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Jun NH_2-terminal Kinase (JNK) Interacting Protein 1 (JIP1) Binds the Cytoplasmic Domain of the Alzheimer's β-Amyloid Precursor Protein (APP)*

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The familial Alzheimer's disease gene product amyloid β precursor protein (APP) is sequentially processed by β- and γ-secretases to generate the Aβ peptide. The biochemical pathway leading to Aβ formation has been extensively studied since extracellular aggregates of Aβ peptides are considered the culprit of Alzheimer's disease. Aside from its pathological relevance, the biological role of APP processing is unknown. Cleavage of APP by γ-secretase releases, together with Aβ, a COOH-terminal APP intracellular domain, termed AID. This peptide has recently been identified in brain tissue of normal control and patients with sporadic Alzheimer's disease. We have previously shown that AID acts as a positive regulator of apoptosis. Nevertheless, the molecular mechanism by which AID regulates this process remains unknown. Hoping to gain clues about the function of APP, we used the yeast two-hybrid system to identify interaction between the AID region of APP and JNK-interacting protein-1 (JIP1). This molecular interaction is confirmed *in vitro, in vivo* by fluorescence resonance energy transfer (FRET), and in mouse brain lysates. These data provide a link between APP and its processing by γ-secretase, and stress kinase signaling pathways. These pathways are known regulators of apoptosis and may be involved in the pathogenesis of Alzheimer's disease.

The amyloid β (Aβ)[1] peptide is the principal component of amyloid plaques in the brain of Alzheimer's disease (AD) patients (1–3). Aβ is derived from APP by two sequential proteolytic events, one in the extracellular domain (β-secretase cleavage) (4) and one in the transmembrane domain (γ-secretase cleavage) (5). APP processing has become firmly associated with the pathogenesis of AD with the identification of missense mutations in three genes associated with familial forms of AD (FAD). The FAD mutations identified to date are found in APP itself, and in two highly homologous genes now known as presenilin 1 and presenilin 2 (PS1, PS2) (6–9). Presenilins are a key component of a multimolecular complex with γ-secretase activity that contains at least one other recently identified protein named nicastrin (10–16). A common feature of all FAD mutations is that they increase the generation of Aβ peptides (especially the Aβ42 form, considered to be more pathogenic than the Aβ40 peptide) by accelerating the rate of APP processing by either β- or γ-secretase (5, 18–20). In addition to the Aβ peptide which is mostly released from the cell, another peptide, AID, is released into the cytoplasm as a result of the γ-secretase cleavage. Although the role of the Aβ peptide in the pathogenesis of AD has been extensively studied, only recently have there been reports as to the role of AID. AID-like peptides have recently been identified in human brains from normal controls and cases of sporadic AD (21). AID has also been implicated in the pathology of AD by data indicating that it can independently trigger apoptosis or enhance other apoptotic stimuli (21). This may represent the mechanism by which APP and the other FAD proteins PS1 and PS2 enhances neuronal apoptosis (21–27). Most recently it has been shown that APP may bind Fe65 and Tip60, and that following γ-secretase cleavage, AID along with Fe65 and Tip60 can travel to the nucleus to act as a transcription factor (28). To identify other proteins which are relevant to the function of APP, and that may mediate the functions of AID, we employed the yeast two-hybrid selection system. Here we describe JIP1 (also known as IB1) as a new binding partner of the APP intracellular domain.

MATERIALS AND METHODS

Yeast Two-hybrid System—The two-hybrid screening was conducted using the Matchmaker system from CLONTECH according to the manufacturers instruction. Yeast strain Y190 was transformed with the corresponding bait plasmids by the lithium acetate/polyethylene glycol 4000 procedure and selected on synthetic drop-out plates lacking tryptophan. Selected colonies were analyzed for expression of the GAL4BD-bait fusion protein by immunoblot analysis. For library screening, Yeast190 expressing GAL4BD-APP fusion proteins were sequentially transformed with a human fetal brain cDNA library cloned in the pACT2 vector (CLONTECH). 2×10^6 clones were analyzed. Transformed yeast were selected in synthetic drop-out plates lacking tryptophan, leucine, and histidine in the presence of 50 mM 3-aminotriazol (Sigma) and grown for 5 days at 30 °C. Colonies positive for growth on selective media were blotted on filter paper (Whatman No. 5), perme-

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[1] The abbreviations used are: Aβ, amyloid β; AID, APP intracellular domain; APP, β-amyloid precursor protein; JIP-1, JNK interacting protein-1; JNK, Jun NH_2-terminal kinase; PTB, phosphotyrosine-binding domain; FRET, fluorescence resonance energy transfer; AD, Alzheimer's disease; BD, binding domain; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.

abilized in liquid nitrogen, and placed on another filter soaked in Z buffer (60 mM Na_2HPO_4, 40 mM $Na_2H_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 37.5 β-mercaptoethanol) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactosidase. Colonies were then scored as positive when a bright color developed in 2–5 h. Assays were done for 5–10 independent transformants.

cDNA Cloning and Constructs—Two GAL4BD-APP baits were constructed using the pAS2 vector (CLONTECH). Construct pAS2-AT and pAS2-LK consisted of the COOH-terminal 58 amino acids or 48 amino acids of APP fused to the DNA-binding domain (BD) of GAL4, respectively. APP, APPNcas, APPCcas, and AID were made as described previously (21). GST fusion proteins were made in pGEX vectors (Amersham Biosciences, Inc.). Mutations were introduced by using the transformer site-directed mutagenesis kit (CLONTECH).

From the cDNAs isolated from the yeast two-hybrid screening, hJIP1 clone AT27 was cloned into Flag-tagged pcDNA3.1 (Invitrogen), pECFP-N1 and pEYFP-N1 (CLONTECH) for expression in mammalian cells or *in vitro*. Human JIP1e was constructed with the same sequence for hJIP1 (GenBank™ accession number AAD20443) with the first 24 amino acids exchanged for an alternative stretch of 34 amino acids derived from EST IMAGE:2545752 (ATCC). This was cloned into Flag-tagged pcDNA3.1, pECFP-N1 and C1, and pEYFP-N1 and C1. Fragments of hJIP1 were cloned by PCR using Pwo polymerase (Roche Molecular Biochemicals) and the following oligonucleotides (Invitrogen): SH_3-forward: AAAAGAATTCTGTTCTCCTGCATCATC; SH_3-reverse: AAACTCGAGTTAGTCACTGTTTTTGGC; PTB-forward: AAAAGAATTCAGTTCCGGGTGAAGTTCCTG; PTB-reverse: AAACTCGAGTTACTCCACAAACTGCTTGTA. HJIP1-SH_3 containing residues 479–562 was cloned using primers SH_3 forward and SH_3-reverse, hJIP1 PTB containing residues 566–700 was cloned using primers PTB-forward and PTB-reverse and hJIP1-SH_3PTB containing residues 479–700 was cloned using primers SH_3 forward and PTB-reverse. Appropriate restriction enzymes were used to splice these fragments into Flag-tagged pcDNA3.1, pECFP-C1, and pEYFP-C1.

