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# Growth Factor Receptor-bound Protein 2 Interaction with the Tyrosine-phosphorylated Tail of Amyloid β Precursor Protein Is Mediated by Its Src Homology 2 Domain*

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**The sequential processing of the familial disease gene product amyloid β precursor protein (AβPP) by β- and γ-secretases generates amyloid β, which is considered to be the pathogenic factor of Alzheimer's disease, and the AID peptide (AβPP intracellular domain). The AID peptide acts as a positive regulator of apoptosis and modulates transcription and calcium release. To gain clues about the molecular mechanisms regulating the function of AβPP and AID, proteins interacting with the AID region of AβPP have been isolated using the yeast two-hybrid system. Recent evidence indicates that AβPP undergoes post-translational modification events in the AID region and that phosphorylation might regulate its affinity for interacting proteins. To test this possibility and to uncover AβPP-binding partners whose interaction depends on AβPP phosphorylation, we used a proteomic approach. Here we describe a protein, growth factor receptor-bound protein 2 (Grb2), that specifically binds AβPP, phosphorylated in Tyr$^{682}$. Furthermore, we show that this interaction is direct and that Grb2 binds to phospho-AβPP via its Src homology 2 region. Together with the evidence that Grb2 is in complex with AβPP in human brains and that these complexes are augmented in brains from Alzheimer's cases, our data indicate that Grb2 may mediate some biological and possibly pathological AβPP-AID function.**

Alzheimer's disease (AD)[1] constitutes approximately two-thirds of all cases of dementia (1, 2) and is the most common neurodegenerative disease. AD is genetically linked to a few molecules, one of which is the amyloid β precursor protein (AβPP), a type I transmembrane protein that undergoes processing by the secretases to produce various fragments. Processing by the β- and γ-secretases generates the Aβ fragment from the β to the γ sites and AβPP intracellular domain (AID) from the γ site to the C terminus (2, 3). Recently, another γ-secretases-dependent cleavage has been described to occur at the "ε" site that lies within AID (4–7), generating a shorter AID fragment. The pathologic cascade, which leads to clinical manifestations of AD, has not been fully characterized although the "amyloid hypothesis" has been used to explain certain aspects of AD pathology. According to this hypothesis, the accumulation of Aβ is the primary event that leads to all subsequent events in the pathology of AD (8).

However, considering that Aβ and AID production is dependent on the γ-secretase, several groups have attempted to understand the biological effects of AID production. Indeed, it has been found that AID is able to trigger apoptosis or lower the threshold of the cell to other apoptotic stimuli (9–17) and to regulate $Ca^{2+}$ release (18, 19) and possibly transcription (12, 16, 20, 21). To gain information about the mechanisms by which AID regulates these biological processes, the yeast two-hybrid screen has been used to identify proteins interacting with the cytoplasmic tail of AβPP. Using this method, several AβPP interactors have been identified. These include a class of cytoplasmic proteins containing a phosphotyrosine-binding (PTB) domain such as Fe65, X11, JIP1, JIP2, ARH, mDab1, Numb, Nbl, and AIDA (22–30). The PTB domain of these proteins interacts in a phosphorylation-independent manner with the YENPTY sequence present in the intracellular domain of AβPP.

Recent data have shown that the cytoplasmic tail of AβPP undergoes post-translational modification events such as Thr and Tyr phosphorylation (31). AβPP Thr$^{668}$ (numbering for the AβPP$^{695}$ isoform) has been found to be constitutively phosphorylated in adult rat brain (32) and in neurons (33). Cdc2 kinase phosphorylates Thr$^{668}$ during the $G_2$/M phase of the cell cycle. In differentiated PC12 cells and in SH-SY5Y cells AβPP Thr$^{668}$ phosphorylation is mediated by Cdk5 (34). Glycogen synthase-3β and more efficiently c-Jun N-terminal kinase-3 also phosphorylate Thr$^{668}$ *in vitro* (35, 36). More recently, it has been shown that c-Jun N-terminal kinase-1 and -2 phosphorylate Thr$^{668}$of AβPP in a JIP1-dependent (36, 37) and -independent manner (37) *in vivo* and *in vitro*. Phosphorylation of Tyr$^{682}$ (Y$^{p}$ENPTY, following the AβPP$^{695}$ isoform numbering) (where

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[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, amyloid β; AβPP, amyloid β precursor protein; AID, AβPP intracellular domain; PTB, phosphotyrosine binding; Grb2, growth factor receptor-bound protein 2; SH, Src homology; GST, glutathione *S*-transferase; fl, full-length; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-light-mass spectrometry; strep, streptactin; YFP, yellow fluorescent protein; RTK, tyrosine kinase receptor; WT, wild type; WB, Western blot; MEF, mouse embryonic fibroblast; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase.

$Y^p$ is phosphotyrosine) can be mediated by a constitutively active form of the tyrosine kinase Abl (2, 38–40) or by overexpression of the nerve growth factor receptor TrkA (41). These phosphorylation events are detected in the brain of normal subjects as well as in patients with Alzheimer's disease. We have found that phosphorylation of $Tyr^{682}$ affects the interaction of AβPP with some binding partners. ShcA and -C, members of a family of cytoplasmic adaptor proteins that also includes ShcB, contain a PTB region that binds to the YENPTY AβPP motif (42). However, unlike the other PTB-containing proteins that interact with AβPP, ShcA and -C associate with AβPP only when $Tyr^{682}$ is phosphorylated. Interestingly, the expression level of ShcA protein is augmented in AD brains as compared with normal brain samples (43, 44). Moreover, ShcAβPP complexes are found in human brains and are increased in AD samples.

