Fyn Modulation of Dab1 Effects on Amyloid Precursor Protein and ApoE Receptor 2 Processing*

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Dab1 is an intracellular adaptor protein that interacts with amyloid precursor protein (APP) and apoE receptor 2 (apoEr2), increases their levels on the cell surface, and increases their cleavage by α-secretases. To investigate the mechanism underlying these alterations in processing and trafficking of APP and apoEr2, we examined the effect of Fyn, an Src family-tyrosine kinase known to interact with and phosphorylate Dab1. Co-immunoprecipitation, co-immunostaining, and fluorescence lifetime imaging demonstrated an association between Fyn and APP. Fyn induced phosphorylation of APP at Tyr-757 of the YENPTY motif and increased cell surface expression of APP. Overexpression of Fyn alone did not alter levels of sAPPα or cytoplasmic C-terminal fragments, although it significantly decreased production of Aβ. However, in the presence of Dab1, Fyn significantly increased sAPPα and C-terminal fragments. Fyn-induced APP phosphorylation and cell surface levels of APP were potentiated in the presence of Dab1. Fyn also induced phosphorylation of apoEr2 and increased its surface levels and, in the presence of Dab1, affected processing of its C-terminal segment. In vivo studies showed that sAPPα was decreased in the Fyn knock-out, supporting a role for Fyn in APP processing. These data demonstrate that Fyn, due in part to its effects on Dab1, regulates the phosphorylation, trafficking, and processing of APP and apoEr2.

The cytoplasmic domain of APP contains a YENPTY sequence that serves as a binding motif for adaptor proteins that possess a phosphotyrosine binding domain, such as members of the Fe65, X11, and Dab protein families. Such adaptor proteins play critical roles in tyrosine kinase-mediated signal transduction, protein trafficking, phagocytosis, cell fate determination, and neuronal development (1). Importantly for Alzheimer disease (AD), interactions between these cytoplasmic proteins and APP also lead to altered processing of APP and production of the Aβ peptide.

The Dab family members Dab1 and Dab2 are important for nervous system development. These proteins possess a phosphotyrosine binding domain and have been shown to interact with APP and apoEr2 (2). In neuronal migration during development, the Dab1 protein functions downstream of the extracellular protein Reelin, binding to the receptors apoEr2 and very low density lipoprotein receptor (LDL) receptor (3), members of the LDL receptor family (4). The Reelin-receptor interaction activates the Src family kinases Src, Fyn, and Yes and stimulates Dab1 tyrosine phosphorylation through a Reelin-induced multimerization of the receptors (5). Recently, we reported that Dab1 interacted with APP and apoEr2 and increased levels of their secreted extracellular domains and their cytoplasmic C-terminal fragments. These effects depended on the NPTY domains of APP and apoEr2 and on the phosphotyrosine binding domain of Dab1 (6).

Tyrosine phosphorylation is known to potentiate downstream signal transduction by fostering interactions between proteins containing Src homology 2 and other phosphotyrosine-dependent domains (7). Fyn-tyrosine kinase is composed of multiple domains: Src homology 1 (SH1; kinase domain), SH2 (phosphotyrosine binding domain), SH3, and a fourth N-terminal domain that is essential for function (8). Fyn has diverse biological functions due to its ability to phosphorylate numerous intracellular signaling molecules. Most notably, Fyn is involved in modulating central nervous system signal transduction processes, and Fyn−/− mice have defects in myelination as well as in learning and memory (8). Fyn plays a role in memory processes mediated by the N-methyl-d-aspartate receptor (NMDAR), affecting regulation of the NR2B subunit of the NMDAR (9). Fyn has been implicated in AD pathogenesis, including Aβ-induced changes in signaling cascades (10, 11). Recent studies have shown that Fyn is important for APP/Aβ-dependent synaptic deficits and exacerbates Aβ-induced neuronal and...
behavioral deficits (11, 12); these effects were blocked by the genetic ablation of Fyn.

Here, we demonstrate that Fyn increases cell surface expression of APP and apoEr2, potentiates the effect of Dab1 on increasing α-cleavage of APP and apoEr2, and decreases Aβ production. The data presented here implicate a role for a Fyn-Dab1 complex in mediating changes in APP processing.

MATERIALS AND METHODS

Vector Construction—ApoEr2 with a C-terminal HA tag was generated as described previously (13). The following Fyn constructs were generous gifts from Dr. Katsuya Nagai (Osaka University): wild-type human Fyn; Fyn-KN (kinase negative form), containing a K299M substitution that is the catalytic site of Fyn; Fyn-CA (constitutively active form), containing a Y531F substitution that cannot be inactivated through phosphorylation of the C-terminal negative regulation site. Dab1 wild-type and mutant (5F) constructs were kind gifts from Dr. Brian Howell (National Institutes of Health).

Cell Lines and Culture Conditions—COS7 and HEK293 cells were maintained in Opti-MEM (Invitrogen) with 10% fetal bovine serum (Invitrogen) in a 5% CO2 incubator. COS7 cells were maintained in Opti-MEM (Invitrogen) with 10% fetal bovine serum (Invitrogen) in a 5% CO2 incubator. COS7 cells were transiently transfected with 0.5–1 μg of each plasmid in FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol and cultured 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For co-transfections, cells were similarly transfected with 0.5–1 μg of each plasmid in FuGENE 6 (Roche Applied Science) and cultured 24 h in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. After 24 h the cells were transferred to Opti-MEM serum-free media (Invitrogen) and treated with indicated compounds. COS7 cells were incubated overnight in conditioned media at a 1:10 dilution in serum-free media.