Mouse JIP1a in pCMV5 was obtained as a kind gift from Dr. Roger Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA) (29). Mouse JIP1b was constructed by cutting EST IMAGE:4506969 (Incyte Genomics) which codes for the 47-amino acid insertion with *Eco*O65I and *Eco*RV and splicing it into mJIP1a already cut with the same enzymes.

The sequences of all constructs were confirmed by the dideoxy termination method using an ABI Prism 377 DNA sequencer (PerkinElmer Life Sciences, Foster City, CA) by Genewiz Inc. (New York), or by the Albert Einstein College of Medicine sequencing core, using their protocols.

Cell Lines and Trasnsfections—Human embryonic kidney (HEK) 293T cells were grown in RPMI 1640 media (Invitrogen) supplemented with glutamine and with 10% heat-inactivated fetal calf serum (Biofluids, Rockville, MD). Transfections were performed in 6-well plates either using LipofectAMINE reagent (Invitrogen), 8 μl per 1 μg of DNA, or FuGENE 6 (Roche Molecular Biochemicals) with 3 μl per 1 μg of DNA.

Protein Expression and Purification and GST Pull-down—Recombinant GST fusion proteins were expressed in *Escherichia coli* strain BL21 (Invitrogen) to make nonphosphorylated proteins and strain TKB1 (Stratagene) to make tyrosine-phosphorylated proteins using the pGEX system (Amersham Biosciences, Inc.). Phosphorylation of fusion proteins was confirmed by Western blotting with the PY20 monoclonal anti-phoshotyrosine antibody (Transduction Labs). Proteins were purified using glutathione-Sepharose beads. [^{3}H]Leucine-labeled proteins were made using the TnT-coupled *in vitro* transcription/translation system (Promega). After synthesis of the radiolabeled protein for 1.5 h, aliquots of the protein were incubated with GST fusion proteins bound to glutathione-Sepharose beads for 2 h at room temperature. The beads were then washed three times with lysis buffer T (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 45 mM NaCl) and boiled with SDS loading buffer with dithiothreitol. The proteins were separated by SDS-PAGE electrophoresis and the gel were fixed with 50% methanol, 40% H_2O, 10% acetic acid. The gel was incubated in Amplify (Amersham Pharmacia Biotech) for 20 min and dried. Pulled down proteins were detected using autoradiography.

Immunoprecipitation and Immunoblot Analysis—Twenty-four to forty-eight h following transfection, cells were washed with ice-cold phosphate-buffered saline, and lysed in lysis buffer T containing a protease inhibitor tablet (Roche Molecular Biochemicals). Lysis was allowed to continue for 10 min on ice and was then spun down at full speed at 4 degrees for 10 min. Some lysate representing the total lysate was removed and boiled with SDS loading buffer with dithiothreitol, while the rest was immunoprecipitated for 2 h at room temperature with either monoclonal antibody directed against the Flag epitope already bound to agarose beads (Sigma) or with 2.5 μl of polyclonal LivingColors antibody (CLONTECH) with 15 μl of protein A/G-agarose beads (Pierce). The beads were washed five times with lysis buffer and boiled with SDS loading buffer with dithiothreitol. The proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Bio-Rad). Membranes were probed with the same antibodies as was used for immunoprecipitation and horseradish peroxidase-conjugated secondary antibodies were used. Proteins were detected using the Supersignal West Pico chemiluminescent system (Pierce).

FRET Analysis—HEK 293T cells were plated in 24-well plates and co-transfected with the CFP and YFP fusion proteins using FuGENE 6 (Roche Molecular Biochemicals). All transfections contained a total of 200 ng of DNA with the ratios between the two determined empirically to yield the best co-expression as follows. All transfections with AID or AID(Y682G) had a DNA ratio of AID to JIP1 of 1:1 while all transfections with APP or APP(Y682G) had a DNA ratio of APP to JIP1 of 4:1. Cells were harvested between 18 and 24 h after transfection in their conditioned media and strained through nylon mesh to separate the cells. A MoFlo Multi Laser Sorter (MLS), (Cytomation, Fort Collins, CO) was configured as follows. An argon laser tuned to 488 nm was used to excite YFP (but doesn't excite CFP) (30, 32),[2] and a krypton laser tuned to 413 nm was used to excite CFP. YFP emission was detected with a 530-nm/40-nm bandpass filter and CFP emission was detected with a 473-nm/12-nm bandpass filter. FRET was detected with a 550-nm/30-nm bandpass filter using the 413-nm excitation. Each cell was first illuminated with the 488-nm laser to detect the presence of the YFP fusion protein, and then with the 413-nm laser to detect both the presence of CFP via the 473-nm filter and FRET via the 550-nm filter. Appropriate neutral density filters were used to minimize the overlap between the emissions of the two fluorophores. Because of residual spectral overlap between the emission spectra of YFP and CFP[3] compensation was adjusted empirically before each FRET session such that no FRET was detected between noninteracting proteins such as free YFP and CFP. The threshold for a positive FRET cell was set using the standard cytometry method of comparing each sample to a negative control which in our case was the Y682G mutant of each APP or AID sample (see "Results" section). Additionally, if a sample did not have a co-transfection profile matching its control it was rejected from our analysis. Acquisition and analysis was done using the Summit version 3.0 software package (Cytomation). Data was displayed to allow for easy visualization of the number of cells that were co-transfected with the two fusion proteins, as well as the percentage of the cells that had the CFP and YFP in close enough proximity to exhibit FRET.

Generation of Antisera against Human and Mouse JIP1—Suitable peptide sequences with high antigenicity, hydrophilicity, flexibility, surface probability, and turns in their secondary structure were chosen. The BLAST program (NCBI/National Institutes of Health) was used to compare the peptide sequence against known sequences in the major data bases (GenBank™ etc.) to void similarity and cross-reactivity with other related and unrelated proteins. Cysteine was added to either end of the peptide (if necessary) and was conjugated to the carrier protein keyhole limpet hemocyanin by Sulfo-SMCC (Pierce-Endogen). Rabbits were immunized, boosted, and bled according to standard protocols. An affinity column was made by conjugating the same peptide used for immunization to aminohexane gel using Sulfo-SMCC. To purify antibodies, antiserum was incubated with the gel for 1–2 h after extensive washing of the column with phosphate-buffered saline. Purified antibodies are first eluted with three 10-ml fractions of KSCN, then with three 10-ml fractions of glycine. The antibodies were finally dialyzed, concentrated and centrifuged at 4,000 rpm to remove antibody aggregates.