These data underscore the biological relevance of AβPP phosphorylation. Moreover, they suggest that these phosphorylation events may control AβPP functions by regulating the affinity of AβPP for distinct binding partners. Because the yeast two-hybrid system is not ideal to identify interactions that are regulated by post-translational modification events, we used a biochemical approach to isolate brain proteins in which the binding to AβPP is modulated by AβPP phosphorylation. In this study, we describe one of the interactors isolated using this strategy, growth factor receptor-bound protein 2 (Grb2). We present data showing that Grb2 directly interacts with AβPP and that this interaction requires phosphorylation of $Tyr^{682}$ in AβPP. Unlike the other interactors that bind the YENPTY AβPP motif via their PTB domain, Grb2 binds to $Y^p$ENPTY via its Src homology (SH) 2 region. These data, together with the finding that a complex of Grb2-AβPP is formed in human brains and that these complexes are increased in AD brains, suggest that Grb2 may mediate some biological and perhaps pathological AβPP-AID function.

## MATERIALS AND METHODS

*Strep-tag Peptides Synthesis*—Strep-tag peptides including the strep-tag alone (control), strep-tag AID, strep-tag AID phosphothreonine (AID-Thr(P)$^{668}$), strep-tag AID phosphotyrosine (AID-Tyr(P)$^{682}$), and strep-tag AID phosphothreonine and phosphotyrosine (AID-Thr(P)$^{668}$/Tyr(P)$^{682}$) were synthesized and purified by Tufts University Core Facility (Boston, MA). The sequences of the peptides used are indicated in Fig. 1*A*.

*cDNA Constructs*—The GST-Grb2 full-length (fl) and GST-Grb2 N- and C-terminal SH3 constructs were generously provided by Dr. Robert A. Weinberg. The GST-Grb2-SH2 construct was prepared by PCR amplification of the SH2 domain. The PCR primers used for the cloning (restriction sites are indicated in boldface) are the following: SH2 sense, 5′-AAAA**GGATCC**GCCATGTGGTTT-3′; SH2 antisense, 5′-AAA-**ACTCGAG**TTATTCTATGTC-3′. The amplified product was cloned into pGEX vector (Amersham Biosciences) and sequenced. The GST-AID constructs have been described previously (42). All of the YFP-Grb2 constructs were prepared by PCR amplification, cloned into YFP-$N_1$ vector (Clontech), and sequenced.

*Cell Culture*—Stock cultures of wild type and ShcA knock-out mouse embryonic fibroblast (MEF) cells (a gift from Dr. G. Pelicci) and human embryonic kidney 293 cells stably transfected with AβPP$^{695}$ were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Biofluids, Rockville, MD) and penicillin/streptomycin in a humidified atmosphere containing 7% (v/v) $CO_2$ at 37 °C.

*GST Fusion Proteins*—Recombinant GST fusion proteins were constructed as described above and expressed in *Escherichia coli* strain BL21 (Invitrogen) to make non-phosphorylated proteins and were expressed in strain TKB1 (Stratagene) to make tyrosine-phosphorylated proteins (42). Proteins were purified using glutathione-Sepharose beads (Amersham Biosciences). Phosphorylation of fusion proteins was confirmed by immunoblotting.

*Antibodies*—Rabbit polyclonal Grb2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal ShcA antibody was from Transduction Laboratories (Lexington, KY). Phosphotyrosine-100 mouse monoclonal antibody was obtained from Cell Signaling Technology (Beverly, MA), and mouse monoclonal P2-1 antibody was a generous gift of W. E. Van Nostrand (45). 22C11 monoclonal antibody was purchased from Chemicon International (Temecula, CA), and horseradish peroxidase-conjugated secondary antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL).

*GST Pulldowns, Immunoprecipitation, and Western Blot Analysis*—For GST pull-down experiments, cells were collected, washed with phosphate-buffered saline, and lysed with lysis buffer (50 mM Tris/HCl, pH 7.4, 70 mM NaCl, 1% (v/v) Triton X-100, 50 mM sodium fluoride, 1 mM sodium vanadate, pH 7.5) to which was added 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (42). 6 μg of each GST fusion protein was incubated with cell lysates for 2 h. Samples were washed four times with lysis buffer and then boiled with SDS-PAGE sample buffer. For immunoprecipitation experiments cells were collected 48 h after transfection, washed with phosphate-buffered saline, and lysed with lysis buffer. After 30 min of precleaning with ImmunoPure Plus immobilized protein AG (Pierce), cell lysates were incubated overnight with the antibodies and then processed as described previously. The eluted proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with the primary antibodies for 1 h or overnight. Immunoblots were revealed using horseradish peroxidase-conjugated secondary antibodies followed by chemiluminescence (Supersignal West Pico, Pierce).

*In Vitro Protein Interaction Assays*—Equivalent molar amounts (3 nmol) of strep-tag AID peptides were incubated with 30 μl of 50% Strep-Tactin matrix (IBA) in a total volume of 400 μl of NET-N buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris/HCl, 1% (v/v) Nonidet P-40, pH 8.0) for 1 h at 4 °C. The beads were washed two times with 400 μl of NET-N buffer and then incubated with 6 μg of each GST fusion protein in 400 μl of NET-N buffer for 2–4 h at 4 °C. The beads were then washed with 1 ml of NET-N four times. The bound proteins were eluted from the beads by boiling the samples at 95 °C in SDS-PAGE loading buffer for 4 min. Proteins were analyzed by NuPAGE® Novex Bis-Tris 4–12% gel (Invitrogen) electrophoresis, and then each gel was stained with Coomassie Blue.

