells, and assayed for binding to the GST-APPC. In this assay, we used the NH2-terminal truncated JIP-1b to avoid the effect of JNK binding. The HA-tagged truncated ΔN (ΔNΔKB) (Fig. 1C) that lacked the KLC binding site was also expressed, and then assayed for binding activity. The TPR domain of KLC1 alone did not bind to the GST-APPC in these assay conditions (Fig. 8A). Coexpression of ΔN resulted in the binding of the TPR domain to the GST-APPC, whereas coexpression of ΔNΔKB lacking the KLC binding site did not although the ΔNΔKB did bind to the GST-APPC, suggesting that JIP-1b acts as a linker of KLC1 to APP.

The interaction of APP with KLC1 through JIP-1b in mammalian cells was examined by coimmunoprecipitation. APP and the FLAG-tagged KLC1 were expressed with the HA-tagged JNK, MLK3, and JIP-1b in HEK293 cells. Lysates of the cells were immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitates were immunoblotted with an anti-phospho-APP antibody. Coimmunoprecipitation of APP with KLC1 through JIP-1b was not significantly changed by coexpression with JNK or with JNK and MLK, although expression of MLK did induce APP phosphorylation (Fig. 8C). The phosphorylated APP was coimmunoprecipitated with KLC1 through JIP-1b. The APP mutant lacking the phosphorylation site (T668A) was coimmunoprecipitated with KLC1 through JIP-1b similarly to the wild-type APP. These results suggest that the

**Fig. 6. Inhibition of the phosphorylation of endogenous APP by JBD.** PC12 cells were transfected with the HA-tagged JBD, differentiated, and unstimulated or stimulated with sorbitol. The cells were fixed, permeabilized, and incubated with anti-HA (a, d, g, and j), anti-APP (G369) (b and h), or anti-phospho-APP (e and k) antibodies followed by Alexa-488 (for APP and phospho-APP) or Alexa-594 (for JBD) conjugated secondary antibodies.

**Fig. 7. APP phosphorylation in NGF-differentiated PC12 cells overexpressing MLK3.** Differentiated PC12 cells were transfected with the HA-tagged MLK3. The cells were fixed, permeabilized, and incubated with an anti-HA antibody (a, d, and g) and anti-phospho-c-Jun (b), anti-APP (G369) (e), or anti-phospho-APP (h) antibodies followed by Alexa-488 (for phospho-c-Jun, APP and phospho-APP) or Alexa-594 (for MLK3) secondary antibodies. Arrows in a–c show the cells that do not express MLK3.
association of APP with KLC1 through JIP-1b is not affected by its phosphorylation.

**DISCUSSION**

JIP family proteins were studied from two aspects. One is that JIP proteins act as scaffolds that organize the JNK signaling pathway. They interact with multiple components of the JNK signaling pathway, including the JNK group of mitogen-activated protein kinases, the mitogen-activated protein kinase kinase isoform MKK7, and members of the MLK group of mitogen-activated protein kinase kinase kinases, leading to the efficient activation of JNK (15). The other is that JIP proteins interact with KLC and function as linkers of conventional kinesins (kinesin-I) to membranous cargo (17, 27). Kinesin-I is a heterotetramer of two kinesin heavy chains and two KLCs. Kinesin heavy chains consist of an NH$_2$-terminal motor domain, a central coiled coil stalk domain for dimerization, and a COOH-terminal domain. KLC contains an NH$_2$-terminal domain that associates with the COOH-terminal domain of kinesin heavy chains, and six TPR domains in the COOH-terminal tail region. JIP proteins interact with the TPR domain of KLC (17). Despite the characterization of these functions for JIP proteins, it was not clear why scaffold proteins for the JNK signaling also function as linkers of a motor protein to its cargos. We have shown here that JIP-1b not only enhances the interaction of APP and KLC1, but also enhances the JNK-dependent phosphorylation of APP, suggesting that a linker protein of the kinesin motor protein to a cargo also scaffolds the signaling pathway that regulates the phosphorylation of the cargo protein.