Mice and Brain Lysates—Adult BALB/c mice (age 3 months) were euthanized, brains were removed and homogenized in lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.5. One mg of protein was used for immunoprecipitations with αAPP (C7 (34) or 1736 (35), both kindly provided by Dr. D. J. Selkoe (Center for Neurologic Diseases, Harvard Medical School, Brigham and Women's Hospital)) JIP1-NT (Zymed) or rabbit anti-mouse IgG antibodies (ICN, Aurora, OH). Immunoprecipitations were performed overnight at 4 °C, followed by incubation of immunoprecipitates with protein A-agarose beads,

[2] Siegel, R. M., Chan F. K., Zacharias, D. A., Swofford, R., Holmes, K., Tsien, R. Y., and Lenardo, M. J. (2000) *Science's STKE* www.stke.org/cgi/content/full/OC_sigtrans; 2000/38/pl1.

[3] Clontech web site, www.clontech.com/gfp/excitation.shtml.

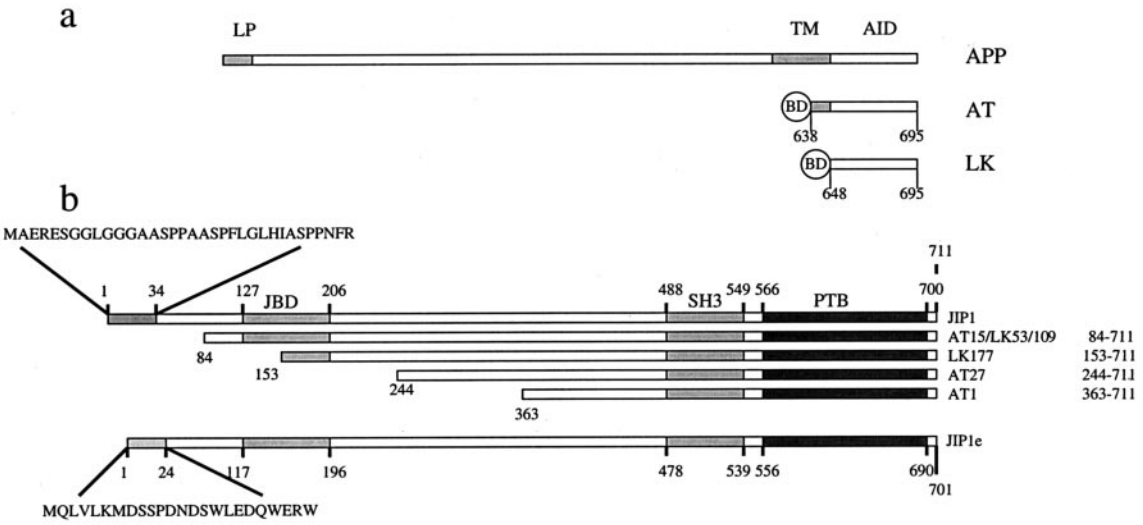

FIG. 1. **Schematic representation of the baits used and the JIP1 clones identified in the yeast two-hybrid screen.** *a,* the two APP baits comprise overlapping regions of the carboxyl-terminal portion of APP. *AT* includes the intracellular APP fragment released upon cleavage of APP by the γ-secretase. *LK* consists of the cytoplasmic domain of APP. The leader peptide (*LP*), transmembrane region (*TM*), and intracellular domain (*AID*) of APP are indicated. *b,* the human JIP1 protein found in GenBank™ is reported. *Boxes* indicate the JNK-binding domain (*JBD*), SH3 domain and PTB domains. The six JIP1 clones recovered from the screening are illustrated. Clones AT1/15/27 were obtained by using AT as the bait. Clones LK53/109/177 were identified using LK as the bait. The *bottom* illustration shows the JIP1e construct used in our experiments.

washing, and immunoblots as described above. Characterization of the polyclonal JIP1 antibodies JIP-NT and JIP-SH3 (Zymed) is discussed under "Results" and the figure legends.

RESULTS

hJIP1 Interacts in Yeast with the Carboxyl-terminal of APP—To identify proteins that interact with AID we screened a fetal human brain cDNA library by means of the yeast two-hybrid system. In two screens, bait plasmids pAS2-AT and pAS2-LK expressing a protein consisting of the GAL4 DNA-binding domain fused to either the terminal 48 or 58 amino acids of APP, respectively, were used (Fig. 1*a*). This construct, which did not lead by itself to the activation of the two reporter genes *HIS3* and *LacZ*, was used to screen ~2×10^6 transformants. Screening for co-activation of both reporter genes resulted in the identification of known APP-binding proteins, such as Fe65 and X11. In addition, we identified 6 independent clones coding for carboxyl-terminal fragments of hJIP1 (Fig. 1*b*).

hJIP1 Interacts with APP in Vitro—To confirm the interaction between hJIP1 and APP, an *in vitro* approach was taken. An ^{3}H-labeled hJIP1 protein containing amino acids 244–711 (AT27, see Fig. 1*b*) was transcribed and translated *in vitro* and added to either a purified GST-AID or GST-APPCcas (Fig. 3*a*) fusion protein, or GST alone (all bound to glutathione-Sepharose beads). The protein-protein complex was allowed to form under appropriate buffer condition, resolved by gel electrophoresis, and visualized by autoradiography. Fig. 2 shows that the hJIP1 fragment AT27 directly binds to GST-AID and GST-APPCcas (but not to GST alone), whereas another unrelated protein, AIP1 (36), does not. The binding of hJIP1 to GST-APPCcas indicate that the point of interaction with APP was localized to the terminal 31 amino acids of APP.

Considering that other proteins which bind APP in its intracellular domain, such as mDab1, Fe65, and X11, do so via their PTB domains (37–42), we attempted to find if the PTB domain of hJIP1 was also responsible for its binding to APP. Additionally, considering that the above proteins bind APP via the Y^{682}ENPTY^{687} motif independent of phosphorylation, we attempted to discover whether this motif was involved in hJIP1s binding and whether phosphorylation of APP was necessary for hJIP1s binding. HEK 293T cells were transfected with con-

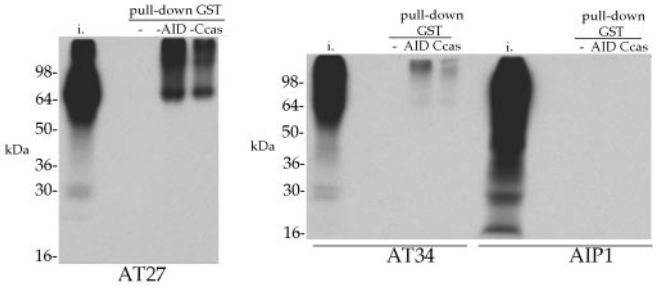

FIG. 2. **JIP1 interacts with APP directly.** *In vitro* transcribed and translated (*i.*) hJIP1 clone AT27, X11 clone AT34 (identified in the same two-hybrid screen) or AIP1 (control protein) were incubated with either GST (−), GST-AID (*AID*), or GST-Ccas (*Ccas*) fusion proteins. These GST fusion proteins are schematically represented in Fig. 3*a*.