*Strep Pulldown from Mice Brain and MEF Cells*—The strep pulldown experiment from MEFs was performed as the GST pull-down procedure described above, except that 3 nmol of strep-tag peptide were used instead of 6 μg of GST proteins. To prepare the mice brain lysates, adult BALB/c mice (age 3 months) were euthanized, and brains were removed and homogenized in buffer containing 100 mM Tris/HCl, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, pH 8.0, protease and phosphatase inhibitors. To preclean the lysate, it was passed through the column containing strep-tag beads, and then equal amounts of precleaned lysate were applied onto each column containing Strep-Tactin matrix incubated previously with the different strep-tag peptides. The beads were washed four times with lysis buffer and then eluted with 10 mM desthiobiotin. The pulled samples were analyzed in parallel by SDS-PAGE followed by ammoniacal silver staining or Western blotting.

*Mass Spectrometry Analysis*—Protein bands from SDS-PAGE were excised from the gel, triturated, and washed with water. Samples were reduced in gel, *S*-alkylated, and digested with trypsin as reported previously (46). Digests were subjected to a desalting/concentration step on μZipTip$C_{18}$ (Millipore Corp., Bedford, MA) before mass analysis. Peptide mixtures were loaded on the MALDI target together with α-cyano-4-hydroxycinnamic acid as matrix using the dried droplet technique. Samples were analyzed with a Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA) (47); spectra were acquired in reflectron mode and elaborated using the Data Explorer 4.1 software provided by the manufacturer. Internal mass calibration was performed with peptides derived from enzyme autoproteolysis. The PROWL software package was used to identify bands unambiguously from independent Swiss Protein and NCBI non-redundant sequence data bases.

*Enzyme-linked Immunosorbent Assay*—The human amyloid β-(1–40) enzyme-linked immunosorbent assay kit was purchased from Immuno-Biological Laboratories (Fujioka, Japan). 48 h after transient transfection of YFP and YFP-Grb2 fusion proteins, Aβ-(1–40) was assayed in triplicates from conditioned media of HEK 293 stably transfected with AβPP$^{695}$. The assay was performed according to the manufacturer's instructions.

## RESULTS

*Identification of Growth Factor Receptor-bound Protein 2 as Binding Protein of Phosphorylated AβPP Tyrosine*—With the

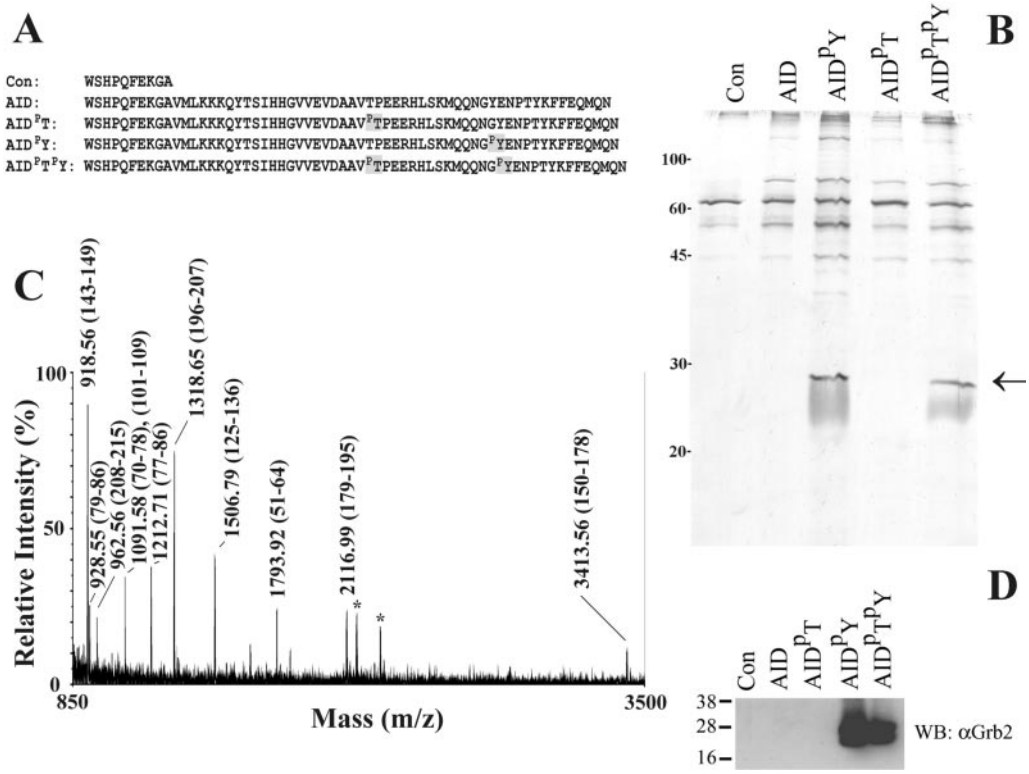


FIG. 1. **Grb2 interacts with AID only upon Tyr$^{682}$ phosphorylation.** *A*, sequence of the synthetic peptides used for the strep pull-down experiments: strep-tag alone (control), strep-tag AID, strep-tag AID phosphothreonine (AID-Thr(P)$^{668}$), strep-tag AID phosphotyrosine (AID-Tyr(P)$^{682}$), and strep-tag AID phosphothreonine and tyrosine (AID-Thr(P)$^{668}$/Tyr(P)$^{682}$). *B*, mouse brain cell lysate was applied in parallel on separate columns coated with the synthetic peptides: control peptide (con), AID, AID-Thr(P)$^{668}$, AID-Tyr(P)$^{682}$, and AID-Thr(P)$^{668}$/Tyr(P)$^{682}$. Columns were washed and then eluted by SDS-PAGE loading buffer. Pulled samples were analyzed in parallel by SDS-PAGE and stained by ammoniacal silver. The species with an apparent mass of ~28 kDa that specifically interacted with AID-Tyr(P)$^{682}$ and AID-Thr(P)$^{668}$/Tyr(P)$^{682}$ but not with control, AID, and AID-Thr(P)$^{668}$ peptides are indicated. These bands were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF-MS for peptide mass fingerprint. *C*, the mass spectrum obtained by MALDI-TOF-MS is reported. Non-redundant sequence data base searching identified these 28-kDa proteins as Grb2. *D*, peptide pulldown from mouse brain lysates. Mouse brain lysates precipitated by the synthetic peptides: control peptide, AID, AID-Thr(P)$^{668}$, AID-Tyr(P)$^{682}$, and AID-Thr(P)$^{668}$/Tyr(P)$^{682}$ (from the *left*) were analyzed by SDS-PAGE and Western blot (*WB*) with the αGrb2 antibody. A 28-kDa component is revealed only in the samples precipitated by AID-Tyr(P)$^{682}$ and AID-Thr(P)$^{668}$/Tyr(P)$^{682}$, indicating that the interaction Grb2-AID is tyrosine phosphorylation-dependent.

