We have isolated a novel protein based on its association with Drosophila APP-like protein (APPL), a homolog of the β-amyloid precursor protein (APP) that is implicated in Alzheimer’s disease. This novel APPL-interacting protein 1 (APLIP1) contains a Src homology 3 domain and a phosphotyrosine interaction domain and is expressed abundantly in neural tissues. The phosphotyrosine interaction domain of APLIP1 interacts with a sequence containing GYENPTY in the cytoplasmic domain of APPL. APLIP1 is highly homologous to the carboxyl-terminal halves of mammalian c-Jun NH2-terminal kinase (JNK)-interacting protein 1b (JIP1b) and 2 (JIP2), which also contain Src homology 3 and phosphotyrosine interaction domains. The similarity of APLIP1 to JIP1b and JIP2 includes interaction with component(s) of the JNK signaling pathway and with the motor protein kinesin and the formation of homo-oligomers. JIP1b interacts strongly with the cytoplasmic domain of APP (APPcyt), as APP interacts with APPL, but the interaction of JIP2 with APPcyt is weak. Overexpression of JIP1b slightly enhances the JNK-dependent threonine phosphorylation of APP in cultured cells, but that of JIP2 suppresses it. These observations suggest that the interactions of APP family proteins with APLIP1, JIP1b, and JIP2 are conserved and play important roles in the metabolism and/or the function of APPs including the regulation of APP phosphorylation by JNK. Analysis of APP family proteins and their associated proteins is expected to contribute to understanding the molecular process of neural degeneration in Alzheimer’s disease.

β-Amyloid precursor protein (APP), implicated in the cause and progression of Alzheimer’s disease, is a receptor-like transmembrane protein consisting of an extracellular domain, a transmembrane domain, and a short cytoplasmic domain (for review, see Ref. 1). APP belongs to the conserved APP family (APPs), which includes amyloid precursor-like protein 1 and 2 (APLP1 and APLP2) in mammals, APP747 in Xenopus, eAPP in electric ray, APP-L1 in Caenorhabditis elegans, and APP-like (APPL) in Drosophila (for review, see Ref. 2). Besides the involvement of APP in the pathogenic process of Alzheimer’s disease, APPs are expected to be functionally important because mice lacking all of the APP family genes die in the early postnatal period (3), but the physiological role of APPs has not been analyzed sufficiently in mammal. Except for mammalian APPs, Drosophila APPL is the most characterized (4). APP is expressed abundantly in neural tissue (5) and has been associated with synaptic differentiation at the neuromuscular junction (6). APP is metabolized in the same manner as mammalian APPs (7), and human APP expressed in fly is transported normally in neurons (8). Furthermore, the behavioral defect of APPL-null flies is partially rescued by the expression of human APP (9). These facts suggest that the molecular mechanism of metabolism and physiological roles of APPs may be basically conserved among species. Revealing the characteristics of APPs which have been conserved among species may be helpful in understanding the character of APP and the molecular mechanism of the pathogenic process of Alzheimer’s disease.

The cytoplasmic domains of APPs are more highly conserved than the other domains among a wide variety of species and are important for intracellular metabolism (10, 11) and for physiological function (12–15). Several proteins have been reported to interact with the cytoplasmic domain of APP (APPcyt) in mammals (16–21), although the importance and physiological function(s) of these interactions are not well understood. In invertebrates the protein interaction of APPs has been scarcely identified. In this study we identified a novel gene in Drosophila, named APLIP1, which encodes a protein that interacts with the cytoplasmic domain of APPL (APPLcyt). APLIP1 was homologous to mammalian scaffold proteins JIP1b and JIP2, which were implicated in the JNK signaling cascade and/or intracellular transport (22–25). They could interact with APPs and in addition shared abundant expression in neural tissue and the properties to bind JNK kinase and kinesin. Moreover, we propose a possible function of mammalian JIP to modulate the phosphorylation of APP. Analysis of this evolutionarily

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conserved interaction of APPs with APLIP1 and JIP contributes to our knowledge of the mechanism of Alzheimer’s disease progression.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid System**—The yeast two-hybrid screening was executed with the MATCH MAKER two-hybrid system (Clontech) as described (18). The pGBK7AAPP.Lcyt “bait” plasmid encoding the cytoplasmic domain (amino acids 834–866) of APPL (4) and a Drosophila whole adult cDNA library cloned into the vector pACT2 (Clontech) were cotransfected into the yeast HP7c strain. Cotransfectants were grown on a selective medium lacking Trp, Leu, and His and were assayed for their activation of lacZ reporter genes. Positive clones were recloned into Escherichia coli HB101, and their nucleotide sequences were determined. Quantification of the β-galactosidase activity of cotransfectants was executed with a liquid assay using o-nitrophenyl galactopyranoside according to the manufacturer’s protocol (Clontech) and was described in Miller units.