structs expressing either JIP1e, JIP1 AT27, JIP1-SH3-PTB, JIP1-SH3, or JIP1-PTB with either Flag or YFP tags. Cell lysates were incubated with GST-AID, GST-AID expressed in bacteria which phosphorylates tyrosine, GST-AID Y682G, and GST alone under the appropriate buffer condition. The beads were washed and the total lysates and pull downs were resolved by gel electrophoresis. Western blot analysis (Fig. 3*b*) using either anti-Flag or anti-YFP antibodies revealed that only the hJIP1 proteins containing the PTB domain (*i.e.* JIP1e, JIP1 AT27, JIP1-SH3-PTB, and JIP1-PTB) were pulled down by AID while the fragment lacking the PTB domain (*i.e.* JIP1-SH3) was not. Furthermore, we found that APP binds JIP1 independent of tyrosine phosphorylation, but requires the presence of tyrosine 682.

To further determine the residues on APP necessary for hJIP1 binding, GST fusion proteins consisting of the final 31 amino acids of APP (APPCcas), APP residues 680–689, or APP residues 682–687 were expressed either in bacteria which phosphorylate or do not phosphorylate tyrosine. These fusion proteins were incubated with lysates from HEK 293T cells transfected with YFP-hJIP1e. Western blot analysis (Fig. 3*c*) using an anti-YFP antibody revealed that although the amount of hJIP1 pulled down by APPCcas appears not to be effected by phosphorylation, when the number of residues flanking the YENPTY motif is decreased to two on either side, there appears to be a phosphorylation dependence. Furthermore, when these

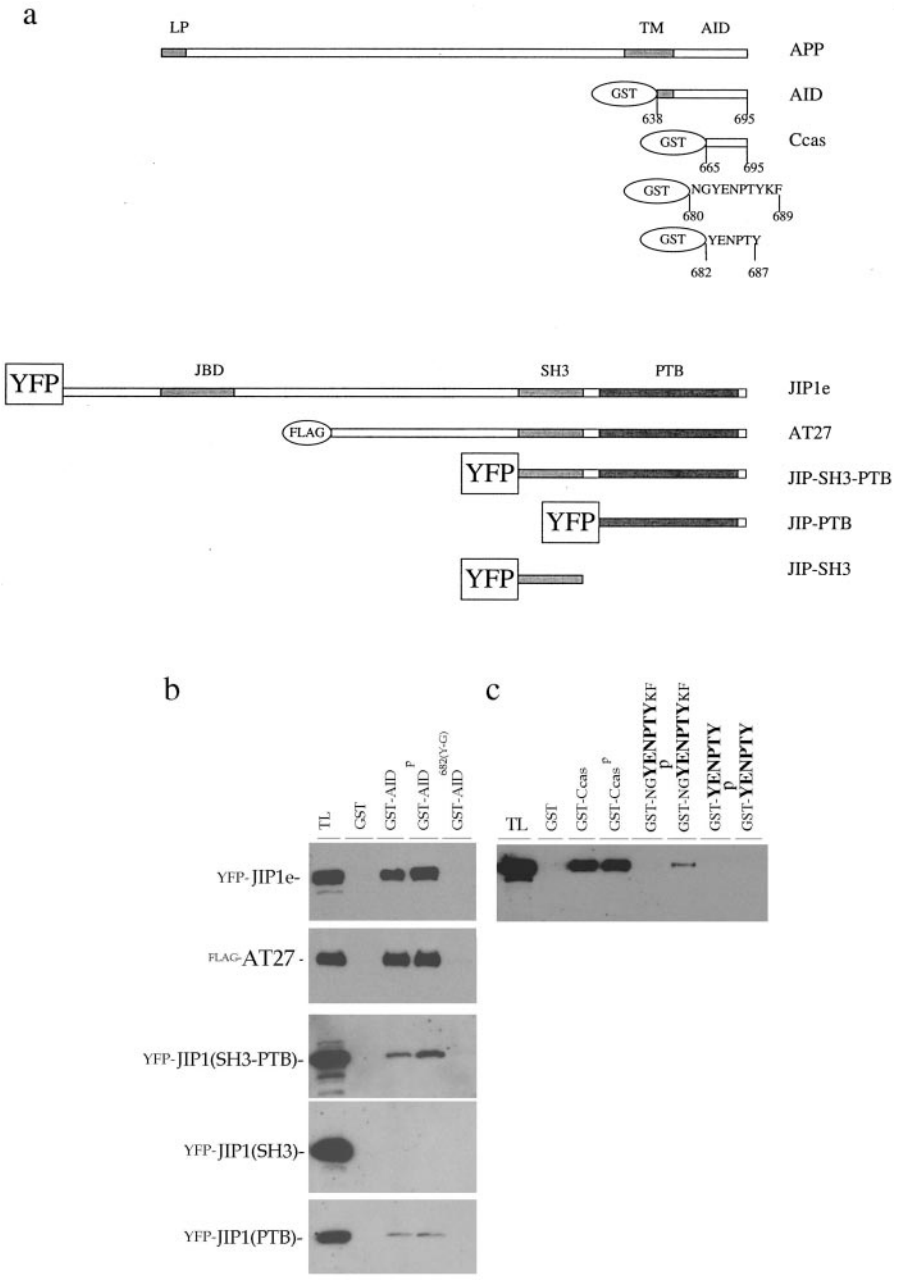

FIG. 3. **Interaction between JIP1 and APP is mediated by the PTB domain of JIP1 and the NGYENPTYKF region of APP (amino acids 680–689).** *a,* schematic representation of the constructs used. HEK293T cells were transfected with constructs coding for YFP fused to either full-length hJIP1 (*JIP1e*), SH3-PTB domains of JIP1 (*JIP1-SH3-PTB*), SH3 domain of JIP1 (*JIP1-SH3*), PTB domain of JIP1 (*JIP1-PTB*), or JIP1 clone AT27 fused to a Flag epitope (*AT27*). Protein lysates were incubated with the following recombinant proteins: *b,* GST, GST-AID, GST-AID^{P}, GST-AID Y682G; *c,* GST-Ccas, GST-Ccas^{P}, APP residues 680–689 (GST-NGYENPTYKF and GST-NGYENPTY^{P}KF), or APP residues 682–687 (GST-YENPTY and GST-YENPTY^{P}). After precipitation and gel separation, samples were transferred onto nitrocellulose membranes and probed with either an anti-YFP or anti-FLAG antibody.