goal of identifying new AβPP interacting proteins in which binding is regulated by phosphorylation events, a direct AID-proteomic approach was used. Five synthetic peptides, *i.e.* control peptide, AID, AID phosphothreonine (AID-Thr(P)$^{668}$), AID phosphotyrosine (AID-Tyr(P)$^{682}$), and AID phosphothreonine and tyrosine (AID-Thr(P)$^{668}$/Tyr(P)$^{682}$) were immobilized on different samples of Strep-Tactin resin (Fig. 1*A*). A mouse brain cell lysate was applied in parallel on separate columns packed with these differently coated solid supports, was washed, and then was eluted by SDS-PAGE loading buffer. Before binding experiments, lysate was passed twice on resin to remove aspecific ligands. The pulled samples were analyzed in parallel by SDS-PAGE and stained by ammoniacal silver (Fig. 1*B*). Several bands were detected that did not occur in control, thus demonstrating the suitability of this methodology to isolate AβPP AID interacting proteins. A species migrating with an apparent mass of ~28 kDa seemed to occur depending on the nature of coated support. In fact, this protein specifically interacted with AID-Tyr(P)$^{682}$ and AID-Thr(P)$^{668}$/Tyr(P)$^{682}$ but not with control, AID, and AID-Thr(P)$^{668}$ peptides, indicating that this binding to AID requires specific AID phosphorylation on Tyr$^{682}$. Different bands were excised from the gel, treated, and digested as described under "Materials and Methods" and analyzed by MALDI-TOF-MS. Peptide mass fingerprint experiments and non-redundant sequence data base searching identified the 28-kDa protein as Grb2 (Fig. 1*C*). These findings were confirmed by analyzing the same samples by SDS-PAGE and Western blot using an anti-Grb2 antibody (*WB*: *αGrb2*) (Fig. 1*D*). The αGrb2 antibody detected a component migrating at 28 kDa only in the samples eluted from the columns coated with AID-Tyr(P)$^{682}$ and AID-Thr(P)$^{668}$/Tyr(P)$^{682}$ peptide and not with control, AID, and AID-Thr(P)$^{668}$ peptides.

*The Grb2-AβPP Interaction Is Not Mediated by ShcA*—Grb2 is an adaptor protein containing an SH2 domain flanked on both sides by an Src homology domain 3 (SH3) called SH3 N and C termini. The SH2 region of Grb2 interacts with phosphorylated tyrosine residues present in several proteins. Well described Grb2 interactors are tyrosine kinase receptors (RTKs) (48–50). Upon activation by specific ligands, RTKs undergo tyrosine autophosphorylation. These phosphotyrosines function as docking sites for the SH2 domain of Grb2 and other cytoplasmic adaptors, which include Shc. Receptor-bound Shc proteins can also be phosphorylated by active RTKs and function as docking sites for an indirect recruitment of Grb2 to RTKs. Therefore, Grb2 can bind RTKs either directly or indirectly through Shc. In view of the finding that Shc family members directly bind to tyrosine-phosphorylated AβPP, we first tested whether ShcA proteins bridge Grb2 to AβPP-Tyr(P)$^{682}$. To this end, we used ShcA knock-out mouse embryonic fibroblasts (kindly provided by G. Pelicci). Protein lysates from ShcA knock-out cells, as well as from wild type MEFs used as a control, were purified by affinity chromatography with control, AID, and AID-Tyr(P)$^{682}$synthetic peptides, as described under "Materials and Methods." Pulled samples were analyzed by SDS-PAGE and Western blot using either an anti-