**Molecular Cloning of APLIP1, JIP1b, and JIP2**—The cDNA encoding full-length APLIP1 was isolated from a Drosophila embryonic cDNA library (a kind gift from Dr. Ueda) using a radiolabeled probe prepared from a partial fragment of APLIP1 (nucleotides 841–1788). Hybridizations were carried out with a standard procedure (28). Positive clones were subjected to excision of the pBluescript phagemid from the AZAPII vector using the ExAssist/SOLR system (Stratagene). Mouse cDNAs were isolated by hybridization in mouse brain cDNA libraries cloned into the UNIZAP XR phage vector (Stratagene) using an APLIP1 probe as a fragment. The 5′-upstream sequence of mouse JIP2 cDNA was obtained by 5′-rapid amplification of cDNA ends (version 2; Invitrogen) from mouse whole brain total RNA using a reverse primer (5′-CTCTTTTCACAGTGTTGCGGAG-3′) and a nested PCR reverse primer (5′-TAAAGCCGACGCGACCTAGT-3′).

**Plasmid Construction**—The cDNA encoding APP.Lcyt was produced by RT-PCR using adult total RNA, and the resulting cDNA fragment encoding amino acid positions 834–866 of APPL (4) was cloned into pGEMT (Clontech) at EcoR1/BamHI sites for yeast two-hybrid screening. Variants of cDNAs were produced by PCR and cloned into pGAD24 (Clontech) at Smal/PasI sites. Other various amino-terminal truncated proteins of APLIP1 (N84 (amino acids 85–490), N207 (208–490), N280 (281–490), N350 (351–490), and N383 (384–490)) were also produced by PCR and cloned into pGAD24 (Clontech) at EcoR1/PvuI sites, cDNAs of APLIP1, N207, and C1A17 were cloned into the mammalian expression vector pcDNA3.1MYc/HisA (Invitrogen) to produce pcDNA3.AMYc/HisA to express proteins tagged with a c-Myc epitope at the carboxyl terminal. The rabbit polyclonal anti-APP antibody G369 has been described previously (28). A rabbit polyclonal anti-APPL antibody raised against the extracellular domain of APPL (Ab952) was a kind gift from Dr. K. White (29). Anti-FLAG (M2; Sigma), anti-FA-12CA5 (Roche Diagnostics), and anti-c-Myc (Invitrogen) monoclonal antibodies were purchased. Polyclonal APP phosphorylation state-specific antibody (pABThr-668) UT-33 was raised against a phosphoryopeptide APP605-677(Cys)PThr-668 of APP605 and described previously (12, 30, 31).

**Expression of Proteins in Cultured Cells**—African green monkey kidney COS-7 and mouse neuroblastoma Neuro-2a cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum. To express APPs, 5×10^3–1×10^6 Neuro-2a cells were transiently transfected with 0.5–2 μg of each plasmid in LipofectAMINE 2000 (Invitrogen) or LipofectAMINE (Invitrogen) according to the manufacturer’s protocol and cultured for 48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. The cells were harvested and lysed in CHAPS lysis buffer (phosphate-buffered saline containing 10 mM CHAPS, 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM NaN<sub>3</sub>, and 1 mM NaF) for 0.5 h at 4 °C. Microcystine-LR (1 μM) was added to the buffer to analyze the phosphorylated forms. The lysed cells were centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant was used for the following analyses.

