The amyloid precursor protein (APP) is proteolytically processed to release a C-terminal domain that signals to the nucleus to regulate transcription of responsive genes. The APP C terminus binds to a number of phosphotyrosine binding (PTB) domain proteins and one of these, Fe65, stimulates APP nuclear signaling. Fe65 is an adaptor protein that contains a number of protein-protein interaction domains. These include two PTB domains, the second of which binds APP, and a WW domain that binds proline-rich ligands. One ligand for the Fe65WW domain is the tyrosine kinase c-Abl. Here, we show that active c-Abl stimulates APP/Fe65-mediated gene transcription and that this effect is mediated by phosphorylation of Fe65 on tyrosine 547 within its second PTB domain. The homologous tyrosine within the motif Tyr-(Leu/Met)-Gly is conserved in a variety of PTB domains, and this suggests that PTB tyrosine phosphorylation occurs in other proteins. As such, PTB domain phosphorylation may represent a novel mechanism for regulating the function of this class of protein.

The amyloid precursor protein (APP) is a type-1 membrane protein with a large ectodomain and a smaller C-terminal intracellular domain. APP undergoes proteolytic processing by enzymes termed secretases. α,β-Secretases cleave at sites that are N-terminal to the membrane-spanning domain, and subsequently γ-secretase cleaves APP within the membrane. The results of these activities are secreted products that include the large APP extracellular domain, the 40–42-amino acid residue Aβ peptide that is deposited in the brains of patients with Alzheimer’s disease, and the remaining intracellular APP C-terminal domain (1). This C-terminal fragment contains a YENPTY motif and through this, APP binds to a number of phosphotyrosine binding (PTB) domain proteins. These include the Fe65s, X11s (also known as munc-18-interacting proteins, mint), c-Jun N-terminal kinase (JNK)-interacting protein-1 (JIP-1), Numb, ShcA, and disabled (2–16).

The molecular mechanisms that control APP/Fe65 nuclear signaling are not properly understood, and indeed, APP can function to inhibit Fe65 nuclear translocation (28). However, the Fe65WW domain is required for potent APP/Fe65-mediated transcription and also for nuclear translocation of Fe65 (19, 28). This suggests that its binding partners may contribute to the transcriptional competency of the complex. One partner, c-Abl, is known to be present within the nucleus (29, 30) and has been shown to phosphorylate APP on tyrosine 682 (27). Here, we demonstrate that active c-Abl also phosphorylates Fe65 and that this phosphorylation functions to stimulate APP/Fe65 transcriptional activity.

EXPERIMENTAL PROCEDURES

Experiments involving immunoprecipitation, glutathione S-transferase (GST) pull-downs, and GSK3β labeling were all performed at least three times with similar results. Reporter gene transcription assays were repeated as indicated in the text.

Cell Culture, Transfection, and Indirect Immunofluorescence—CHO and COS-7 cells were grown in F-12 (HAM) or Dulbecco’s modified Eagle’s medium, respectively, containing 10% (v/v) fetal bovine serum supplemented with 2 mM glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. For immunofluorescence staining, cells were grown and transfected on glass coverslips, and then fixed and processed for immunocytochemistry as described (31). Captured images were analyzed for fluorescent signal intensity using Metamorph image analysis software.

Plasmids and Mutagenesis—Plasmids for expression of wild-type and C-terminal Myc-tagged Fe65 and for the APPα isoform were described (32). Tyrosines 117, 234, 269, 270, 403, 467, 546, 547, and 658 in Fe65 were mutated to phenylalanine using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutagenic oligonucleotides were: Y117F, 5′-GCTCTGATACACTGTTCTCTCCTGAGCTTGAGGCTC-3′ and 5′-GAGTCGAAGCTAGAGAAAAGACAGGTCGATACCAGGC-3′; Y234F, 5′-CAGGGACGCCCCCTTTTGGCTCCTCAGAGAGGAC-3′ and 5′-GTCCTGCTGGGAAAGGGAGGCCGTC-3′; Y269F, 5′-GGGCGCTCCAGACGAGCTATTCCATTCTCGGACATCCCACAGGGG-3′ and 5′-CCTCGGTGTGGGATGCGCTATTCCATTCTCGGACATCCCACAGGGG-3′; Y270F, 5′-GGGCGCTCCAGACGAGCTATTCCATTCTCGGACATCCCACAGGGG-3′ and 5′-CCTCGGTGTGGGATGCGCTATTCCATTCTCGGACATCCCACAGGGG-3′; Y403F, 5′-CATCCGTCAGCCTCTCCT-
c-Abl Phosphorylates Fe65 to Enhance APP/Fe65 Transcription