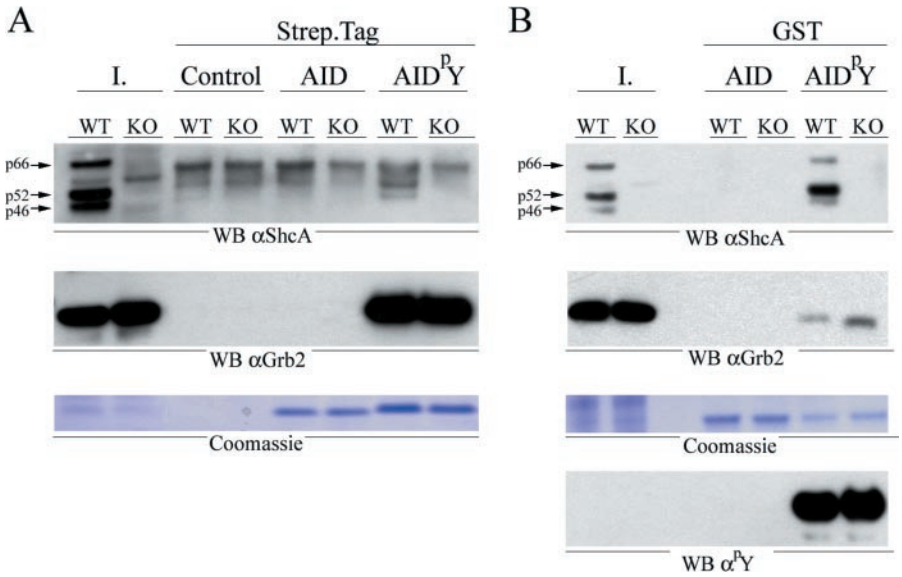


FIG. 2. **Pull-down experiments from ShcA WT and knock-out MEFs.** *A,* the same amount of total protein was precipitated from ShcA WT and knock-out (KO) MEFs using control, AID, and AID-Tyr(P)$^{682}$ synthetic peptides. The samples were then analyzed by WB and revealed by αShcA (*top panel*) and αGrb2 (*middle panel*) antibodies. All ShcA isoforms, 46-, 52-, and 66-kDa, are detected in the ShcA WT total lysates only (*I.*). The p52 and p46 ShcA isoforms interact specifically with AID-Tyr(P)$^{682}$ and not with control or AID peptides. The αGrb2 WB reveals that AID-Tyr(P)$^{682}$ peptide precipitates Grb2 protein in both ShcA WT and knock-out cells suggesting that ShcA is not necessary for the formation of Grb2-AID-Tyr(P)$^{682}$ complex. The Coomassie Blue staining in the *lower panel s*hows the amount of peptides used for each pulldown. *B,* total lysate from ShcA WT and knock-out cells was pulled down using GST-AID and GST-AID-Tyr(P)$^{682}$ proteins. The WB with the α Tyr(P) antibody verifies the phosphorylation state of the GST-AID-Tyr(P)$^{682}$ proteins (*bottom panel*). The αShcA WB reveals that all ShcA isoforms are precipitated specifically by GST-AID-Tyr(P)$^{682}$ and not by GST-AID proteins (*top panel*). In accord with the strep-tag pull-down experiment Grb2 interaction with GST-AID-Tyr(P)$^{682}$ proteins does not require ShcA as shown by the αGrb2 WB (*second panel*). The apparent size differences in the bands detected by the αShcA and αGrb2 antibodies are caused by the gel distortion during the electrophoresis. In the *third panel* Coomassie Blue staining indicates the amount of GST proteins used for each pulldown.

ShcA (αShcA) or an anti-Grb2 antibody. The lower part of the gel was stained with Coomassie Blue to verify that the same amount of peptides was used for each pulldown (Fig. 2*A*, *lower panel*). The three ShcA isoforms of 46, 52, and 66 kDa were identified in the total lysate of wild type but not ShcA knock-out MEFs (*lane I*). As shown in the *top panel o*f Fig. 2*A*, the 52- and 46-kDa ShcA isoforms interacted with AID-Tyr(P)$^{682}$ but not with control and AID peptides. Grb2 protein, which is highly expressed in both wild type and ShcA knock-out MEFs, also interacted specifically with AID-Tyr(P)$^{682}$ peptide (Fig. 2*A*, *WB αGrb2*). Interestingly, AID-Tyr(P)$^{682}$ was still able to recruit Grb2 from ShcA knock-out MEFs, suggesting that ShcA is not required for the formation of the Grb2-AID-Tyr(P)$^{682}$ complex.

To further confirm these data we repeated the pull-down experiment using GST-AID fusion proteins. We expressed GST-AID in BL21 and TKB1 *E. coli* strains. The TKB1 strain is able to produce tyrosine-phosphorylated recombinant proteins (Fig. 2*B*), and this phosphorylation occurs on Tyr$^{682}$ (42). To verify that the same amount of GST protein was used in the experiment, we analyzed the samples by SDS-PAGE and Coomassie Blue staining, as shown in the *lower panel* of Fig. 2*B*. We analyzed the precipitated samples by Western blot using αShcA and αGrb2 antibodies. The GST pull-down experiments were cleaner than the strep pull-down assays, and all ShcA isoforms were clearly pulled down by GST-AID-Tyr(P) but not by GST-AID (Fig. 2*B*, *WB αGrb2*). Consistent with the data obtained using synthetic peptides, Grb2 interacted specifically with GST-AID-Tyr(P), and this interaction was apparent in samples from both ShcA knock-out and wild type MEFs. These data indicate that the interaction between Grb2 and AID-Tyr(P) is not mediated by ShcA.

*Grb2 Interacts Directly with AID-Tyr(P) via Its SH2 Domain*—The above data indicate that ShcA is not necessary to bridge Grb2 to AβPP-Tyr(P)$^{682}$. This suggests that the interaction between Grb2 and AβPP-Tyr(P)$^{682}$ is direct. Alternatively, it is possible that ShcA knock-out cells may express low levels of ShcB and/or ShcC proteins sufficient to link Grb2 to AβPP. In addition, other proteins may mediate the binding of Grb2 to AID-Tyr(P). To distinguish among these possibilities, we performed *in vitro* interaction assays using recombinant proteins. For this purpose, we used the GST-Grb2 fusion proteins and AID synthetic peptides. We expressed and produced GST fused either to full-length Grb2 (Grb2 fl) or to the single Grb2 domains: the SH3 N-terminal domain (Grb2 SH3-N) and the SH2 (Grb2 SH2-C) and SH3 C-terminal (Grb2 SH3-C) domains (Fig. 3*A*). We incubated the GST-Grb2 proteins with AID, AID-Tyr(P)$^{682}$, and the control peptides bound to the strep beads as described under "Materials and Methods." The samples were eluted with SDS-PAGE loading buffer and were analyzed by SDS-PAGE followed by Coomassie Blue staining. In the *left panel* of Fig. 3, the input of the following GST fusion proteins is shown. When the GST fusion proteins were bound to the control peptide, a weak signal was revealed in each lane indicating an aspecific interaction with the control peptide. Low level of interaction, similar to the background aspecific binding seen with the control peptide, was also found between the AID peptide and GST-Grb2fl. Of note, GST-Grb2fl was strongly bound by the AID-Tyr(P)$^{682}$ peptide, indicating that Grb2 can specifically and directly interact with Tyr-phosphorylated AID. Furthermore, whereas both of the GST-Grb2 SH3 domain fusion proteins did not interact with AID-Tyr(P)$^{682}$, the GST-Grb2 SH2 recombinant protein was still able to interact with AID-Tyr(P)$^{682}$, albeit less efficiently than GST-Grb2fl. These data indicate that Grb2 is a binding partner of AβPP-AID; this interaction is direct and involves the SH2 domain of Grb2.