Antibodies—We used antibodies anti-HA (Abcam), anti-GFP (Sigma), anti-Fyn (Upstate), anti-c-Myc (Abcam), phosphotheonine APP (Chemicon), 4G10 (Upstate), anti-β-actin (Chemicon), 2B3 (IBL), and anti-Dab1 (from Dr. Andre Goffinet). Anti-apoEr2 antibody 5810 was a kind gift of Dr. Uwe Beffert; apoEr2 C-terminal antibody α-20 was the kind gift of Dr. Johannes Nimpf. For analysis of APP, we used 22C1 (Chemicon), 6E10 (identifying sAPPα) (Signet), C1/6.1 (recognizing the C terminus of APP), provided by Dr. Paul M. Mathews, and antibody 369 (recognizing the C-terminal of APP) provided by Dr. Sam Gandy.

Quantification of APP and ApoEr2 Proteolytic Fragments—After transfection, COS7 cells were maintained for 48 h, and conditioned media and cell lysates were collected. secreted fragments were identified by Western blot analysis of the media (sAPPα, 6E10 antibody; sapor2, 5810 antibody). CTFs were measured by Western blots of cell lysates (APP-CTF, C1/6.1; apoEr2-CTF, HA antibodies). C1/6.1 recognizes both α- and β-CTFs; however, α-CTFs are the form predominantly detected in our system. The samples were separated by SDS-PAGE on 4–15% polyacrylamide gels, transferred electrophoretically to polyvinylidene difluoride membranes, and blocked with 5% nonfat dry milk. The blots were incubated with antibodies at room temperature for 1 h. Horseradish peroxidase-conjugated secondary antibodies were visualized by an ECL detection system and exposed to film. Aβ-(1–40) levels in the conditioned media were determined using 82E1/1A10 ELISA, which recognizes both mouse and human Aβ (Immunobio-Biological Laboratories) (14). Aβ-(1–40) levels in mouse brain lysates were determined using 14F1/1A10 ELISA (15). Levels of sAPPα in mouse brain lysates were determined by ELISA using 2B3, a monoclonal antibody specific for α-site-cleaved APP (15), as the capture antibody and a polyclonal N terminus APP antibody as the detection antibody.

Primary Neuronal Cell Culture—Primary mouse embryonic cortical neuronal cultures were prepared from embryonic day 16 Swiss-Webster mice as previously described (16). Brain cortices were chopped and trypsinized for 10 min at 37 °C. After trypsinization, 0.4 μg/ml trypsin inhibitor, 0.025% DNase, and 12 mM MgSO4 were added and mixed until tissue was thoroughly homogenized. Cells were then transferred to Neurobasal medium containing B27 serum supplement, 1 mM glutamine, 10 μg/ml gentamycin, and 1:1000 10 μM Ara-C. Neurons were seeded on 50 μg/ml poly-γ-lysine-coated 12-well tissue culture plates at a density of 2 × 10⁶ cells.

Primary Hippocampal Neuronal Culture and Cell Surface Immunostaining—Primary hippocampal neurons from E18-19 Sprague-Dawley rats were cultured at 150 cells/mm² as described (17). Neurons were transfected at 10–12 days in vitro by calcium phosphate precipitation (4–5 μg of DNA/well). 6–8 days after transfection, cell surface expression levels of APP were analyzed. Surface immunostaining was performed as described previously (18). Briefly, live neuron cultures were incubated with anti-GFP antibody (10 μg/ml in conditioned medium) for 10 min to specifically label surface APP, then lightly fixed for 5 min in 4% paraformaldehyde (non-permeabilizing conditions). After fixation, the surface-remaining antibody labeled APP was measured with Alexa Fluor 555-linked α-mouse secondary antibodies for 1 h. To measure total levels of APP, cells were then permeabilized with 20 °C methanol and incubated with anti-GFP antibody overnight. After incubation, total APP was detected using secondary antibody linked to Alexa Fluor 488. Images were collected using a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Confocal z-series image stacks encompassing entire neurons were analyzed using Metamorph software (Universal Imaging Corp., Downingtown, PA) (18). Levels of surface APP were normalized by total APP for analysis (19).

Co-immunoprecipitations—Transfected COS7 cells were washed with phosphate-buffered saline and lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1% Nonidet P-40, phosphatase inhibitors (Sigma), and protease inhibitors (Roche Applied Science). For immunoprecipitation, the lysates were incubated for 2 h at 4 °C with the anti-HA antibody or the C1/6.1 antibody bound to protein G- Sepharose beads (Amersham Biosciences). As a negative control, the experiment was conducted with an irrelevant antibody (α-P-JNK). The precipitates were then washed three times with lysis buffer and resuspended in SDS sample buffer. The samples were separated by SDS-PAGE as previously described.
Biotin-labeled Cell Surface Proteins—COS7 cells were transiently transfected with APP and Fyn or Dab1 constructs or with apoEr2 and Fyn or Dab1 constructs in FuGENE 6 (Roche Applied Science) and cultured 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After 24 h, cells were washed twice with phosphate-buffered saline, and surface proteins were labeled with Sulfo-NHS-SS-Biotin, 500 μl at 500 μg/ml phosphate-buffered saline (Pierce) under gentle shaking at 4 °C for 30 min. 50 μl of quenching solution (Pierce) was added to cells at 4 °C, which were washed twice with Tris-buffered saline. Cells were lysed in 500 μl of lysis buffer, collected with a cell scraper, disrupted by sonication on ice, incubated for 30 min on ice, and clarified by centrifugation (10,000 × g, 2 min). To isolate biotin-labeled proteins, lysate was added to immobilized NeutroAvidin TM Gel (50 μl) and incubated for 1 h at room temperature. Gels were washed 5 times with wash buffer and incubated for 1 h with SDS-PAGE sample buffer including 50 mM dithiothreitol. Surface proteins were then analyzed by immunoblotting.

Immunocytochemistry—Cells were fixed in 4% paraformaldehyde for 10 min, washed in phosphate-buffered saline (pH 7.4), permeabilized with 0.5% Triton X-100 for 20 min, and blocked with 1.5% normal goat serum for 1 h. Cells were transfected with full-length APP (APP770-V5) and the constitutively active form of Fyn. Transfected cells were immunostained using monoclonal antibody V5 (Sigma) and rabbit C-terminal anti-Fyn antibody. Cells were then incubated with Alexa488- and cyanine 3 (Cy3)-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature. Immunostained cells were coverslipped using glycerol vinyl alcohol mounting solution (Zymed Laboratories Inc., San Francisco, CA) before fluorescence lifetime imaging microscopy (FLIM) measurements or confocal imaging with appropriate filters using a Bio-Rad 1024 confocal microscope.