flanking residues are missing, the amount of hJIP1 pulled down is below the limit of detection. This indicates that other residues in the cytoplasmic tail of APP as well as phosphorylation of tyrosine 682 play complementary roles in stabilizing the interaction between APP and hJIP1. Also, although we have demonstrated that phosphorylation of APP is not necessary for hJIP1 binding, it may be important in APP sorting (43) or in modulating which of the various proteins which bind the YENPTY motif will do so most strongly.

hJIP1 Interacts with APP in Vivo—To assess the interaction of hJIP1 and APP in mammalian cells, HEK 293T cells were co-transfected with constructs expressing either APP-GFP, APPNcas-GFP, AID-GFP, or APPCcas-GFP along with Flag-hJIP1 AT27 (Fig. 4*a*). Cell lysates were immunoprecipitated with a monoclonal antibody directed against the Flag epitope (Sigma) followed by immunoblot analysis with anti-GFP antibody. Fig. 4*b* shows that although Flag-hJIP1 AT27 immunoprecipitates APP, AID, and APPCcas, all of which contain the YENPTY motif, it does not immunoprecipitate APPNcas which lacks the YENPTY motif. Additionally, the reverse immunoprecipitation with a polyclonal antibody directed against the GFP epitope and Western blotting with anti-Flag antibody revealed that only APP, AID, and APPCcas can immunoprecipitate hJIP1 AT27 while APPNcas cannot.

mJIP1b But Not mJIP1a Interacts with APP in Vivo—In the mouse, two forms of mJIP1 have been identified, mJIP1b containing a complete PTB domain, and mJIP1a which lacks 47 amino acids at the beginning of its PTB domain (Fig. 4*a*) (29, 44). This gave us the opportunity to test *in vivo* if the lack of a complete PTB domain could abolish mJIP1s interaction with APP. HEK 293T cells were co-transfected with constructs expressing either APP-GFP or APPNcas-GFP along with Flag-mJIP1a or Flag-mJIP1b. Cell lysates were immunoprecipitated with a monoclonal antibody directed against the Flag epitope followed by Western blot analysis with GFP antibody. Fig. 4*c* shows that although Flag-mJIP1b, which contains a complete PTB domain, immunoprecipitates APP, mJIP1a does not. Correspondingly, APPNcas, which lacks the YENPTY motif, is not immunoprecipitated by either form of mJIP1. Furthermore, the reverse immunoprecipitation with a polyclonal antibody di-

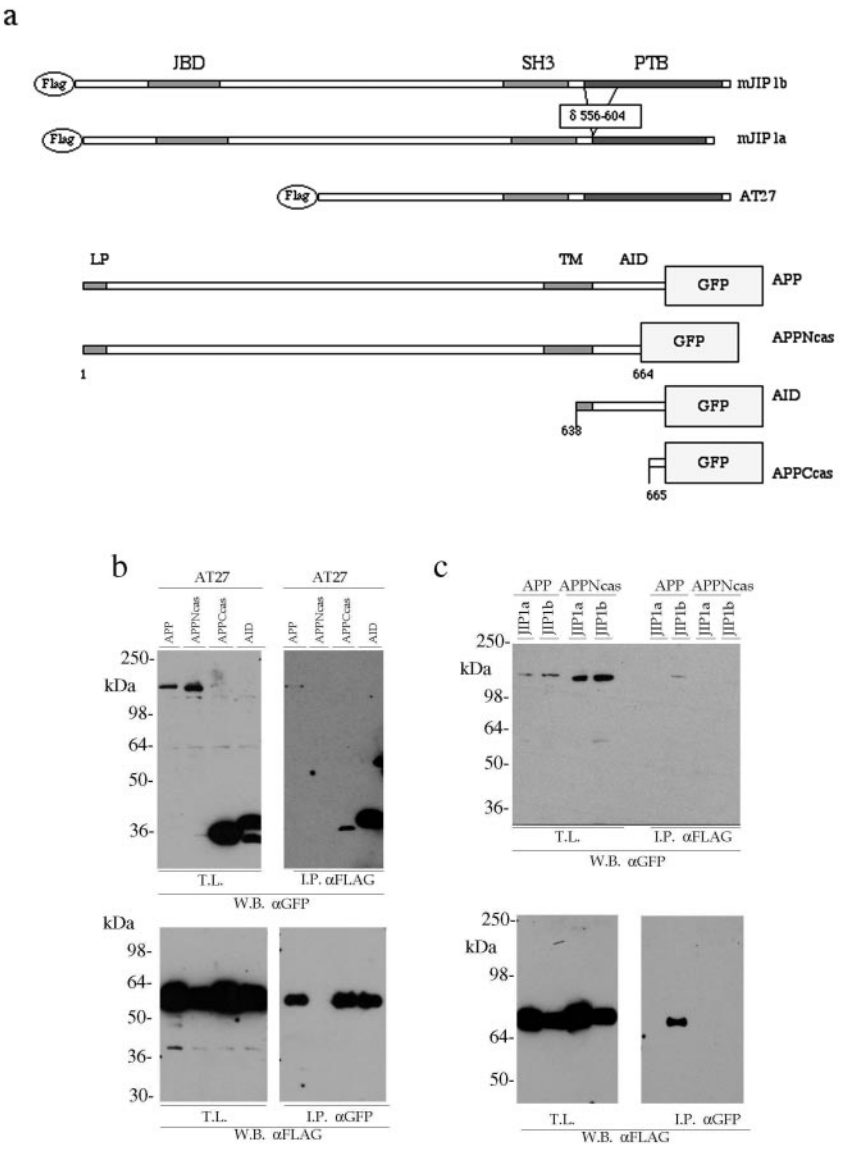

FIG. 4. **JIP1 coimmunoprecipitates with APP.** *a,* schematic representation of mouse JIP1a and JIP1b isoform. The 47-amino acid deletion in the PTB domain of mJIP1a is indicated. Also, the APP-GFP fusion constructs used are depicted. *b,* immunoblot analysis of APP and JIP1 proteins. Equal amounts of cell lysate (*T.L.*) from HEK293T cells transfected with the Flag-tagged JIP1 clone AT27 and APP-GFP (*APP*), APPNcas-GFP (*APPNcas*), AID-GFP (*AID*), or APPCcas-GFP (*APPCcas*). Expression of APP constructs was monitored by Western blot analysis with an anti-GFP antibody (*W.B. αGFP*), while expression of mJIP1a and 1b was assessed by hybridizing immunoblots with and anti-Flag epitope antibody (*W.B. αFLAG*). Lysates from transfected cells were immunoprecipitated with either the anti-FLAG antibody (*I.P. αFLAG*) or an anti-GFP antibody (*I.P. αGFP*). After SDS-PAGE, immunoprecipitated samples were analyzed by Western blot using either an anti-GFP or anti-FLAG antibody. The *lower band* in the total lysate of the AID transfection that is not present in the immunoprecipitation represents a degradation product that lacks the amino-terminal YENPTY containing portion of the AID-GFP fusion protein. *c,* HEK293T cells were co-transfected with the indicated combinations of APP-GFP (*APP*), APPNcas-GFP (*APPNcas*), Flag-mJIP1a (*JIP1a*), and Flag-mJIP1b (*JIP1b*) expression vectors, were resolved by SDS-PAGE. Immunoprecipitations and Western blots were performed as in *b*.

rected against the GFP epitope and Western blotting with anti-Flag antibody revealed that APP can immunoprecipitate only mJIP1b but not mJIP1a. This showed that the 47-amino acid sequence in mJIP1b is essential to its binding APP.