TCCACAAAAAACACTG-3′ and 5′-CAGGGTGGTTTTTGGAGAAAAGAGGCTCGAGGATG-3′; Y467F, 5′-GAGGGACCTCTGTCTGAGGAAAAATGTACCTG-3′ and 5′-CAGGACATTCTCCCAGGTAAAAAGACCTTGGAAAATGTACCTG-3′; Y546F, 5′-GAGGAAAGGCTTACTATTTTCTCTGAGGAAATGTACCTG-3′ and 5′-CAGGACATTCTCCCAGGTAAAAAGACCTTGGAAAATGTACCTG-3′; Y547F, 5′-GAGGAAAGGCTTACTATTTTCTCTGAGGAAATGTACCTG-3′ and 5′-CAGGACATTCTCCCAGGTAAAAAGACCTTGGAAAATGTACCTG-3′; Y658F, 5′-GCGGTGATGCTCTCCTTGGCAGAAGTGCTGTG-3′ and 5′-CATCCAGACACTCTGGAGGACAGCTAGCAGCGC-3′. Tyrosine 682 in APP was mutated to phenylalanine in a similar fashion using oligonucleotides 5′-GATGACACGACACGGCTTCGAAAATCCAAACTCAACCTTGC-3′ and 5′-GGCTCAGTCTACGAGGAGGATTTTGCAGG-3′ which truncates the GAL4 sequence so that it contains only the DNA binding domain. Mutagenic oligonucleotides were 5′-CAGGGTGGTTTTTGGAGAAAAGAGGCTCGAGGATG-3′ and 5′-CAGGGTGGTTTTTGGAGAAAAGAGGCTCGAGGATG-3′, and 5′-GAGGGACCTCTGTCTGAGGAAAAATGTACCTG-3′ and 5′-GAGGGACCTCTGTCTGAGGAAAAATGTACCTG-3′.

FIG. 1A. Fe65 binds to c-Abl and c-AblΔXB through its WW domain, and Fe65 and c-Abl are both present in the nucleus. A, the presence of c-Abl and c-AblΔXB in Fe65 immunoprecipitates from Fe65 + c-Abl- and Fe65 + c-AblΔXB-co-transfected cells. Fe65 was immunoprecipitated using antibody 9B11 to the Myc tag, and the immunoprecipitates were then probed on immunoblots for Fe65 (using a polyclonal Fe65 antibody), c-Abl, or c-AblΔXB as indicated. Samples of the lysates are also shown. (+) and (−) refer to the presence or absence of antibody 9B11 to the Fe65 Myc tag in the immunoprecipitations. B, GST pull-downs from c-Abl- or c-AblΔXB-transfected cells using equimolar amounts of GST or GST-Fe65WW domain baits as indicated. Captured proteins were probed for c-Abl on immunoblots. Samples of the lysates are also shown. Note that Fe65 binds a greater proportion of c-AblΔXB than c-Abl in both assays. C, GST pull-downs from c-AblΔXB-transfected cells using baits equimolar amounts of GST, GST-Fe65WW domain, or GST-Fe65WW mutant involving altering the Tyr-Tyr-Trp motif to Ala-Ala-Ala (GST-Fe65WW). Captured proteins were probed for c-Abl on immunoblots. A sample of the lysate is also shown. GST-Fe65WW binds less c-AblΔXB than GST-Fe65WW. D, immunofluorescent staining of Fe65 and c-Abl in Fe65 + + c-Abl- and Fe65 + c-AblΔXB-co-transfected cells. Scale bar in C is 10 μm.