Fluorescence Resonance Energy Transfer Measurements Using FLIM—The proximity of proteins were assessed by FLIM, a fluorescence resonance energy transfer-based technique using multiphoton microscopy (20). Lifetime of a donor fluorophore attached to one protein decreases due to nonradiative energy transfer if an acceptor fluorophore attached to another protein is positioned within a range of 1–10 nm of the donor. Thus, a decreased donor lifetime is related to the proximity of the two fluorophores (and the proteins labeled with these fluorophores). Alexa488 was used as a donor fluorophore and Cy3 as an acceptor fluorophore. Images of Alexa488 fluorescence lifetimes on a pixel-by-pixel basis were acquired using a Bio-Rad Radiance 2000 multiphoton microscope, Ti:sapphire laser (Spectra Physics, San Jose, CA), a high-speed detector MCP R3809 (Hamamatsu, Japan), and a time correlated single-photon counting acquisition board (SPC 830, Becker and Hickl GmbH, Germany) (20). The images were analyzed using SPC-Image V2.8 (Becker and Hickl GmbH). Donor fluorophore lifetimes were fit using this software to two exponential decay curves to determine a fraction of the donor interacting with an acceptor. Statistical analysis of FLIM data were performed by Student’s t test after determination of normal distribution and variance. The results are also presented in pseudocolored images representing color-coded donor lifetime in pixel-by-pixel basis.

Fyn Knock-out Mice—Fyn knock-out mice were obtained from The Jackson Laboratory as well as wild-type B6129S controls. Age-matched mice aged 3–6 months were used for analysis of secreted proteins and CTFs. Mouse brains were homogenized in tissue homogenization buffer containing 250 mM sucrose, 20 mM Tris base, 1 mM EDTA, 1 mM EGTA, and protease inhibitors. For detection of sAPPα and sAPPβ, the crude homogenate was mixed with 0.4% diethylamine, sonicated, and centrifuged at 135,000 × g for 1 h at 4 °C. For detection of APP and apoEr2 CTFs, proteins were further extracted with the addition of a 10-fold volume of radioimmune precipitation assay buffer and centrifuged for 10 min at 4 °C.

Statistical Analyses—All data were analyzed using analysis of variance with Graphpad Prism 4 software using Tukey’s Multiple Comparison test for post hoc analyses with significance determined as p < 0.05. Descriptive statistics were calculated with StatView 4.1 and displayed as an expressed mean ± S.E. All experiments were conducted a minimum of three times unless otherwise noted.

RESULTS

Fyn Interacts with APP in COS7 Cells and Primary Neurons—To test whether Fyn interacts with APP, we used the following constructs of Fyn: Fyn-WT (wild-type); Fyn-KN (kinase negative) containing a K299M substitution; Fyn-CA (constitutively active) containing a Y531F substitution (Fig. 1A) (21). We transfected COS7 cells with APP and Fyn-KN or APP and Fyn-CA, immunoprecipitated with the anti-Fyn antibody, and probed for APP with the C1/6.1 antibody. Fyn-CA co-precipitated with APP (Fig. 1B, upper panel). Western blot analysis of COS7 cell extracts confirmed levels of total APP (Fig. 1B, middle panel). Interestingly, co-transfection with Fyn-KN resulted in a dramatically lower level of total APP. Thus, there was no co-precipitation of APP with Fyn-KN (Fig. 1B, first lane); this may have been due to the reduction of APP levels. To test the specificity of our findings, we conducted the reverse experiment and immunoprecipitated with C1/6.1 and probed with anti-Fyn, which similarly demonstrated an interaction between Fyn-CA and APP (data not shown).

To test whether the interaction between Fyn-CA and APP was merely the result of an interaction between overexpressed proteins, we examined whether endogenous APP and Fyn co-immunoprecipitated in primary neuronal cultures. We incubated extracts of primary neurons with C1/6.1 and probed the precipitates with Fyn antibody. Immunoprecipitation of APP from primary neurons resulted in precipitation of Fyn (Fig. 1C, second lane). As a negative control, the experiment was conducted with an irrelevant antibody (α-P-JNK) (Fig. 1C, first lane). APP and Fyn were measured (middle and lower panels) to demonstrate similar inputs into the immunoprecipitations. Conversely, we incubated primary neuron extracts with anti-Fyn and probed the precipitates with C1/6.1 antibody. Immunoprecipitation of Fyn resulted in precipitation of APP (Fig. 1D, second lane). The negative control with α-P-JNK did not demonstrate precipitation of APP (Fig. 1D, first lane).

To determine whether Dab1 was required for the interaction between APP and Fyn, Dab1 knock-out and wild-type brain
not required for this interaction, it greatly enhances the association between APP and Fyn.

**Fyn Co-localizes with APP in Primary Hippocampal Neurons**—To further test the interaction between APP and Fyn, we utilized FLIM, a morphology-based fluorescence resonance energy transfer technique that can reveal close protein-protein proximity in intact cells. Fluorescence lifetime is influenced by the surrounding microenvironment and is shortened in the immediate vicinity of a fluorescence resonance energy transfer acceptor molecule. For these experiments, COS7 cells were transfected with APP-V5, APP-V5 and Fyn-CA, or APP-V5, Fyn-CA, and Dab1 constructs. The cells were then immunostained with anti-V5 visualized by Alexa488 (donor) and anti-Fyn visualized by Cy3 (acceptor) (Fig. 3, A and B). In the presence of Fyn, anti-V5 fluorescence had a signal emitting a shorter half-life. Shorter fluorescence lifetime indicates a closer association between APP and Fyn. Fluorescence lifetimes for all groups were significantly different from control at \( p < 0.001 \). Lifetime for APP and Fyn-CA in the presence of active Dab1 was significantly different from APP and Fyn-CA in the presence of a form of Dab1 in which the 5 tyrosine residues were replaced with phenylalanine (Dab1(5F)) \( (p < 0.05) \). Thus, FLIM analysis showed a close association between Fyn and APP throughout the cell, and this association was stronger in the presence of active Dab1.