*Src Promotes AβPP-tyrosine Phosphorylation and Increases the Formation of AβPP-Grb2 Complexes in Vivo*—To evaluate the role of tyrosine phosphorylation in the formation of the AβPP-Grb2 complex *in vivo*, we overexpressed the constitutive active form of the tyrosine kinase Src (Src$^{Y527F}$) (47, 51) and the

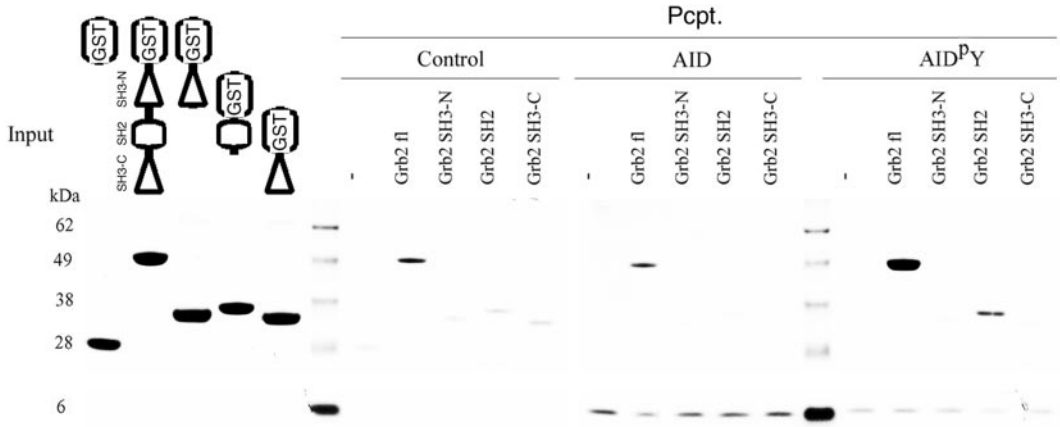


FIG. 3. ***In vitro* interaction assays with GST-Grb2 recombinant proteins and AID synthetic peptides.** The schematic structure of the GST-Grb2 fusion proteins is indicated: GST-Grb2 fl (1–218 amino acids), GST-Grb2 SH3-N (5–61 amino acids), GST-Grb2 SH2 (62–145 amino acids), and GST-Grb2 SH3-C (163–218 amino acids). The different GST-Grb2 fusion proteins were incubated with control, AID, and AID-Tyr(P)$^{682}$ peptides. The samples were then analyzed by SDS-PAGE and Coomassie Blue staining. The *first panel* shows the input of the GST-Grb2 fusion proteins: GST alone (28 kDa), GST-Grb2 fl (55 kDa), GST-Grb2 SH3-N (35 kDa), GST-Grb2 SH2 (38 kDa), and GST-Grb2 SH3-C (35 kDa). Although the GST proteins are incubated with the control peptide, a weak signal is shown in all the lanes, indicating a low aspecific interaction between the GST proteins and the strep-peptide. The same level of interaction is still detected between the GST-Grb2 fl protein and the AID peptide. When the GST proteins are incubated with AID-Tyr(P)$^{682}$ peptide, GST-Grb2 fl is strongly interacting. Furthermore, although both the GST-Grb2 SH3 fusion proteins do not interact with AID-Tyr(P)$^{682}$ peptide, GST-Grb2 SH2 is still able to interact, signifying that the Grb2-SH2 domain is sufficient to bind tyrosine-phosphorylated AID. The bands migrating around 6 kDa correspond to the AID peptides used in the assays.

empty vector as a control in HEK 293 cells stably transfected with the AβPP$^{695}$ isoform. Forty-eight h after transfection, cells were harvested, lysed, and immunoprecipitated with the P2-1 antibody, which recognizes an epitope at the AβPP N terminus. The samples were eluted with SDS loading buffer and analyzed by Western blot with the anti-tyrosine-phosphorylated antibody (Fig. 4*A*), the anti-AβPP (22C11) antibody, and the anti-ShcA and the anti-Grb2 antibodies (Fig. 4*B*, *top*, *middle*, and *bottom panels*, respectively). The Western blotting with the anti-tyrosine-phosphorylated antibody clearly showed an increase in the total level of tyrosine phosphorylation in the cells transfected with Src$^{Y527F}$ compared with the control. Furthermore, in the sample overexpressing Src$^{Y527F}$, the P2-1 antibody immunoprecipitated a 98-kDa phosphorylated component, which could be either AβPP or a protein interacting with AβPP.

Interestingly, Western blot analysis with the anti-ShcA antibody indicated that, despite the fact that the level of ShcA proteins was the same in the total lysates of both the samples, Src$^{Y527F}$ overexpression caused an increase in the amount of all the ShcA isoforms co-immunoprecipitated by the anti-AβPP antibody compared with the control. The same increase was observed for Grb2 as well. Since similar amounts of AβPP were immunoprecipitated from each sample, these results indicate that the regulation of AβPP tyrosine phosphorylation modulates the formation of AβPP-Grb2 and AβPP-Shc complexes *in vivo*.