**Association of APP with Components of the JNK Signaling Pathway through JIP-1b**—JIP-1b contains multiple domains, a JBD in the NH$_2$-terminal region, a SH3 domain in the middle region, as well as a PID and a KB domain in the COOH-terminal region. Mutagenic analysis revealed that the PID of JIP-1b mediates the interaction with APP through the NPTY motif. This interaction is also shown by other groups (20, 23). The GST pull-down assay showed that the association of APP with JNK is achieved only in the presence of JIP-1b, and that JIP-1b lacking the JBD does not mediate this association. Thus, APP and JNK are assembled on JIP-1b through the PID and JBD, respectively. MKK7 binds to the region between the JBD and the SH3 domain of JIP-1b (15). Because MLK3 binds to the COOH-terminal region of JIP-1b (15), the binding of APP to JIP-1b may compete with that of MLK3. Expression of MLK3 together with APP and JNK enhanced both the JNK activation and APP phosphorylation, while additional expression of JIP-1b further enhanced both responses. Recently, a model for MLK-dependent signal transduction to JNK on JIP3 scaffolds was proposed (28). JIP maintains members of the MLK group in a monomeric, unphosphorylated, catalytically inactive state. Upon stimulation, the JNK-JIP binding affinity increases, whereas the JIP-MLK interaction affinity is attenuated. Dissociation of MLK from JIP results in subsequent MLK dimerization, autophosphorylation, and JNK activation. According to this model, the binding of JNK to JIP-1b may lead to the dissociation of MLK from JIP-1b and the subsequent activation of MLK followed by the JNK activation, resulting in the APP recruitment to JIP-1b and the enhancement of APP phosphorylation. JIP proteins are known substrates of JNK (28, 29). We also detected the phosphorylation of JIP-1b by in vitro kinase assay. Thus, the phosphorylation of JIP-1b may also regulate the interaction of JIP-1b with APP and JNK.

**Phosphorylation of APP**—In vitro kinase assay using purified JNK and JIP-1b showed that JIP-1b linking APP to JNK enhances the in vitro phosphorylation of APP by JNK. In HEK293 cultured cells exogenously expressing JNK, APP, and JIP-1b or its mutants, the JIP-1b mutant, which lacked the APP binding site, decreased the APP phosphorylation by JNK when compared with that in the presence of the wild-type JIP-1b. Furthermore, the JNK activation by MKK7 or MLK3 on the JIP-1b scaffold enhanced APP phosphorylation, and the
JIP-1b mutant that lacked the APP binding site reversed this enhancement. These results suggest that JIP-1b can function as a scaffold protein for the APP phosphorylation catalyzed by JNK.

Because the phosphorylation of APP appears to regulate its function and localization in neurons, it is important to elucidate the mechanism of this phosphorylation in neurons. In the present study, we examined the phosphorylation of endogenous APP by JNK in NGF-differentiated PC12 cells, and found that sorbitol treatment of the cells strongly enhanced JNK activity and induced the APP phosphorylation. Overexpression of the JBD region of JIP-1b, which was shown to suppress JNK activity, inhibited APP phosphorylation induced in the cells treated with sorbitol. Thus, it is most likely that endogenous APP is phosphorylated in the NGF-differentiated PC12 cells, and that this phosphorylation is catalyzed by JNK. Because a subcellular localization study showed that APP phosphorylated by JNK localized to the similar fractions as those of JIP-1b, it is possible that the phosphorylation of endogenous APP by JNK occurs through the JIP-1b scaffold. Thus, the JNK signaling pathway probably plays an important role in the APP phosphorylation in neurons.

JNK is activated in response to cellular stress, and contributes to the apoptotic response (for a review see Ref. 16). JNK is implicated in neuronal death following exposure to excitotoxins (30) and neurodegenerative diseases (31). JIP-1b is also involved in neuronal death caused by JNK (14, 32). Thus, the JNK activation, which leads to neuronal death, could induce APP phosphorylation. Overexpression of MLK3, which induces the JNK activation leading to neuronal apoptosis (22, 26), induced APP phosphorylation in PC12 cells, suggesting that APP phosphorylation is correlated with neuronal death induced by JNK. Although the functional significance of APP phosphorylation in neuronal apoptosis is not clear, it is tempting to speculate that APP phosphorylation is related to the pathogenesis of Alzheimer’s disease. However, to clarify this relationship, it will be necessary to compare the level of APP phosphorylation between healthy and Alzheimer’s disease brains.