**APP, Fyn, and Dab1**

**FLIM Assay**—To further test the interaction between APP and Fyn, we utilized FLIM, a morphology-based fluorescence resonance energy transfer technique that can reveal close protein-protein proximity in intact cells. Fluorescence lifetime is influenced by the surrounding microenvironment and is shortened in the immediate vicinity of a fluorescence resonance energy transfer acceptor molecule. For these experiments, COS7 cells were transfected with APP-V5, APP-V5 and Fyn-CA, or APP-V5, Fyn-CA, and Dab1 constructs. The cells were then immunostained with anti-V5 visualized by Alexa488 (donor) and anti-Fyn visualized by Cy3 (acceptor) (Fig. 3, A and B). In the presence of Fyn, anti-V5 fluorescence had a signal emitting a shorter half-life. Shorter fluorescence lifetime indicates a closer association between APP and Fyn. Fluorescence lifetimes for all groups were significantly different from control at \( p < 0.001 \). Lifetime for APP and Fyn-CA in the presence of active Dab1 was significantly different from APP and Fyn-CA in the presence of a form of Dab1 in which the 5 tyrosine residues were replaced with phenylalanine (Dab1(5F)) \( (p < 0.05) \). Thus, FLIM analysis showed a close association between Fyn and APP throughout the cell, and this association was stronger in the presence of active Dab1.
**APP, Fyn, and Dab1**

**FIGURE 2.** Fyn co-localizes with APP in primary hippocampal neurons. Primary hippocampal neurons cultured from E18 Sprague-Dawley rats were immunostained at day in vitro 14 with antibodies against Fyn and secondary anti-goat rabbit Alexa488 (left panel, green) and APP and secondary anti-goat mouse Alexa594 (middle panel, red). Co-localization of Fyn and APP (right panel) is seen in the neuronal cell body and throughout dendritic processes. The lower panels show a higher magnification for visualization of co-localization of Fyn and APP in dendrites.

**A.**

| TF | Donor | Acceptor | Life time (mean±SD) N |
|----|-------|----------|----------------------|
| 1. APP | APP-V5 | None | 2219±53 | 15 |
| 2. APP/Fyn-CA | APP-V5 | Fyn | 1837±284 | 12 |
| 3. APP/Fyn-CA/Dab1 | APP-V5 | Fyn | 1667±292 | 11 |
| 4. APP/Fyn-CA/Dab15F | APP-V5 | Fyn | 1917±86 | 14 |

**B.**

**FIGURE 3.** APP and Fyn interaction by FLIM assay. A, COS7 cells were transiently transfected with APP and vector, APP and Fyn-WT, APP and Fyn-KN, or APP and Fyn-CA. After transfection, sAPPα and APP CTF were measured from the conditioned media and cell lysate (20 μg/lane), respectively. Fyn-WT and Fyn-CA did not alter sAPPα or APP CTF compared with control. However, Fyn-KN decreases total APP, sAPPα, and APP CTF. B, COS7 cells were transiently transfected with APP and vector (n = 5), APP and Fyn-WT (n = 5), or APP and Fyn-CA (n = 5) (left). Aβ-(1–40) levels in the conditioned media were determined by ELISA. Fyn-WT and Fyn-CA significantly decreased secreted Aβ-(1–40) levels (by 62 and 86%, respectively, p < 0.05) compared with vector. COS7 cells were transfected with APP and vector (n = 6) or APP and Fyn-KN (n = 6), and Aβ-(1–40) levels in the conditioned media were determined by ELISA (right). Fyn-KN significantly decreased Aβ-(1–40) levels by 94% (p < 0.05). CTRL control. C, COS7 cells were transiently transfected with APP and vector (first and fourth lanes), APP and Fyn-KN (second and fifth lanes), or APP, Fyn-KN, and Dab1 (third and sixth lanes) and treated with vehicle (first through third lanes) or MG-132 (10 μM, fourth through sixth lanes), a reversible proteasome inhibitor, for 12 h. Co-transfection with Dab1 resulted in recovery of total APP, but not sAPPα or APP CTF (third lane). However, MG-132 treatment significantly inhibited Fyn-KN-induced decreases of all APP species (fifth and sixth lanes).

Fyn Effects on APP Processing and Aβ Production—We tested whether the interaction of Fyn with APP affected APP processing. We transfected COS7 cells with APP and Fyn constructs and measured secreted APP, total APP, and APP CTF. We found that Fyn wild type and Fyn-CA did not alter sAPPα and APP CTF levels compared with transfection with APP and vector (Fig. 4A, second and fourth lanes). Consistent with our previous results (Fig. 1B, first lane), co-expression of APP and Fyn-KN (third lane) significantly decreased all APP species (Fig. 4A, third lane). To further test whether Fyn affects Aβ production in COS7 cells, we transfected cells with APP and vector, APP and Fyn-WT, or APP and Fyn-CA and measured Aβ-(1–40) levels by ELISA. We found that Fyn-WT and Fyn-CA significantly decreased secreted Aβ-(1–40) levels by 65–80% (Fig. 4B, left side). We also tested whether Fyn-KN affected Aβ production and transfected COS7 cells with APP and vector or Fyn-KN. Consistent with its effects on the other APP-derived species, Fyn-KN significantly reduced Aβ-(1–40) levels by 94% (Fig. 4B, right side).