**In Vitro Binding Assays Using GST Fusion Proteins**—A pGEX-4T-1 cDNA construct was introduced into E. coli BL21, and a GST fusion protein was prepared. The protein was purified with glutathione-Sepharose 4B (Amersham Biosciences) as described (18), and the purity of the protein was examined by staining with Cooamsil Brilliant Blue R-250 after SDS-PAGE. The purified GST fusion proteins (~1 nmol) coupled to glutathione-Sepharose 4B were incubated for 2 h at 4 °C with cell lysates derived from COS-7 cells transiently expressing the recombinant cDNAs. The beads were washed with 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 0.1% Tween 20, and a splicing variant of JIP1b, which is also known as a splicing variant of JIP1 (GenBank G91680), was added to the beads with a 1 μM radioactive probe (10 μCi) coupled to glutathione-Sepharose 4B (PerkinElmer Life Sciences). For Northern blotting, 1 μg of poly(A)^+ RNA was loaded onto a 1% (w/v) agarose gel under denaturated conditions, electrophoresed, and transferred to a membrane. Radiolabeled probes (>10^6 cpm/μg DNA) of APLIP1 and ribosomal protein 49 (rp49) were prepared with [α-32P]dCTP (3,000 Ci/mmol, PerkinElmer Life Sciences) and a random primer labeling kit (Roche Diagnostics). Hybridization was carried out using ExpressHyb hybridization solution (Clontech) according to the user’s manual, and signals were detected by autoradiography. A cDNA of rp49 (GenBank U92345) was obtained by RT-PCR with adult head poly(A)^+ RNA. For RT-PCR analysis, poly(A)^+ RNA, prepared from adult heads and bodies separated using glass beads and a sieve, were reverse transcribed with SuperScript II (Invitrogen) using an oligo(dT) primer 2 h at 37 °C and treated with RNase H (Takara). A PCR was performed with Ex-Taq (Takara) using [α-32P]dCTP and primer sets specific for APLIP1 and rp49 as follows: APLIP1, 5′-ATAGCCGCTAATCTTGTAG-3′ and 5′-GTGTGTCGCGCAGACAAA-3′; rp49, 5′-AGTCCGATGGATAGCTGTAAG-3′ and 5′-AGTAAAGCCGGTCTGCTGATG-3′. The resulting products were electrophoresed on 5% (w/v) acrylamide gels and quantified with a Fuji BAS image analyzer.

**In Situ Hybridization**—A digoxigenin-labeled RNA probe was prepared with T7 RNA polymerase (Roche Diagnostics), digoxigenin-11-UTP, and APLIP1 cDNA in plasmid vector SKII digested with EcoRI as a template for the antisense probe. Embryos were dechorionated and fixed as described previously (27). Hybridization was performed with DABN20071 and with APLIP1 antisense probes visualized using an anti-digoxigenin antibody conjugated to alkaline phosphatase and an nitro blue tetrazolium/5-bromo-4-chlor-3-indolyl-phosphate substrate (Roche Diagnostics). The probe bound to the GST fusion protein was eluted with SDS sample buffer and analyzed by Western blot with ECL (Amersham Biosciences) or 125I-protein A (Amersham Biosciences).
RESULTS

Isolation of APLIP1—APPs are thought to function through interaction of their cytoplasmic domains with cytoplasmic proteins. Therefore, we screened a Drosophila adult cDNA library with the yeast two-hybrid system using APPLcyt as bait and obtained clones encoding part of a novel protein. The full-length cDNA was isolated from an embryonic cDNA library by plaque hybridization using partial cDNA as a probe. The full-length cDNA obtained encoded a protein consisting of 490 amino acids and containing putative SH3 and PI domains in its carboxyl-terminal half (Fig. 1, A and B). We called this novel protein APLIP1, for APP-like protein interacting protein 1.

From an analysis of the Drosophila genomic data base (32), the APLIP1 gene was revealed to be composed of five exons and to be located on region 61F1–61F4 of chromosome 3L. No other gene resembling APLIP1 was found in the data base. In this two-hybrid screening, a cDNA encoding the carboxyl-terminal fragment of the Drosophila homolog of X11L (dX11L) (GenBank AF208839)2 and a putative Ser/Thr protein kinase, Bin4 (GenBank AF096886), were also selected as positive clones. The binding activities of these clones with APPL examined in yeast are comparable with that of APLIP1.

Expression of APLIP1—APPs is expressed specifically in neural tissues during development (4, 7). If APLIP1 interacts with the cytoplasmic domain of APPL in vivo, we would expect APLIP1 to be expressed in neural tissues as well. Therefore, we examined the expression profile of APLIP1. Northern blot analysis was performed against poly(A)\(^+\)/H11001 RNA prepared from various developmental stages (Fig. 2A). A 2.4-kb transcript showed strong expression during late embryonic (12–18 h after egg deposition (E12–18)) to adult stages. No signal could be detected in the lane containing RNA from an E0–6 embryo, and only a weak signal was observed in E6–12 embryos. This expression profile is similar to that of APPL, although expression of APPL is weak in larval stages (4). Next we examined whether the expression of APLIP1 is enriched in the nervous system. We prepared poly(A)\(^+\)/H11001 RNA from the heads and bodies of adult flies and quantified the amount of transcript of the APLIP1 and rp49 by RT-PCR using specific primer sets (Fig.