Our immunofluorescence staining study showed that phosphorylated APP localizes to the nucleus in differentiated PC12 cells that are not treated with sorbitol. Recently, it was shown that γ-secretase generates the cytoplasmic fragment of APP, which was named APP intracellular domain (AICD) (33), and that the AICD is transported to the nucleus with FE65 where it is involved in gene transcription (34). Thus, it is possible that the AICD is phosphorylated at the nucleus, and then detected by the anti-phospho-APP antibody. However, we cannot exclude the possibility that it may be nonspecific staining. Further characterization is necessary to clarify whether the AICD is phosphorylated at the nucleus.

Association of APP with KLC1 through JIP-1b—Because neuronal overexpression or deletion of APP, the Drosophila functional homolog of APP, disrupted axonal transport (6, 7), APP appears to function as a cargo receptor for kinesin-I. Thus, it is important to elucidate the mechanism of the association of APP with kinesin-I. It was previously shown that APP directly interacts with KLC (5). Our GST pull-down assay showed that the association of the COOH-terminal cytoplasmic region of APP with the TRP domain of KLC1 is only achieved in the presence of JIP-1b. The communoprecipitation study showed that a small amount of APP was communoprecipitated with KLC1 from cells overexpressing these proteins, and that coexpression of JIP-1b strongly enhances this communoprecipitation. These results suggest that JIP-1b plays a crucial role in the association of APP with kinesin-I. Two possibilities are considered concerning the JIP-1b-mediated interaction of APP and KLC. One is that JIP-1b enhances the direct interaction (Fig. 9A). The x-ray crystallographic analysis of the TPR domains has shown that at least three repeats are required to bind the partner protein (35). JIP-1b and APP could simultaneously bind to KLC by sharing the six TPRs. The other is that JIP-1b mediates an indirect interaction (Fig. 9B). Although it is not clear at present which interaction is involved, JIP-1b may regulate APP transport by kinesin-I. Other linker-mediated interactions have been reported between the kinesin superfamily member KIF17 and the N-methyl-D-aspartate receptor and between KIF13A and the mannose 6-phosphate receptor (36, 37).

Because JIP-1b acts as a scaffold protein for JNK signaling, axonal transport by kinesin-I might be regulated by this signaling. Mutations in the Sunday Driver protein, Drosophila homolog of mammalian JIP-3/J SAP1, cause aberrant accumulations of axonal cargoes, which are similar in phenotype to kinesin-I mutants of Drosophila (26). Furthermore, mutations in the UNC-16 protein, Caenorhabditis elegans homolog of JIP-3/J SAP1, result in the mislocalization of synaptic vesicle and glutamate receptor markers. Deletion mutations in C. elegans JNK and JNK kinases result in a similar mislocalization of synaptic vesicle markers (38). These observations suggest that JIP-3/J SAP1 and JNK signaling regulates the localization of vesicular cargo by kinesin-I transport. Although JIP-1b and JIP-3/J SAP1 are structurally unrelated, both proteins are involved in JNK signaling and directly bind to kinesin-I via the TRP domain of KLC. Thus, the JIP-1b-mediated transport is likely to be regulated by JNK signaling. APP phosphorylation by JNK through JIP-1b may affect the kinesin-I transport. Our results suggest that APP phosphorylation by JNK does not affect the interaction of APP with KLC through JIP-1b. Thus, APP phosphorylation may possibly regulate axonal transport of APP or its function in growth cones.

Our results in this study raise the possibility that the JIP-1b-dependent axonal transport of APP may be regulated by the JNK signaling pathway. Thus, it is possible that a disorder of the JNK signaling pathway results in a disturbance of the axonal transport of APP, which may be implicated in the pathogenesis of Alzheimer’s disease. Further characterization of the relationship between the JNK signaling pathway and axonal transport of APP will help us to understand the pathogenesis of Alzheimer’s disease.

Acknowledgments—We thank Dr. J. Yamauchi and Dr. A. Armin for providing MKK7 cDNA and KLC1 cDNA, respectively.
A Scaffold Protein JIP-1b Enhances Amyloid Precursor Protein Phosphorylation by JNK and Its Association with Kinesin Light Chain 1
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J. Biol. Chem. 2003, 278:22946-22955.
doi: 10.1074/jbc.M212160200 originally published online March 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212160200

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