The dramatic effects of Fyn-KN on APP levels led us to investigate its mechanism of action. To determine whether reduction of APP by Fyn-KN was mediated by proteasomal
degradation of APP, we transfected COS7 cells with APP and vector (Fig. 4C, first and fourth lanes), APP and Fyn-KN (second and fifth lanes), or APP, Fyn-KN, and Dab1 (third and sixth lanes) and treated the cells with vehicle control (first through third lanes) or the proteasome inhibitor MG132 (fourth through sixth lanes). Consistent with previous results (Fig. 4A), Fyn-KN dramatically reduced total APP, sAPPα, and APP CTF (second lane). Interestingly, in the presence of Fyn-KN, co-transfection with Dab1 restored levels of total APP but not sAPPα and APP CTF (third lane). MG132 treatment inhibited Fyn-KN-induced APP degradation both in the absence and presence of Dab1 (fifth and sixth lanes).

Fyn Affects APP Processing in the Presence of Dab1—Previous studies have shown that Fyn interacts with Dab1 (23). Recently, we and another group have shown that Dab1 affects APP processing and Aβ6 production (6, 24). This led us to hypothesize a synergistic effect of Fyn and Dab1 on APP processing.

To test whether Fyn affects APP processing in the presence of Dab1, we transfected COS7 cells with APP, Fyn-CA, and either wild-type Dab1 or Dab1(5F). Consistent with our previous findings (6), we found that Dab1 increased sAPPα and APP CTF, whereas Dab1(5F) did not (Fig. 5A, first through third lanes). As seen previously (Fig. 4A), Fyn-CA alone did not alter sAPPα or APP CTF (Fig. 5A, fourth lane). Interestingly, we found that co-transfection with both Fyn-CA and Dab1 (Fig. 5A, fifth lane) had a synergistic effect and increased sAPPα and APP CTF compared with Dab1 alone (Fig. 5A, second lane) or Fyn alone (Fig. 5A, fourth lane). However, co-transfection with Fyn-CA and Dab1(5F) did not alter APP processing (Fig. 5A, sixth lane).

Importantly, Fig. 5A showed that active Fyn and Dab1 together had a greater effect on APP processing compared with Fyn or Dab1 alone. To further determine whether Fyn activation is important for its effects on APP processing in the presence of Dab1, we transfected COS7 cells with APP, Dab1, and Fyn constructs. We found that co-transfection with Dab1 and Fyn-CA (Fig. 5B, third lane) significantly increased sAPPα (79%) and APP CTF (105%) compared with vector (fourth lane).

Consistent with previous data (Fig. 4C), co-transfection with Dab1 and Fyn-KN did not result in a dramatic decrease in total APP as seen with transfection with Fyn-KN alone (Fig. 5B, middle panel). These data suggest that the active form of Fyn can potentiate the effects that Dab1 has on APP processing.

Fyn Effects on ApoEr2 Processing—We tested whether Fyn affected processing of apoEr2, which shares intracellular and extracellular binding partners with APP (6, 25, 26). We transfected COS7 cells with apoEr2 and vector or apoEr2 and Fyn constructs (Fig. 6A). Secreted apoEr2 was measured in conditioned media, and apoEr2 CTFs was measured in cell lysates. We found that Fyn-WT and Fyn-CAN did not alter total levels of apoEr2 and apoEr2 CTF (second and fourth lanes). However, Fyn-KN decreased total cellular apoEr2 as well as secreted apoEr2 and apoEr2 CTFs (third lane). Consistent with previous data (Fig. 4C, first and second lanes), apoEr2 CTF was measured in cell lysates. We transfected COS7 cells with apoEr2 and Dab1(5F), and treated with vehicle (second lane) or MG-132 (10 μM, third lane), a reversible proteasome inhibitor. MG-132 treatment significantly inhibited Fyn-KN-induced apoEr2 degradation (third lane).
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Dab1, and Fyn constructs. We found that Fyn-WT (Fig. 6B, second lane) and Fyn-CA (fourth lane) increased sapoEr2 and apoEr2 CTF in the presence of Dab1 compared with cells transfected with apoEr2 and vector (first lane). We also found that Fyn-KN did not reduce cell-associated apoEr2 expression level in the presence of Dab1 (Fig. 6B, middle panel, third lane). However, co-transfection with Dab1 and Fyn-KN decreased sapoEr2 and apoEr2 CTF levels.

Next, we used a pharmacological inhibitor to investigate whether the Fyn-KN mediated decrease in apoEr2 expression was also due to proteasomal degradation. For these experiments, we transfected COS7 cells with apoEr2 and vector (Fig. 6C, first lane) or apoEr2 and Fyn-KN and treated the cells with vehicle (second lane) or MG132 (third line). We found that MG132 treatment efficiently inhibited Fyn-KN-induced apoEr2 degradation (Fig. 6C), as with APP (Fig. 4).

Fyn Promotes Phosphorylation of APP, Dab1, and ApoEr2—Tyrosine phosphorylation of Dab1 and Fyn are important for their signal-transducing effects during development (27). To test whether Fyn affects APP phosphorylation, we expressed APP and Fyn constructs in COS7 cells. We immunoprecipitated APP and probed for phosphorytosine. We found that Fyn-CA induced phosphorylation of APP (Fig. 7A, third lane). We then asked whether Fyn affects APP phosphorylation in the presence of Dab1. We expressed APP, Dab1, or Dab1(5F) and Fyn constructs in COS7 cells. Both Fyn-WT and Fyn-CA induced tyrosine phosphorylation of APP in the presence of Dab1 (Fig. 7B, second and fourth lines), but Fyn-CA did not induce tyrosine phosphorylation of APP in the presence of Dab1(5F) (Fig. 7B, fifth lane). Levels of Dab1, Fyn, and APP were consistent across transfections (Fig. 7B, lower panels), including the presence of Dab1(5F) in the fifth lane, which is recognized by the Dab1 antibody. Fyn-KN again reduced APP levels in the absence of Dab1.