The above data suggests an interesting role for the two splice variants of JIP1 in the mouse, and more recently with the two forms being identified in the rat (45). JIP1b may function to bring APP into contact with the other components bound to JIP1. Alternatively, APP may act to isolate JIP1b and its bound components within a certain subcellular compartment. The JIP1a isoform, which does not bind APP, may instead regulate signaling pathways in an APP-independent manner. Further work is needed to determine whether these two splice forms are present in humans and if APP is involved functionally in the JIP1/JNK pathway.

hJIP1 Interacts with APP in Living Cells Using FRET Analysis—To further characterize the interaction between hJIP1 and APP, we used fluorescence resonance energy transfer (FRET) to detect interaction between hJIP1 and APP in living cells by fluorescence activated cell sorting (30, 32, 46).[2] In FRET on living cells, a fusion protein is made between one protein and cyan fluorescent protein (CFP) and the other protein and yellow fluorescent protein (YFP). The two fusion proteins are then co-transfected into mammalian cells. If the proteins are in close proximity, on the order of 10 nm or less, the energy from the excitation of CFP will be transferred to YFP and emission at the wavelength of YFP will be detected. If the proteins are not within this proximity, excitation of CFP is not transferred and only emission at the wavelength of CFP is detected.

HEK 293T cells were co-transfected with constructs as shown in Fig. 5*a*. When JIP1 constructs containing the JIP1 PTB domain were co-transfected with APP or AID, FRET was detected. Importantly, when they were co-transfected with APP or AID with the Y682G mutation the interaction was abolished (Fig. 5, *d* and *e*). Each sample and its Y682G negative control had similar co-transfection profiles, which allowed us to have a nearly ideal control for each sample. As predicted based on previous experiments, hJIP1-SH_3 did not interact with APP or AID. Surprisingly, in our initial experiments we noticed that when we co-transfected hJIP1e with an amino-terminal fluorescent tag along with the four APP constructs we were not able to detect the FRET signal that we saw with the JIP1-PTB (Fig. 5*e*) and JIP1-SH_3PTB (Fig. 5*d*) constructs. This was unexpected considering that hJIP1e contains a complete PTB domain. A possibility was that since FRET efficiency is extremely sensitive to fluorophore distance and orientation, the

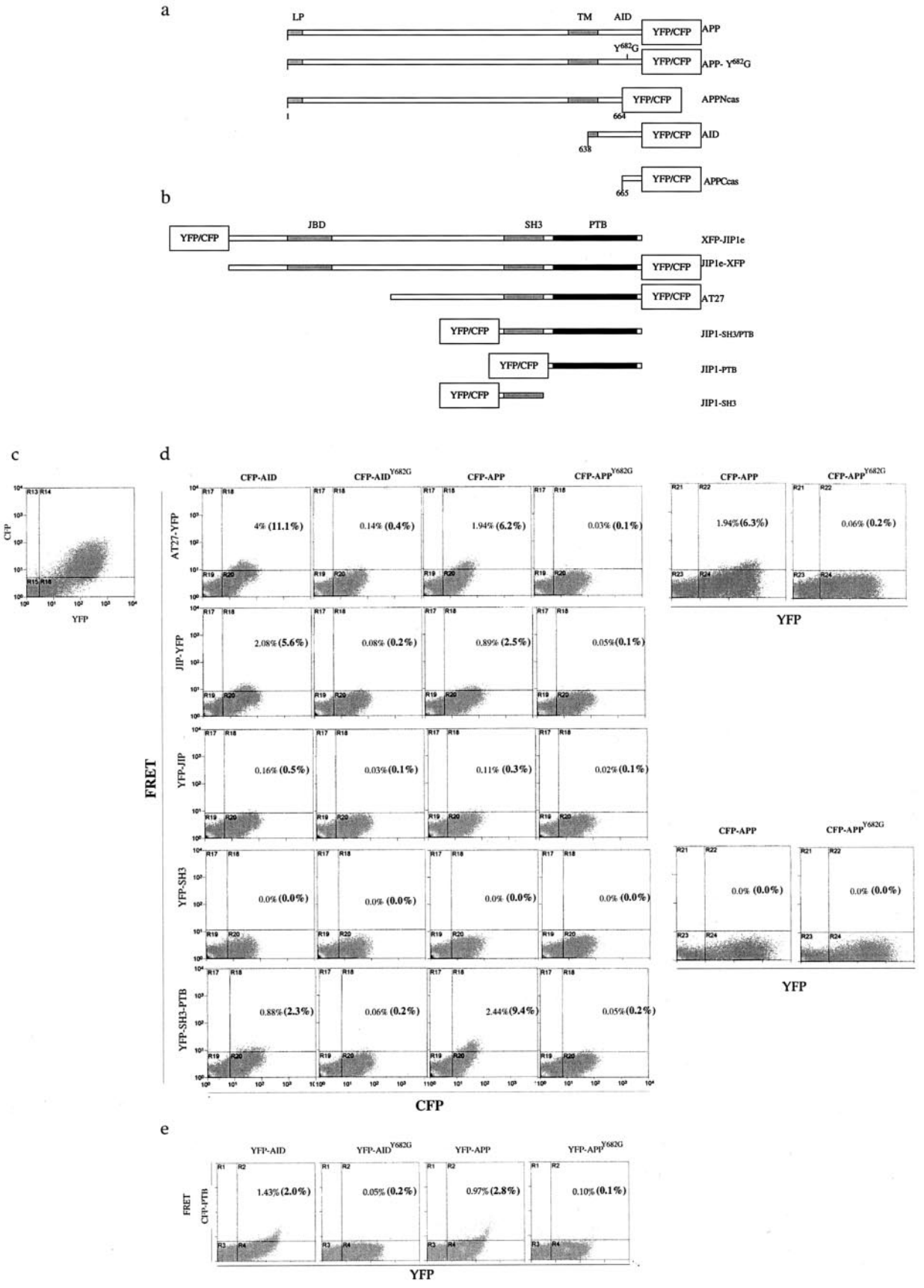

FIG. 5. **Dot plots demonstrating relationships between CFP, YFP and FRET.** *a* and *b* illustrate the APP and JIP1 constructs utilized for the FRET experiments. *c,* representative plot of CFP *versus* YFP to check for co-transfection efficiency. The R14 gate of each sample was used to calculate the percentage of co-transfected cells exhibiting FRET. *d,* dot plots of 24 representative samples showing the presence or lack of FRET. Each plot represents a single co-transfection between the fusion proteins listed at the *left* and *top* of the rows and columns, respectively. Each *dot*,