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3 H. Taru, K. Iijima, M. Hase, Y. Kirino, Y. Yagi, and T. Suzuki, unpublished observation.
FIG. 2. Expression of Drosophila APLIP1. A. Northern blot analysis of APLIP1. Membranes containing 1 μg of poly(A)+ RNA were hybridized with APLIP1 (upper panel) or rp49 (lower panel) probes. E0–6, E6–12, E12–18, and E18–24 indicate embryos 0–6, 6–12, 12–18, and 18–24 h after egg deposition, respectively. L3, third instar larva; PP, pre-pupa; P, pupa; A, adult flies. A 2.4-kb transcript is the most prominent on the autoradiogram (upper panel). Numbers indicate molecular sizes of the RNA standard (kb). B, semiquantitative RT-PCR analysis of APLIP1. Poly(A)+ RNA from the heads and bodies of adult flies was analyzed by RT-PCR with specific primer sets for APLIP1 (upper panel) and rp49 (lower panel). The resulting PCR products were detected and quantified with a Fuji BAS 2000 imaging analyzer. C, expression of APLIP1 in an embryo. Whole mount in situ hybridization of embryos was carried out with antisense probes against the APLIP1 gene. The arrow designates the brain region, and arrowheads point to the central nervous system.

A larger amount of the APLIP1 PCR product was obtained from the heads than from the bodies, whereas comparable amounts of the PCR product derived from the rp49 RNA were observed in the heads and bodies. This result suggests that APLIP1 is expressed largely in neural tissues, as is APPL. The abundant expression of the APLIP1 in the neural tissues was confirmed by in situ hybridization (Fig. 2C). APLIP1 expression was detected in the brain and central nervous system of late embryos (Fig. 2C), whereas no signal was observed in early embryos (data not shown). These observations suggest the possibility that APLIP1 interacts with APPL in neural tissues.

Interaction between APLIP1 and APPL—The APLIP1 protein interacted with APPL in yeast cells. To investigate whether they interact in other systems, we examined protein-protein interactions between APLIP1 and APPL in vitro using a GST fusion protein. A GST-APLIP1 fusion protein was prepared in bacteria, purified, immobilized on glutathione-Sepharose, and incubated with a lysate of COS-7 cells expressing APPL. APPL expressed in COS-7 cells exhibits several bands probably differing in glycosylation. APPL attached to the beads was washed, eluted together with GST-APLIP1, and detected by Western blot analysis using an anti-APPL antibody (Fig. 3A). APPL bound to the beads coupled with the GST-APLIP1 fusion protein but not to GST alone.

We examined the interaction of APLIP1 with APPL in cells (Fig. 3B). APPL tagged with FLAG epitope (FLAG-APLIP1) was expressed in COS-7 cells together with or without APPL (left panel of Fig. 3B). The proteins were immunoprecipitated from the cell lysate with an anti-FLAG antibody and analyzed by immunoblot with anti-APPL and anti-FLAG antibodies. We observed that APPL was coimmunoprecipitated from the cell lysate with the FLAG-APLIP1 (middle panel of Fig. 3B). Conversely, when we immunoprecipitated APPL from the cell lysates with anti-APPL antibody, FLAG-APLIP1 was recovered with APPL (right panel of Fig. 3B). These results indicate that APLIP1 binds to APPL both in vitro and in the cell.

Identifying the Binding Domains of APLIP1 and APPL—The region required for the interaction between APLIP1 and APPL was determined. The ability of various truncated protein constructs derived from APLIP1 to bind to the APPLct was examined using the yeast two-hybrid system. Growth in a selective medium and β-galactosidase activity were analyzed (Fig. 4A). Proteins that were truncated but still contained the putative SH3 and PI domains in the carboxyl-terminal half (N384, NΔ207, and N2280) were able to bind APPLct. A transmembrane harboring a protein lacking the SH3 region (NΔ330) showed no significant difference in β-galactosidase activity compared with the control (mock) but still could grow in the selective medium. However, N3387, which lacked the amino-terminal side of the PI domain, and CΔ137, which lacks the carboxy-terminal region including the PI domain, exhibited neither β-galactosidase activity nor growth in the selective medium. This result suggests that the carboxyl-terminal region containing the PI domain of APLIP1 is essential for binding to APPLct and that the SH3 domain may be involved in the preservation of the structure of the PI domain necessary for binding to APPL.