We then asked whether Fyn affected Dab1 phosphorylation using the same cells. We immunoprecipitated Dab1 with an anti-FLAG antibody and probed for phosphorytosine. Fyn-WT and Fyn-CA induced tyrosine phosphorylation of Dab1 (Fig. 7C, middle panel, third lane). We then asked whether Fyn affects Dab1 phosphorylation in the presence of Dab1. We expressed APP, Dab1, or Dab1(5F) and Fyn constructs in COS7 cells. Both Fyn-WT and Fyn-CA induced tyrosine phosphorylation of Dab1 (Fig. 7C, third line). We then asked whether Fyn affects Dab1 phosphorylation in the presence of Dab1. We expressed APP, Dab1, or Dab1(5F) and Fyn constructs in COS7 cells. Both Fyn-WT and Fyn-CA induced tyrosine phosphorylation of Dab1 (Fig. 7C, third line). We then asked whether Fyn affects Dab1 phosphorylation in the presence of Dab1. We expressed APP, Dab1, or Dab1(5F) and Fyn constructs in COS7 cells. Both Fyn-WT and Fyn-CA induced tyrosine phosphorylation of Dab1 (Fig. 7C, third line). We then asked whether Fyn affects Dab1 phosphorylation in the presence of Dab1. We expressed APP, Dab1, or Dab1(5F) and Fyn constructs in COS7 cells. Both Fyn-WT and Fyn-CA induced tyrosine phosphorylation of Dab1 (Fig. 7C, third line).
whereas APP mutant 1 contained an alanine substitution at tyrosine 757 (Y757A), whereas APP mutant 2 contained an alanine substitution at tyrosine 762 (Y762A). We then transfected COS7 cells with Fyn-CA and APP wild-type (Fig. 7E, first lane) or APP mutant constructs (second and third lanes). Cell lysates were immunoprecipitated with antibody 369 for APP and immunoblotted for phosphotyrosine. Consistent with our previous findings, Fyn led to tyrosine phosphorylation of wild-type APP (first lane). Similarly, Fyn induced phosphorylation of APP mutant 2 (third lane), but Fyn did not induce phosphorylation of APP mutant 1 (second lane). Thus, we conclude that Fyn phosphorylates the first tyrosine in the E757YENPTY762 sequence of APP. Levels of cellular APP and Fyn were consistent across conditions (bottom panels).

We also asked whether Fyn affects apoEr2 phosphorylation and transfected COS7 cells with apoEr2 and vector (Fig. 7F, first lane) or apoEr2 and Fyn constructs (second through fourth lanes). We immunoprecipitated apoEr2 and probed for phosphotyrosine. Fyn-WT and Fyn-CA induced tyrosine phosphorylation of apoEr2 (second and fourth lanes).

**Fyn Increases Levels of Cell Surface APP and ApoEr2—**To examine the effect of Fyn on APP and apoEr2 trafficking, we co-transfected COS7 cells with APP or apoEr2 and various Fyn constructs. We measured cell surface proteins by biotin-labeling live cells, isolating biotin-labeled proteins from lysates with avidin beads, and immunoblotted for apoEr2. Levels of cell surface APP and apoEr2 were increased by co-expression of Fyn-WT or Fyn-CA and Dab1 constructs. We found that Fyn did not increase cell surface levels of APP compared with vector control (first lane), but Dab1(5F) did not (fourth lane). Fyn-CA and Dab1 together increased the cell surface levels of APP (fifth lane), but Fyn-CA and Dab1(5F) did not (sixth lane). These data suggest that both Fyn and Dab1 alone, as well as active Fyn and Dab1 together, are capable of increasing surface APP. Further-

**FIGURE 8.** Fyn alone and together with Dab1 increases cell surface expression of APP and apoEr2. A, COS7 cells were transfected with APP and Fyn constructs. Cell surface proteins were biotin-labeled, isolated with avidin beads, and immunoblotted with 6E10 for APP. Fyn-WT and Fyn-CA increased surface levels of APP by 400% (p < 0.01). Immunoblots of cell lysates showed similar levels of total APP. IP, immunoprecipitation. B, COS7 cells were transfected with apoEr2 and vector or Fyn-CA. Cell surface proteins were biotin-labeled, isolated with avidin beads, and immunoblotted with 5B10 for apoEr2. Fyn-CA increased surface levels of apoEr2 by 200% (p < 0.01). Immunoblots of cell lysates showed similar levels of total apoEr2. C, COS7 cells were transfected with APP, Fyn-CA, and Dab1 constructs. Cell surface proteins were biotin-labeled, isolated with avidin beads, and immunoblotted with 6E10 for APP. Fyn-CA (second lane), Dab1 (third lane), and Fyn-CA with Dab1 (fifth lane) increased surface levels of APP, but Dab1(5F) alone (fourth lane) and in the presence of Fyn-CA (sixth lane) did not. D, primary hippocampal neurons (day in vitro 14) were transfected (Tfx) with GFP-APP and vector (top panel), Fyn CA (middle panel), or Fyn CA and Dab1 (bottom panel). Cells were incubated with anti-GFP, and surface APP was visualized with secondary antibody Alexa Fluor 594 (middle panels). Total APP was detected after permeabilization of the cells and detection with anti-GFP and Alexa Fluor 488 (left panels). The right panels show a higher magnification of dendritic processes shown in middle panels. E, fluorescence-activated cell sorting and Western blots show a higher magnification of dendritic processes shown in middle panels. Fyn and Fyn together with Dab1 increased cell surface APP intensity as demonstrated with confocal laser microscopy. E, quantification of cell surface staining as represented in D. Cell-surface APP intensity in neurons (n = 13) was analyzed using Metamorph and normalized by total APP levels. Quantification showed that cells transfected with Fyn or Fyn and Dab1 significantly increased surface levels of APP by 29% (p < 0.01) and 41% (p < 0.01), respectively. CTRL, control.
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more, inactive Dab1 inhibited the Fyn-mediated increase of surface APP.