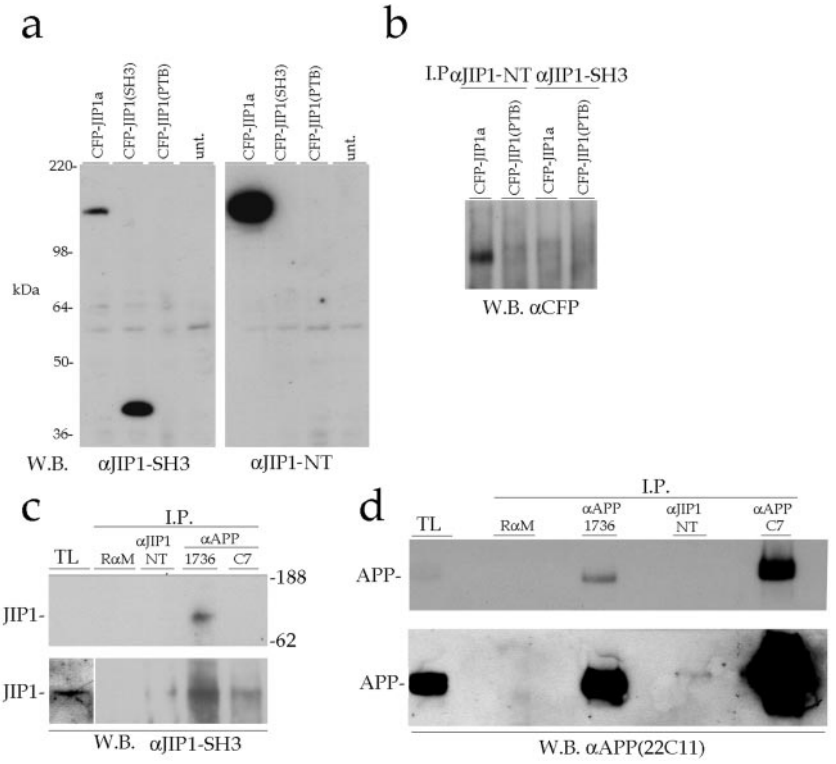

FIG. 6. *a,* two polyclonal anti-JIP1 antibodies were raised, one against an amino-terminal epitope (*JIP1-NT*) and one against an epitope in the SH3 domain of JIP1 (*JIP1-SH3*). While the JIP1-SH3 antibody recognized both full-length JIP1 and the JIP1-SH3 domain, JIP1-NT only reacted with the full-length protein. Neither antibody detected the JIP1-PTB domain. *b*, JIP1-NT can immunoprecipitate overexpressed JIP1, however, JIP1-SH3 does not. *c* and *d,* endogenous APP and JIP1 interact in the adult mouse brain. Immunoprecipitations (*IP*) were done with rabbit anti mouse IgG, antibodies C7, and 1736 against APP, and JIP1-NT and Western blotting (*WB*) was done with either JIP1-SH3 in *c* and anti-APP 22C11 in *d*. Two exposures are displayed so that bands of both strong and weak intensity can be better appreciated.

amino-terminal tag of hJIP1 was too distant from the carboxyl-terminal tag of APP or AID if indeed the proteins interacted via their carboxyl termini (as predicted above). To test this hypothesis we constructed fusion proteins with the fluorescent tags of hJIP1e and JIP1-(244–711)/AT27 on the carboxyl-terminal. As can be seen from Fig. 5*d*, when these constructs were co-transfected with the four APP constructs, FRET was seen with the non-mutated forms of APP and AID but not with the ones with the Y682G mutation. This analysis indicates that the carboxyl termini of hJIP1 and APP are within 10 nm of one another.

In most samples analyzed, the percentage of cells showing FRET was greater for the APP sample than the AID sample. This is compatible with the idea that APP, a type 1 transmembrane protein, can hold hJIP1 securely while AID is free in the cytoplasm where other interactions can interfere with AID-hJIP1 association.

When comparing our study to other FRET studies on preassembled complexes (30, 32),[2] we have observed that the percentage of cells exhibiting FRET in ours was considerably lower. It can be speculated, that for proteins in preassembled complexes the time of interaction is relatively long once the complex is formed. Therefore, at any point in time there will always be a great number of FRET donors and acceptors in close proximity to one another. In our case, however, we believe that we are observing short lived interactions between proteins involved in signaling cascades where the interaction times would be shorter and therefore the FRET signal weaker. This relatively low FRET signal can be used to our advantage because it provides a large range over which FRET increase can be measured when screening for factors that strengthen APPs interaction with hJIP1.

APP Interacts with JIP1 in the Adult Mouse Brain—JIP1 is expressed predominantly in neural tissues. We therefore sought to determine whether APP interacts with JIP1 in the adult mouse brain. To this end, we made homogenates of mouse brains and performed immunoprecipitations with two distinct anti-APP antibodies or a polyclonal antibody raised against a JIP1 amino-terminal peptide (JIP1-NT Fig. 6, *a* and *b*). As shown in Fig. 6*c*, which is representative of data from two independent experiments, APP was immunoprecipitated with both anti-APP as well as JIP1-NT antibodies, while APP was not immunoprecipitated with a control rabbit anti-mouse IgG antibody. Similar findings were obtained with the reverse experiment, that is, JIP1 was immunoprecipitated by the JIP1-NT and the two APP antibodies (Fig. 6*d*) while not by the rabbit anti-mouse IgG control. Interestingly, the anti-APP antibody 1736 was more efficient than the C7 antibody in precipitating APP-JIP1 complexes, although the 1736 immunoprecipitate contained less total APP than the C7 immunoprecipitate. This is noteworthy because the epitope recognized by C7 (generated against the 20 carboxyl-terminal amino acids) include the YENPTY motif while the 1736 antibody raised against amino acids 595–611 (of APP^{695}) does not. Thus, it could be argued that this C7 epitope is less accessible to antibodies when APP is complexed with JIP1 and therefore less total APP is immunoprecipitated. Altogether, these experiments indicate that endogenous APP and JIP1 associate in the adult mouse brain.

representing a single cell, is plotted based on the intensity of the FRET signals intensity *versus* its CFP emission, or for the four on the extreme right FRET *versus* YFP. Cells falling in the R18 gate (or R22 for the four on the extreme right) were scored as FRET positive cells. Percentages are calculated as the number of FRET positive cells divided by the total number of cells or, in *parentheses*, as the number of FRET positive cells divided by the number of co-transfected cells. *e,* dot plots of four representative samples displayed as in *c*, but in this figure with the APP constructs fused to YFP and the JIP1 fragment (*PTB*) fused to CFP. Cells in gate R2 were scored as FRET positive. Representative plots of FRET *versus* both YFP and CFP were shown to illustrate that the intensity of YFP and CFP of each sample and its Y682G mutant control were equal. This ensured that the signal seen as FRET in the positive samples, and not in the control, was a result of real FRET and not just bleed through of intense CFP emission or differences in transfection profiles.