Conversely, we examined the region of the APPLct required for the interaction with APLIP1 (Fig. 4B). Truncated proteins lacking the amino-terminal side of APPLct (ΔN12 and ΔN21) interacted with APLIP1 as well as APPLct did. However, deletion of the carboxy-terminal half of APPLct (ΔC25) resulted in the complete loss of both the β-galactosidase activity and the growth in the selective medium. We further showed that an APPLct construct with an internal deletion of 10 amino acids (Δ873–882) around the GYENPTY motif lost com-
Interaction of APPs with JIPs

**Fig. 4. Identification of regions that are responsible for the interaction between APLIP1 and the cytoplasmic domain of APP.** A, protein constructs derived from APLIP1 (indicated in the schematic structure) were expressed in yeast, and their ability to bind to APPLcyt was examined. APLIP1, full-length APLIP1; NΔ34, amino acids 85–490; NΔ207, 208–490; NΔ280, 281–490; NΔ330, 331–490; NΔ387, 388–490; CΔ137, 1–353. The ability to grow in the selective medium was observed (indicated as + or − under Growth), β-Galactosidase activity (β-gal) was quantified by liquid assay and is indicated in Miller units ± S.D. (n = 4). * indicates p < 0.01; **, p < 0.005; ***,***, p < 0.001 compared with the value of the mock treated study with plasmid alone. B, protein constructs derived from APPLcyt, indicated in the schematic structure (left), were expressed in yeast, and their ability to bind to APLIP1 was examined. APPLcyt, amino acids 834–886; ΔN12, 846–886; ΔN21, 855–886; ΔC25, 834–861; Δ873–882, the internal deletion of amino acids 873–882 of APPLcyt. The ability to grow in the selective medium was observed as above. β-Galactosidase activity was quantified by liquid assay and is indicated in Miller units ± S.D. (n = 3). * indicates p < 0.01; **, p < 0.005; ***, p < 0.001 compared with the value of the mock treated study with plasmid alone. C, binding of APLIP1 protein constructs to the APPLcyt in vitro. A whole cell lysate of COS-7 cells expressing the protein constructs derived from APLIP1 tagged with c-Myc (crude) was incubated with glutathione beads bearing the GST-APPLcyt fusion protein (APPLcyt), GST-APPLcyt deleted in 873–882 (Δ873–882), or GST protein alone (GST). The protein constructs attached to beads were detected by Western blot analysis with an anti-c-Myc monoclonal antibody. APLIP1-myc, full-length APLIP1 tagged with Myc; NΔ207-myc, amino acids 208–490 of APLIP1 tagged with Myc; and CΔ137-myc, amino acids 1–353 of APLIP1 tagged with Myc. Numbers indicate the molecular masses (kDa) of the protein standards.

**Figure 4.** Identification of regions that are responsible for the interaction between APLIP1 and the cytoplasmic domain of APP. The binding of the Myc-tagged CΔ137 protein to the GST-APPLcyt fusion protein. Furthermore, we found that APLIP1 did not interact with the GST-APPLΔ873–882 fusion protein. The in vitro protein binding assays agreed with the results obtained from the yeast two-hybrid assay, which confirmed that the carboxyl-terminal region containing the PI domain of APLIP1 interacts with a sequence containing the GYENPTY motif in APPLcyt.

**Identification of Mammalian Counterparts of APLIP1—**The region around the GYENPTY motif of APP, which was required for the interaction with APLIP1, is almost completely conserved in mammalian APP. Because we observed that human APP interacts with APLIP1 in a yeast two-hybrid assay (data not shown), we suspected that genes corresponding to APLIP1 are conserved in mammal, although no homologous gene could be found in the data base at the time we cloned the APLIP1 gene. Therefore, we screened a mouse brain cDNA library by plaque hybridization using a fragment of APLIP1 as a probe. This screen resulted in the isolation of two cDNAs homologous to APLIP1. A BLAST search revealed that one is identical to JIP1b, a variant protein of the scaffold protein JIP1 (22, 23), which was also reported as islet brain-1 (34). The other is mouse JIP2, which is almost identical to the recently cloned human JIP2 (24). JIP1b and JIP2 are expressed abundantly in brain (data not shown) (22, 24). When the primary structures of APLIP1, JIP1b, and JIP2 were aligned (Fig. 1, A and B), the mammalian JIP1b and JIP2 were found to resemble APLIP1, especially in the carboxyl-terminal structure containing the SH3 and PI domains. This structural resemblance of JIP1b and JIP2 to APLIP1 is expected to correlate with functional similarities.