To test these biochemical findings from COS7 cells, we conducted live cell surface immunostaining of primary hippocampal neurons. We transfected cells with N-terminal GFP-tagged APP with vector (Fig. 8D, top panel), Fyn CA (middle panel), or Fyn-CA and Dab1 (lower panel). Two days after transfection, we immunostained neurons with anti-GFP and secondary goat anti-rabbit Alexa594 to detect cell-surface APP (center column). To measure total APP, we permeabilized cells at −20 °C with methanol and immunostained again with anti-GFP, detected with secondary goat anti-rabbit Alexa488 (left column). The right column shows a higher magnification of the presence of surface APP in dendritic neuronal processes. We quantified these data and analyzed surface APP in cell bodies and neuronal processes after normalization to total APP (n = 15). Quantification of data showed a significant increase in surface APP in neurons transfected with Fyn (29%, p < 0.05) or with Fyn and Dab1 (41%, p < 0.01) compared with cells transfected with vector (Fig. 8E).

Tyrosine Phosphorylation of APP Is Altered in Fyn Knock-out Mice—To test whether Fyn affects APP phosphorylation in vivo, mouse brain lysates from wild-type or Fyn knock-out mice aged 3 months were immunoprecipitated for APP, and the precipitate was probed with an anti-phosphotyrosine antibody. Fyn knock-out brains exhibited dramatically reduced levels of tyrosine-phosphorylated APP compared with wild-type controls (Fig. 9A). Quantification showed that there was a significant decrease (60%, p < 0.001; n = 8) in tyrosine-phosphorylated APP in Fyn knock-out mice compared with controls (Fig. 9B, left). As a control, we also tested whether levels of threonine-phosphorylated APP are altered in Fyn knock-out animals. As expected, we did not find a significant difference in phosphothreonine APP in knock-out animals (12% decrease, not significant) compared with wild-type controls (Fig. 9, A, second panel, and B).

We then asked whether other Src-family tyrosine kinases are altered in Fyn knock-out animals. We immunoblotted brain lysates of knock-out or wild-type mice with antibodies against total Src, phosphorylated Src, and Yes (Fig. 9C, top three panels). We found that Src and phospho-Src levels were unaltered in knock-out animals compared with wild type; however, Yes was dramatically reduced in the Fyn knock-out. We also measured levels of Dab1 and found that Dab1 is increased in Fyn knock-out mice (fourth panel), consistent with previous literature (5). As a loading control, levels of β-actin were found to be similar across samples (bottom panel). We also measured total APP and APP CTF in wild-type and Fyn knock-out animals (Fig. 10B). We found that Fyn knock-out mice exhibited a non-significant 18% decrease in APP CTF compared with wild-type controls. Total APP expression was not altered in knock-out mice.

To determine whether Fyn affected Aβ levels in vivo, we analyzed endogenous Aβ-(1–40) in Fyn knock-out and wild-type mice by ELISA (Fig. 10C). We found a slight but non-significant increase in Aβ-(1–40) in Fyn knock-out mice compared with controls (17%). These data suggest that Fyn knock-out mice show a significant decrease in sAPPα but only slight, non-significant increases in sAPPβ and Aβ and a decrease in APP CTF.

We also measured whether Fyn affected apoEr2 processing in vivo. We found that full-length apoEr2 was not altered in Fyn knock-out mice (Fig. 10D, top panel) and that the unspliced form of apoEr2 CTF was decreased in Fyn knock-out brains, but a smaller band, the alternatively spliced form of apoEr2 CTF, was markedly increased compared with wild-type mice (middle panel). We also conducted live cell surface immunostaining of primary hippocampal neurons. We transfected cells with N-terminal GFP-tagged APP with vector (Fig. 8D, top panel), Fyn CA (middle panel), or Fyn-CA and Dab1 (lower panel). Two days after transfection, we immunostained neurons with anti-GFP and secondary goat anti-rabbit Alexa594 to detect cell-surface APP (center column). To measure total APP, we permeabilized cells at −20 °C with methanol and immunostained again with anti-GFP, detected with secondary goat anti-rabbit Alexa488 (left column). The right column shows a higher magnification of the presence of surface APP in dendritic neuronal processes. We quantified these data and analyzed surface APP in cell bodies and neuronal processes after normalization to total APP (n = 15). Quantification of data showed a significant increase in surface APP in neurons transfected with Fyn (29%, p < 0.05) or with Fyn and Dab1 (41%, p < 0.01) compared with cells transfected with vector (Fig. 8E).

Tyrosine Phosphorylation of APP Is Altered in Fyn Knock-out Mice—To test whether Fyn affects APP phosphorylation in vivo, mouse brain lysates from wild-type or Fyn knock-out (KO) mice were immunoprecipitated for APP, and the precipitate was probed for phosphotyrosine. Levels of tyrosine-phosphorylated APP were decreased in Fyn knock-out mice compared with wild-type controls (top panel). Brain lysates from wild-type or knock-out mice showed similar levels of phosphothreonine APP and total as controls (second and third panels). The absence of Fyn expression was confirmed in the knock-out mice (bottom panel) (the asterisk denotes the 55-kDa heavy-chain immunoglobulin antibody band). 8, quantification of Western blot analysis of tyrosine- and phospho-Src were not altered in Fyn knock-out mice compared with wild-type mice. Levels of Yes were significantly decreased in Fyn knock-out mice, whereas levels of Dab1 were increased.
DISCUSSION

In this study we demonstrate that Fyn-tyrosine kinase mediates the phosphorylation, cellular localization, and processing of APP and apoEr2. Transient transfection in COS7 cells, immunoprecipitation and immunostaining of primary neurons, and FLIM analysis in COS7 cells show that Fyn interacts with APP both in overexpressed systems and endogenously (Figs. 1–3). Fyn induces tyrosine phosphorylation of APP at Tyr-757 in the Y757E67T762 motif (Fig. 7) and alters the presence of APP on the cell surface (Fig. 8). Additionally, the interaction between Fyn and APP decreased Aβ production (Fig. 4). We also observed parallel effects of Fyn on apoEr2 phosphorylation (Fig. 7), localization (Fig. 8), and processing (Fig. 6). We also tested whether Fyn had an indirect effect on APP processing through interaction with Dab1, which alone is known to alter processing and localization of APP and apoEr2 (6). We found that in the presence of Dab1, Fyn induced tyrosine phosphorylation of APP (Fig. 7) and increased cell-surface levels of APP (Fig. 8). Interestingly, Fyn altered APP processing in the presence of Dab1 but not in the presence of Dab1(5F), demonstrating the importance of Dab1 phosphorylation for these effects to occur (Figs. 3, 5, 7, and 8). These data suggest that Fyn and Dab1 collaborate to exert their effects on the processing, phosphorylation, and localization of APP and apoEr2.