*Overexpression of Grb2-SH2 Domain Causes an Increase in Generation of Aβ-(1–40)*—Several reports indicate the importance of the AβPP $^{682}$YENPTY$^{687}$ motif interacting proteins in AβPP trafficking and processing (52). To address whether Grb2 could regulate Aβ production, we overexpressed Grb2 full-length and the single Grb2 domains fused to the YFP in HEK 293 stably transfected with AβPP$^{695}$. Forty-eight h after transfection we measured the accumulation of Aβ in the conditioned medium from the transfected cells. As shown in Fig. 5, the overexpression of YFP-Grb2 full-length (*Grb2 fl*) and Grb2 SH3 C- and N-terminal domain (*Grb2 SH3-C* and *Grb2 SH3-N*, respectively) fusion proteins did not alter the production of Aβ-(1–40). However, the overexpression of the YFP-Grb2 SH2 domain, which can interact with AβPP, caused a significant increase in the production of Aβ-(1–40). Because HEK 293 cells express high levels of endogenous Grb2 protein, it is possible

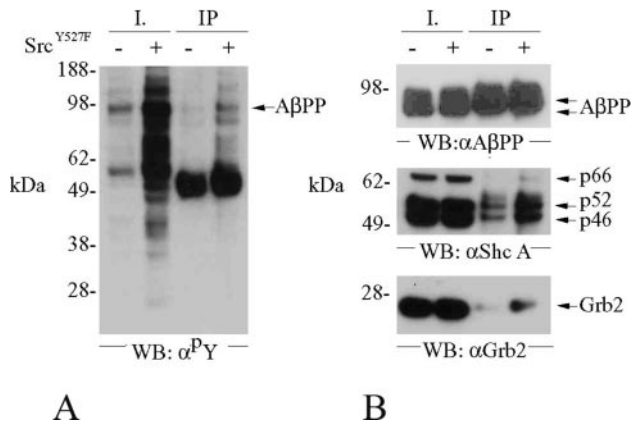


FIG. 4. **Overexpression of Src$^{Y527F}$ in HEK 293 stably transfected with AβPP increases the formation of AβPP-Grb2 and AβPP-Shc complexes *in vivo*.** HEK 293 cells stably expressing AβPP$^{695}$ were transiently transfected with either the constitutive active form of the tyrosine kinase Src (Src$^{Y527F}$) or the empty vector as a control. Src$^{Y527F}$ increases the levels of proteins phosphorylated on tyrosine in total cell lysates (Fig. 4*A*, *lane I*, WB with the anti-phosphotyrosine-100 antibody). The anti-AβPP antibody (P2-1) immunoprecipitated a 98-kDa phosphorylated component, which could be either AβPP itself or an AβPP-interacting protein (Fig. 4*A*, immunoprecipitate (*IP*) *lanes*). In Fig. 4*B*, the same samples were analyzed by WB with the anti-AβPP (22C11), the anti-ShcA, and the anti-Grb2 antibodies (*top*, *middle*, and *bottom panels*, respectively). Although the levels of ShcA and Grb2 proteins are the same in the total lysates of both the samples, Src$^{Y527F}$ overexpression causes an increase in the quantity of ShcA and Grb2 proteins co-immunoprecipitated with AβPP as compared with the control. Because an equal amount of AβPP is immunoprecipitated by the P2-1 antibody from cells overexpressing Src$^{Y527F}$ and the control, the differences observed for Grb2 and ShcA proteins are probably a consequence of the enhanced tyrosine phosphorylation of AβPP.

that overexpression of exogenous Grb2 did not enhance the functional role of Grb2 in AβPP processing. Nonetheless, the evidence that overexpression of the Grb2-SH2 domain considerably enhanced Aβ-(1–40) production might suggest a role for Grb2 in Aβ production. Alternatively, Grb2-SH2 might either compete with other AβPP interactors that reduce Aβ production or enhance AβPP cleavage independently of its ability to bind AβPP.

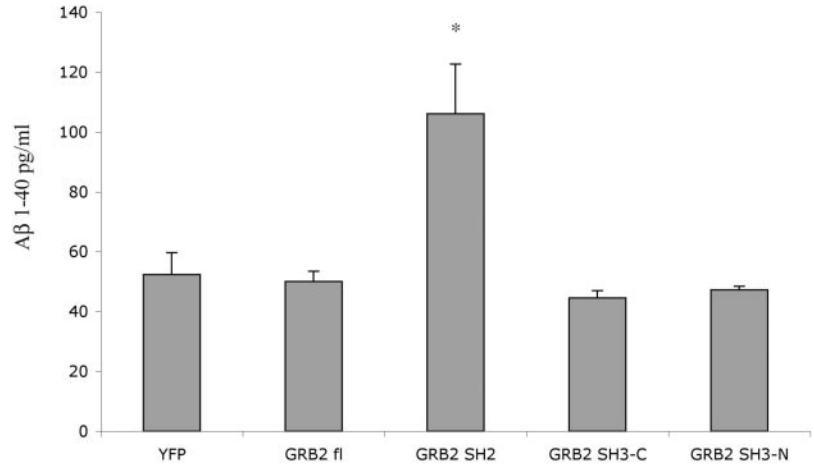


FIG. 5. **Aβ-(1–40) measurement in HEK 293 stably expressing AβPP$^{695}$ after transient transfection of several YFP-Grb2 fusion proteins.** We overexpressed YFP, YFP-Grb2 full-length (*Grb2 fl*), Grb2 SH2, Grb2 SH3-C, and N-terminal domains (*Grb2 SH3-C* and *Grb2 SH3-N*, respectively) constructs in HEK 293 stably transfected with AβPP$^{695}$. Forty-eight h after transfection we measured the accumulation of Aβ-(1–40) in the conditioned medium of cultured cells (see "Material and Methods"). Standard deviations of triplicate experiments are reported. *, mean value is significantly different ($p < 0.02$) from that of the YFP control transfected cells.