**Characterization of APLIP1—**The structural similarity between APLIP1 and the JIPs suggests that they have a conserved function. Mammalian JIP1 and JIP2 have already been reported to bind several proteins to act as scaffold proteins in the JNK signaling pathway and/or as cargos for intracellular protein transport (23–25). JIP1 and JIP2 selectively bind components of the JNK signaling cascade such as JNK (MAP kinase), MKK7 (MAP kinase kinase), and mixed lineage kinase family proteins (MAP kinase kinase kinase) (23, 24). The JNK signaling pathway is basically conserved in Drosophila. The Drosophila JNK pathway consists of Drosophila JNK/basket (DJNK) and JNK kinase Hop, which correspond to JNK and MKK7 in mammals, respectively (for review, see Ref. 35). Thus, we examined whether APLIP1 could bind these protein kinases in the Drosophila JNK pathway as well as JIP1 and JIP2 can bind to the homologous proteins in mammals. First we investigated whether APLIP1 could bind DJNK. A GST-DJNK fusion protein was prepared, immobilized on glutathione beads, and incubated with a lysate of COS-7 cells expressing FLAG-tagged APLIP1, Drosophila Hop, or mouse JIP1b protein (Fig. 5A). Both Drosophila Hop, which is known to interact with DJNK (36), and mouse JIP1b bound to the GST-DJNK fusion protein, but binding of APLIP1 to GST-DJNK could not be detected in the same experiment. We also could not detect an interaction between APLIP1 and DJNK by communoprecipitation analysis (data not shown). Mammalian JIPs share the JNK binding domain consensus sequence in the amino-terminal region (37), which also exists in other JNK-binding proteins such as e-Jun and is responsible for the binding with JNK (22). However, consistent with the results of the in vitro binding assay (Fig. 5A), we did not observe the sequence corresponding to this consensus in APLIP1 (Fig. 1B). We conclude that APLIP1 does not bind to DJNK as mammalian JIPs bind to JNK. On the other hand, we found that Hep bound to the GST-APLIP1 fusion protein but
not to GST alone in vitro (upper panel of Fig. 5B). The interaction of APLIP1 with Hep was also observed in cells. COS-7 cells expressing HA-tagged APLIP1 and FLAG-tagged Hep were lysed and subjected to immunoprecipitation with an anti-FLAG antibody. The immunoprecipitates were analyzed by Western blot with anti-HA and anti-FLAG monoclonal antibodies. The expression of proteins is indicated as \( + \), and the asterisks (*) indicate the heavy chain of IgG. Arrows indicate proteins, and numbers indicate the molecular masses (kDa) of the protein standards.

Finally, the interaction of APLIP1 with the DKLC was examined. JIP1 and JIP2 were recently reported to bind the tetratricopeptide repeat of the kinesin light chain, a component of the motor protein kinesin, at its carboxyl terminus (Fig. 1B) (25). APLIP1 and a fragment of DKLC containing a tetratricopeptide repeat were expressed in COS-7 cells, and a coimmunoprecipitation assay was performed (Fig. 6). APLIP1 was coimmunoprecipitated with HA-tagged APLIP1 from the cell lysate with FLAG-tagged APLIP1. These results suggest that APLIP1 can form homo-oligomers as JIP1 and JIP2 do.

Thus, APLIP1 is not only similar to JIP1b and JIP2 structurally but also shares most of the features of their protein interactions, with the exception of binding to JNK.
proteins. These facts suggest that JIP1b and JIP2 may be able to bind APP. In fact, in our laboratory, JIP1b was isolated in a yeast two-hybrid screening of a human fetal cDNA library using the APPcyt as bait.3 To examine this possibility, we performed coimmunoprecipitation analysis using COS-7 cells expressing FLAG-tagged APP (APP) together with HA-tagged JIP1b (JIP1b), JIP1a (JIP1a), JIP2 (JIP2), or X11L (X11L), or plasmid alone (mock) were immunoprecipitated (IP) with anti-FLAG monoclonal antibody. The crude lysate (crude, middle panel) and the immunoprecipitates (top and bottom panels) were analyzed by immunoblot with an anti-HA (top and middle panels) or anti-FLAG (bottom panel) antibody. B, whole cell lysates of COS-7 cells expressing the HA-tagged JIP1b, JIP1a, JIP2, or X11L were incubated with glutathione beads bearing the APPcyt fused with GST (APPcyt) or GST protein alone (GST). The proteins in the crude lysate and those attached to beads were detected by immunoblot analysis with anti-HA monoclonal antibody.