DISCUSSION

In this study we have demonstrated the interaction between APP and JIP1 *in vitro*, *in vivo*, in living cells and in adult mouse brain lysates. During submission of this paper, a similar report by Matsuda *et al.* (47) was published in which they found JIP1 interacts with APP, APLP1 (amyloid precursor-like protein 1), and APLP2, and with APP molecules containing FAD mutations. These complementary reports indicate that the interaction between JIP1 and APP is real and could potentially be important in understanding a biological function of APP and in shedding light on the role APP in the pathogenesis of Alzheimer's disease.

APP is a ubiquitous single pass transmembrane protein and is the precursor from which Aβ is released. APP can be cut sequentially by the β-secretase (4) and γ-secretase (5) with the release of Aβ or by the α- and γ-secretases which release a smaller peptide termed p3 which is not amyloidogenic (3). In addition to these peptides which are released extracellularly or into the lumen of organelles, another peptide, AID, stretching from the γ-cleavage site to the carboxyl-terminal is released into the cytoplasm. The APP intracellular domain is known to bind Fe65 (39, 41), mDab1 (42), X11 (40), and kinesin (48), but the function of these interactions is not fully understood. Hints to the function of APP processing has recently come to light by the demonstration that AID can induce programmed cell death (21).

There are three main MAP (mitogen-activated protein) kinase pathways in eukaryotes involved in transducing signals to trigger adaptation, survival or apoptosis: JNK, ERK, and p38 (49, 50). There are also three major pathologies in AD:amyloid β plaques consisting mostly of aggregated Aβ (51), neurofibrillary tangles consisting mainly of hyperphosphorylated tau protein (52, 53), and degenerating neurons (54). The three activated MAP kinases have been found to be associated with Aβ plaques, neurofibrillary tangles, and degenerating neurons (55–62). Recently, a study was published indicating that activation of the three MAP kinase pathways happens in a defined temporal order (63). The JNK or ERK pathways are activated initially, and with progression of the disease both the JNK and ERK pathways followed by the p38 pathway is activated.

Of the MAP kinase pathways, the JNK cascade is most closely associated with activation when the cell is stimulated by cytokines, anoxic stress, or environmental stresses (64). There are three members of the JNK family; JNK1 and JNK2 are expressed ubiquitously while JNK3 expression is limited mostly to the brain, testes, and heart. Dual phosphorylation of JNK on a conserved Thr-Pro-Tyr motif activates its kinase activity and it is able to phosphorylate c-Jun as well as JunB, JunD, and ATF2. JNK itself is activated by the upstream MAPKKs, either MKK4 or MKK7 (65), which have been activated by MAPKKKs. Using gene knock out studies, it was shown that the JNK signaling pathway, especially JNK3, is necessary for neuronal apoptosis (66).

Considering the large number of kinases at the various levels of the MAP kinase pathways, it was believed that some mechanism must exist so that certain kinases would only activate certain other downstream kinases along defined pathways. For the JNK kinase pathway, one mechanism came in the form of JIP1, a 660-amino acid protein that scaffolds JNK, MKK7, MLK, or DLK and upstream kinases such as HPK1 to facilitate JNK activation (29, 44). Since then other scaffolds such as JIP2 and JIP3 have been described (67, 68). Recently it was found that when the *JIP1* gene is disrupted, there is normal JNK activation in response to UV and anisomycin-induced stress but there is decreased JNK activation in response to kainite, and oxygen-glucose deprivation (69). This knock out study indicated that JIP1 is important in scaffolding components for JNK activation only in response to specific stresses. They also found that although it had been reported that JIP1 interacts with the reelin receptor-apolipoprotein E receptor 2 (ApoER2) (70) and has been found to be involved in glucose regulation and diabetes (71–73), there was no phenotype of altered reelin signaling or diabetes in these mice.

Our report suggests a novel role for APP, tying together JNK being involved in AD, the processing of APP and the APP-JIP1 interaction. Considering the presence of APP-JIP1 complexes in normal brain tissue (Fig. 6, *c* and *d*), it is tempting to speculate that processing of APP and release of AID may play a role in the redistribution of JIP1 and the activation of stress kinase pathways. In the normally functioning neuron, APP (48, 74) and JIP1 (75) are transported to nerve terminals by kinesin. Once there, APP may bind JIP1 either tightly or perhaps as our FRET data indicates in a looser fashion in which JIP1 associates and dissociates in a more transient way. In response to cellular stress, secretase processing of APP may begin and when γ-secretase cleaves APP, AID along with JIP1 would be released into the cytoplasmic compartment. Alternatively, cleavage of APP by caspases (76–79) may cause JIP1 along with the terminal 31 amino acids of APP (APPCcas) to be released. The now free JIP1 would be able to scaffold and cause activation of the JNK signaling pathway as it does in response to anoxic and excitotoxic stress. This may result in dystrophic changes to neurites (80) and neuronal apoptosis.

This mechanism of regulation would parallel that of Notch which undergoes cleavage by the γ-secretase as does APP. When the Notch intracellular domain (NICD) is released it translocates to the nucleus leading to expression of downstream target genes (31, 33). This sort of mechanism involving APP has in fact been theorized and supported in recent reports of AID and Fe65 acting as a transcription factor (17, 28). Along the same lines, we have recently found a molecular interaction between APP and Shc, which regulates the ERK signaling pathway.[4] Interestingly this interaction is phosphorylation dependent and may also be regulated by APP processing.

In conclusion, both our report showing the interaction between APP and JIP1, and reports by others indicating active JNK in the brains of patients with AD, implicates the JNK signaling cascade as a fundamental pathway in the progression of AD. The physical association between APP and JIP1 in neurons may provide a molecular framework linking altered processing of APP and AID release, to JNK activation, tau hyperphosphorylation, and neurodegeneration. Further work must be done to better clarify how these components interact to lead to the pathology seen in Alzheimer's disease.

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Jun NH_2-terminal Kinase (JNK) Interacting Protein 1 (JIP1) Binds the Cytoplasmic Domain of the Alzheimer's β-Amyloid Precursor Protein (APP)

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