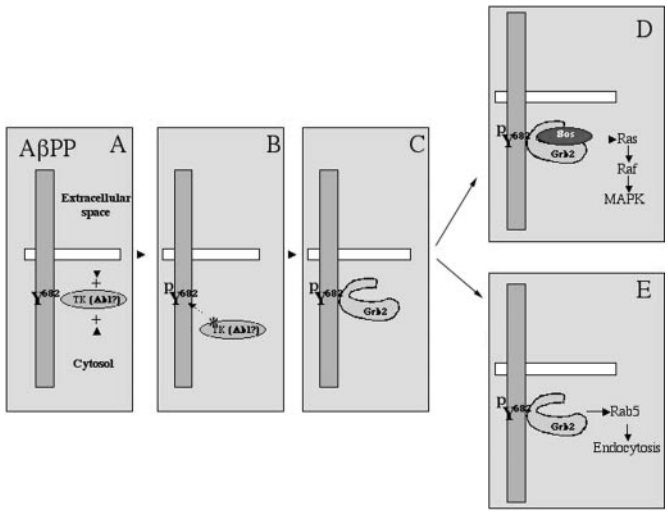


FIG. 6. **Model illustrating the potential role(s) of AβPP-Tyr(P)$^{682}$-Grb2 interaction.** *A*, extracellular or intracellular stimuli activate a tyrosine kinase (such as Abl or Src). *B*, AβPP is phosphorylated on Tyr$^{682}$ by activated tyrosine kinase (*). *C*, AβPP-Tyr(P)$^{682}$ recruits Grb2 (via its SH2 domain). AβPP-Tyr(P)$^{682}$-bound Grb2 engages Sos via its SH3 domain, thereby activating the Ras/MAPK pathway (*D*). Alternatively, Grb2 can signal through Rab5 to promote endocytosis (*E*).

## DISCUSSION

The cytoplasmic tail of AβPP contains a PTB-binding motif ($^{682}$YENPTY$^{687}$) that interacts with the PTB domain of various cytoplasmic proteins. It is important to note that the interaction of the cytoplasmic tail of AβPP with all of the proteins identified to date (Fe65, X11, JIP1, JIP2, ARH, mDab1, Numb, Nbl, and AIDA) does not require the phosphorylation of AβPP (22–27, 29, 30). The only exception is represented by Shc, which binds to AβPP upon phosphorylation of the first tyrosine of the AβPP PTB domain-binding motif (Tyr$^{682}$) (42). In this report, we describe Grb2 as a protein that directly interacts with the cytoplasmic tail of AβPP. However, although both Shc and Grb2 are recruited to AβPP upon phosphorylation of Tyr$^{682}$, different modules mediate these interactions: the PTB domain of Shc and the SH2 region of Grb2, respectively.

Expression of either a constitutively active form of the tyrosine kinase Abl (38), Src (this report), or the nerve growth factor receptor TrkA is associated with phosphorylation of AβPP (41); moreover, Tyr phosphorylation of AβPP C-terminal fragments has been detected in the brains of elderly subjects. Also, Shc and Grb2 have been shown to associate with AβPP fragments in brain samples. It is noteworthy that the levels of Shc proteins and AβPP-Shc-Grb2 complexes are increased in AD cases as compared with control (53). However, these *in vivo* data did not clarify whether Grb2 was directly interacted with AβPP and whether post-translational modifications of AβPP were required to modulate this interaction. Our data clearly determine that Grb2 binding to AβPP is direct and that it requires phosphorylation of Tyr$^{682}$ *in vitro* and *in vivo*.

The physical association of endogenous AβPP and Grb2 in the adult human brain and the augmentation in AβPP-Grb2 complexes in AD patients underscore the biological and perhaps pathological relevance of these findings and prompt us to speculate about the possible functional consequences that this interaction has. The Grb2 SH3 domains interact with proline-rich regions of RAS-specific guanine nucleotide exchange factor Son of sevenless (Sos) (48), Dab2 (54), or GAB2 (55). Thus, through the formation of the phosphoprotein complexes, Grb2 links RTKs to Ras and to the activation of the mitogen-activated protein kinase (MAPK) pathways cascade. It is worth noting that MAPK activation is increased in AD brains (56) and that activated MAPKs can participate in the abnormal hyperphosphorylation of tau in Alzheimer's disease (57–62). Interestingly, the docking site for Grb2 generated by phosphorylation of Tyr$^{682}$ in AβPP has the potential to mechanistically link AβPP to Ras and MAPK activation and suggests that the increase in AβPP-Grb2 complexes found in AD brains might participate in the hyperactivation of MAPKs in AD and possibly tau hyperphosphorylation. More recently, it has been shown that Grb2 activates the Rab5-mediated epidermal growth factor receptor endocytosis (63). Interestingly, Rab5 overexpression also up-regulates the endocytic pathway and increases AβPP intracellular cleavage and Aβ production (64); in fact, it has been reported that AβPP endocytosis modulates Aβ formation (65–68). Thus, it is tempting to speculate that the docking site for Grb2 generated by phosphorylation of Tyr$^{682}$ in AβPP has the potential to mechanistically link AβPP to Ras and MAPK activation as well as endocytosis and AβPP processing (Fig. 6). The plausibility of this hypothesis and whether the increase in AβPP-Grb2 complexes found in AD brains favors the activation of MAPKs, tau hyperphosphorylation and aberrant AβPP processing typical of AD pathology remains to be determined.

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# Growth Factor Receptor-bound Protein 2 Interaction with the Tyrosine-phosphorylated Tail of Amyloid β Precursor Protein Is Mediated by Its Src Homology 2 Domain

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