3, also have the ability to phosphorylate APP at Thr-668 in vitro and in cultured cells (39). Therefore, we assumed that JIP1 and JIP2 could affect the phosphorylation of APP induced by the activation of JNK cascades. In cultured cells, phosphorylation of APP was induced by the overexpression of DLK, one of the upstream kinases of JNK (40), after the activation of JNK cascade (Fig. 8). APP was expressed together with or without DLK, JIP1b, JIP1a, and JIP2 in Neuro-2a cells, and the phosphorylation of APP at Thr-668 was detected and quantified by immunoblot with the APP phosphorylation state-specific antibody. This antibody selectively recognizes APP phosphorylated at Thr-668. The phosphorylated APP was detected in the cells expressing APP together with DLK but not in the cells expressing APP alone or DLK alone. In the cells expressing JIP1b the phosphorylation level of APP increased slightly when compared with it in the cell not expressing JIPs. Conversely, expression of JIP1a, a splicing variant of JIP1 lacking part of the PI domain, resulted in a decreased level of the phosphorylation. Moreover, expression of JIP2 also induced the decrease of APP phosphorylation. It seems interesting that the effects of JIP1b, JIP1a, and JIP2 on APP phosphorylation were different, although these proteins have been reported to play a similar role in JNK signaling (24). Because JIP1b can bind both APP and JNK, it is probable that JIP1b recruits JNK to the APPcyt and contributes to the phosphorylation of APP at Thr-668. Conversely, JIP1a and JIP2 have the ability to bind JNK as well as JIP1b, but they lack the ability to associate with APPcyt efficiently. Most likely, JIP1a and JIP2 isolated JNK from APPcyt.

Phosphorylation of APP at Thr-668 causes a conformational

**Fig. 7. Interaction of APP with JIPs and X11L.** A, coimmunoprecipitation of APP with JIP1b, JIP1a, JIP2, and X11L. The lysate of COS-7 cells transiently expressing FLAG-tagged APP (APP) together with HA-tagged JIP1b (JIP1b), JIP1a (JIP1a), JIP2 (JIP2), or X11L (X11L), or plasmid alone (mock) were immunoprecipitated (IP) with anti-FLAG monoclonal antibody. The crude lysate (crude, middle panel) and the immunoprecipitates (top and bottom panels) were analyzed by immunoblot with an anti-HA (top and middle panels) or anti-FLAG (bottom panel) antibody. B, whole cell lysates of COS-7 cells expressing the HA-tagged JIP1b, JIP1a, JIP2, or X11L were incubated with glutathione beads bearing the APPcyt fused with GST (APPcyt) or GST protein alone (GST). The proteins in the crude lysate and those attached to beads were detected by immunoblot analysis with anti-HA monoclonal antibody.

**Fig. 8. Effect of JIP1 and JIP2 on APP phosphorylation.** FLAG-tagged JIP1b, JIP2, or JIP1a was transiently expressed together with APP and DLK in Neuro-2a cells (expression is indicated as +), and the lysates of these cells were analyzed by immunoblot with anti-phospho-APP antibody UT-33 (top panel, P-APP), and anti-APP antibody G369 (middle panel, APP). The amount of phosphorylated APP was quantified, standardized to the amount of total APP, and is represented as relative ratio to the value (1.0) of the cells not expressing JIPs (bottom panel, graph). Bars indicate the means ± S.D. (n = 4). * p < 0.05, *** p < 0.005.
change of APPcyt and affects the interaction with the binding proteins such as Fe65 (31). Thus, the phosphorylation of APP at Thr-668 is suggested to be important for the metabolism and/or putative function(s) of APP. It is possible that JIPs are involved in APP metabolism and/or function(s) by regulating APP phosphorylation.

**DISCUSSION**

APPs possess a membrane-associated receptor-like structure, and the amino acid sequence of their short cytoplasmic region is highly conserved among a wide variety of species (for review, see Refs. 1 and 2). Protein interactions between the cytoplasmic domains of APPs and cytoplasmic proteins are thought to be important for regulating the metabolism of APPs and/or for putative physiological function of APP (6, 10–15). Here, we isolated a novel gene in *Drosophila* named APLIP1 and indicated that APLIP1 and its putative mammalian homologs JIP1b and JIP2 could interact with the cytoplasmic domain of the APPs. These proteins, APLIP1, JIP1b, and JIP2, resemble each other in their structure, especially in their carboxyl-terminal regions that contain SH3 and PI domains. We revealed that they also share properties such as interactions with APPs, MAP kinase kinase, and kinesin; an abundant expression in the nervous system; and the formation of oligomers. These similarities suggest that APLIP1, JIP1b, and JIP2 belong to same protein family functionally conserved in various species. In mammals, JIP3/JSAP was reported as another member of the JIP family of proteins (37, 41), which displayed scaffold function in the JNK signaling pathway as JIP1 and JIP2 did. In *Drosophila*, a putative homolog of JIP3, designed Sunday Driver protein (SYD), has been reported (42). However, they differ from APLIP1, JIP1b, and JIP2 in their domain structure; they do not possess the SH3 and PI domains that are important regions for binding several proteins including APPs (43–45), and they may have some different roles from APLIP1, JIP1, and JIP2.