Based on these data, we present a model in which Fyn alters APP trafficking and mediates its processing through Dab1 (Fig. 11). Fyn interacts with APP at the YENPTY motif, known to be an internalization sequence (when the tyrosine residues of this sequence are mutated, endocytosis of APP is decreased (28)). Therefore, binding to the YENPTY motif by Fyn may increase retention of APP on the cell surface, possibly by masking this internalization sequence. This model is supported by the finding that Fyn alone can increase cell-surface expression levels of APP (Fig. 8). Furthermore, after binding to Fyn, APP can be phosphorylated by Fyn at Tyr-757 in the Y757E67T762 sequence, which may increase its ability to interact with Dab1. Fyn can then be replaced by Dab1, and the interaction of APP with Dab1 may further promote the retention of APP on the cell surface through indirect anchoring to membrane phosphoinositides (27). We and others have previously demonstrated that Dab1 may help traffic APP to membrane regions with α-secretases (6, 24). Fyn may mediate the effects of Dab1 on APP processing by recruiting Dab1 to phosphorylated APP. This model is supported by the data that Dab1 had a greater effect on APP processing in the presence of Fyn (Fig. 5). Further refinement of this model will rely on experiments with the various specific APP mutants described in this work.
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As we pursued these studies, we made the unexpected finding that Fyn-KN leads to degradation of APP through the ubiquitin-proteasome system (Fig. 4). Phosphorylation of some receptors by Fyn has been implicated in preventing their proteasomal degradation (29), although phosphorylation of the inositol 1,4,5-trisphosphate receptor by Fyn promoted its proteasomal degradation (30). Ubiquilin, a protein that regulates proteasomal degradation, mediates trafficking of APP from intracellular compartments to the cell surface (31), leading to the possibility that in the absence of phosphorylation the APP-Fyn-KN complex is unable to translocate to the cell surface and is, thus, susceptible to interactions with proteins mediating proteasomal degradation. Fyn KN-mediated degradation of holo-APP was inhibited in the presence of Dab1 (Figs. 4 and 5), demonstrating that Dab1 interferes with an APP degradative pathway, possibly by competing for binding of APP by Fyn KN. However, co-expression of Dab1 and Fyn KN did not restore levels of sAPPα or APP CTF unless a proteasomal inhibitor was added. Thus, Fyn KN still altered the normal proteolysis of APP even in the presence of Dab1 through the proteasome (Fig. 4). Impairment of the ubiquitin-proteasome system occurs in AD and in other neurodegenerative disorders (32), and these data suggest that Fyn and Dab1 could alter its effects.

We observed consistent effects of Fyn and Dab1 on APP trafficking and processing in various in vitro models. We tested these effects in vivo, examining Fyn−/− mouse brains. Fyn−/− mice demonstrated lower levels of sAPPα but only slightly lower APP CTF, higher sAPPβ, and higher Aβ-(1–40) compared with wild-type controls (Fig. 10). However, Fyn−/− mice did not provide a simple comparison to our in vitro studies. Several studies show that knock-out of Fyn kinase greatly affects the development of these mice and greatly impairs myelination (33), phosphorylation of N-methyl-d-aspartate receptors (22), and impairs LTP (34) and learning and memory (12). We and other groups have also found increases in Dab1 (5 Fig. 9C) and decreases in Yes levels (Fig. 9C) in these knock-out mice. It is, therefore, difficult to identify whether alterations in levels of various APP fragments (most notably Aβ) are due to the absence of Fyn alone or are the combined effect resulting from changes to several other proteins in these mice. Future studies utilizing acute knock-down of Fyn may prove more useful in the determination of the specific effect of Fyn on APP in the whole brain.

In our present study we also examined whether Fyn also affects apoEr2 phosphorylation, trafficking, and processing. We found that Fyn-WT and Fyn-CA induced apoEr2 phosphorylation (Fig. 7F). In contrast, only Fyn-CA induced tyrosine phosphorylation of APP (Fig. 7A). These differences may be due to differences in the phosphorylation sites present in APP and apoEr2. Although we have identified the tyrosine phosphorylated by Fyn on APP, the apoEr2 C-terminal sequence differs from that of APP in the number of tyrosine residues (35) and may be phosphorylated at multiple sites. Patterns in apoEr2 cell surface expression (Fig. 8) and processing (Fig. 6) were similar to those observed with APP. In addition, we asked whether Fyn affected apoEr2 processing in vivo in Fyn−/− mice and found that total levels of apoEr2 were unchanged compared with wild-type mice. However, we observed two apoEr2 CTF bands in the Fyn knock-out mice, in contrast to a single splice form in the wild type (Fig. 10D), suggesting that Fyn may indirectly regulate apoEr2 alternative splicing.

In summary, we provide the first evidence that Fyn alters the trafficking and phosphorylation of APP and mediates Dab1 effects on APP processing, which may be critical in the pathogenesis of AD. Studies have shown increased levels of Fyn in the brains of AD patients (36), and Fyn kinase interacts with and phosphorylates tau (37), implicating Fyn in another pathological process of AD. Because Aβ accumulation precedes tau pathology in AD, it is possible that up-regulation of Fyn in response to Aβ toxicity may be a triggering event for the onset of tau pathology. Overall, our study suggests that modulation of Fyn may be an attractive disease-modifying therapeutic approach for AD.

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