In mammalian several proteins bind the APPcyt (16–21), whereas the physiological role(s) of these interaction have not been sufficiently revealed. Here we showed that JIP1b and JIP2, mammalian counterparts of APLIP1, could interact with APPcyt. Regarding JIP1b, the binding to APPcyt was also reported recently to be relatively lower than the binding of other APP-binding proteins, such as dDab1, X11, and Fe65 (46). We also observed that the binding of JIP1b was slightly lower than that of the other binding protein X11L *in vitro* but not in the cell. However, the faint differences in the binding activities do not necessarily deny the physiological importance of JIP1b for APP. In fact, we found a novel function of JIP1 and JIP2 to be the modulation of the phosphorylation of APP at Thr-668 residue induced by the activation of JNK. Expression of JIP1b slightly enhanced the phosphorylation of APP, whereas the expression of JIP2 or JIP1a suppressed the phosphorylation. From the previous reports that JIP1b, JIP1a, or JIP2 could equally facilitate the activation of JNK signaling (23, 24), it was expected that these proteins similarly regulate the phosphorylation of APP when JNK was activated. Nevertheless, only JIP1b facilitated the phosphorylation, and others decreased the level of the phosphorylation of APP. We indicated that the interaction of JIP2 and JIP1a with APP was remarkably weaker than that of JIP1b in the cell. Therefore, it is conceivable that the effect of JIP1a or JIP2 to decrease the level of the phosphorylation of APP reflects their weaker binding properties to APP rather than their properties of regulating the JNK signaling cascade. It is assumed that formation of the complex between JIP1a or JIP2 and JNK may suppress JNK to approach the phosphorylation site of APP, whereas the complex of JIP1b with JNK can easily approach APPcyt. Indeed a recent report showed that formation of the tripartite complex composed of JIP1b, JNK, and APP could be observed in cultured cells (46). Phosphorylation of APP at Thr-668 has been implicated in the metabolism and/or putative function(s) of APP (12, 31), and modulation of the phosphorylation level of APP by JIPs in mammal possibly has physiological importance.

In invertebrates only this APLIP1 and dX11L have been reported to interact with APPs (for dX11L, GenBank AF208839) except kinesin interacts genetically with APPL (47, 48). They may be implicated in evolutionarily conserved roles relative to metabolism and/or function of APPs, besides the role of mammalian JIP in the phosphorylation of APP.
Interaction of APPs with JIPs

**Drosophila**, APPL does not have a phosphorylation site corresponding to the Thr-668 residue of mammalian APP695. In addition, there are some differences in the function of APLIP1 on the JNK signaling pathway from that of mammalian JIP1 and JIP2 because APLIP1 could not interact with DJNK, whereas it could interact with Drosophila JNK kinase Hef (Fig. 5). Thus APLIP1 cannot form a complex with DJNK and facilitate JNK activation in *Drosophila* in the same manner as JIPs do in mammals, whereas a possibility of regulating JNK signaling through an interaction with Hef still remains. Therefore, the effect modulating the phosphorylation of APP by JIP1 and JIP2 may be acquired in the evolutionary process. Questions of what the evolutionarily conserved role(s) of the interaction of APLIP1, JIP1b, and JIP2 with APPs are remain to be elucidated. Several physiological roles for the mammalian JIP family proteins have been proposed other than acting as scaffold molecules of JNK cascades (23, 24): as a transactivator of the GLUT2 gene (34) and as cargo for kinesin to mediate the transportation of several transmembrane proteins (25). In *Drosophila* APLIP1 interacts with the kinesin light chain as well as mammalian JIP1 and JIP2 do, but interaction with the molecules of the JNK cascades is only partly conserved. The metabolic scheme of APPs is basically conserved between *Drosophila* and mammal. Kinesin is involved in intracellular transport and metabolism of APP in mammal and is associated with APPL in *Drosophila* (47, 48). Accordingly, we assume that *Drosophila* APLIP1 and mammalian JIP share a role in the intracellular metabolism of APPs.

In conclusion, we identified a novel protein in *Drosophila* named APLIP1 and mammalian JIP1b and JIP2 as binding proteins of APPs. APLIP1, JIP1b, and JIP2 comprise an evolutionarily conserved protein family and share properties in their domain structure, expression pattern, and interaction profiles with proteins such as APPs, kinesin, and JNK kinase, although a few exceptions are observed (Fig. 9). We propose that a novel function of mammalian JIP1 and JIP2 is to modulate the phosphorylation of APP. Further analysis of conserved or different roles of APLIP1, JIP1b, and JIP2 may contribute to our understanding of the mechanisms of APPs metabolism and the pathogenesis of Alzheimer’s disease.

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Interaction of Alzheimer's β-Amyloid Precursor Family Proteins with Scaffold Proteins of the JNK Signaling Cascade
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