GAL4UAS-dependent firefly luciferase reporter pFR-Luc and transfection efficiency Renilla luciferase pHRL-TK plasmids were obtained from Stratagene and Promega, respectively. Plasmid expressing the GAL4 DNA binding domain fused to the C-terminal 50 amino acids of APP (GAL4-APPc) and which corresponds to an in vivo fragment generated by ϕC31 cleavage (34–36) was created by subcloning APP-encoding sequences into pM1 (37). Full-length APP into which the GAL4 DNA binding domain had been engineered (GAL4-APP) was as described (19). Full-length APP containing the GAL4 DNA binding domain at its C terminus was created by mutating residue 148 of the GAL4 sequence to a stop codon in plasmid APP-GAL4 (kind gift of Tomasso Russo). Plasmid APP-GAL4 encodes a chimeric protein comprising full-length GAL4 fused to the C terminus of APP (38). Mutation of GAL4 residue 148 truncates the GAL4 sequence so that it contains only the DNA binding domain. Mutagenic oligonucleotides were 5′-C- TATCAGATTGACTAGCGCAGTCTAG-3′ and 5′-CATATGAGGTTCTCTGATCAGCAG-3′. To express the C-terminal domain of APP, sequences encoding the last 50 residues of APP were cloned into the vector pCMV-Tag2 (Stratagene).

SDS-PAGE and Immunoblotting—Samples were processed for SDS-PAGE by addition of SDS-PAGE sample buffer and heating in a boiling water bath. Samples were separated on either 8.5 or 12.5% (w/v) acrylamide gels and transferred to Protran nitrocellulose membranes (Schleicher & Schuell) using a Bio-Rad TransBlot system. Following blocking and probing with primary antibodies, the blots were washed and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig (Amersham Biosciences) and developed using an enhanced chemiluminescence system (Amersham Biosciences) according...
to the manufacturer's instructions. The Fe65 and APP (APPhab recognizing the C terminus of APP) antibodies have been described previously (32, 39, 40). Anti-Myc antibody 9B11 (that recognizes Myc-tagged Fe65) was obtained from Cell Signaling Technology, c-Abl antibody (24–11) was from Santa Cruz Biotechnology, APP antibody 22C11 (recognizing the N terminus of APP) was from Roche Applied Science, phosphotyrosine antibody 4G10 was from Upstate Cell Signaling, anti- GSK3β was from BD Transduction Laboratories, and antibody DmA1 to tubulin was from Sigma.

Immunoprecipitation and GST Pull-down Assays—Immunoprecipitation and GST pull-down assays were performed as previously described (39, 41). Briefly, for immunoprecipitation assays, cells were lysed in ice-cold lysis buffer comprising 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium orthovanadate, 30 mM sodium fluoride, and protease inhibitors (Complete, Roche Applied Science) and then cleared by centrifugation at 14,000 × g for 30 min at 4 °C. Cell lysates were then precleared with either protein A- or protein G-Sepharose beads (Sigma) and the target protein immunoprecipitated. Following washing of the beads in lysis buffer, immunoprecipitated proteins were resolved by SDS-PAGE and detected by immunoblotting.

GST and GST-Fe65WW domain proteins expressed in Escherichia coli BL21(DE3) were prepared essentially according to the manufacturer's instructions (Amersham Biosciences). Equimolar amounts of GST or GST-Fe65WW domain baits were used in pull-down assays from transfected cell lysates. Cell lysates were prepared by harvesting cells into lysis buffer as described above. Captured proteins were then isolated by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting.

Mass Spectrometric Sequencing of Fe65—Fe65 was sequenced by on-line liquid chromatography tandem mass spectrometry (LC/MS/MS) as recently described by us (42). Briefly, Fe65 was isolated by immunoprecipitation from Fe65 and Fe65 + c-AblXXB-co-transfected cells using antibody 9B11 to the Myc tag on Fe65 and resolved by SDS-PAGE. The bands corresponding to Fe65 were excised, reduced, alkylated, and digested with either trypsin, Asp-N, Lys-C, or chymotrypsin (Roche Applied Science) and then cleared by centrifugation at 15,000 × g for 30 min at 4 °C. Cell lysates were then precleared by harvesting cells into lysis buffer as described above. Captured proteins were then isolated by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting.

Chromatographic separations were performed using an Ultimate LC system (Dionex). Peptides were ionized by electrospray ionization using a Z-spray source fitted to a QToF-micro (Micromass). The instrument was set to run in automatic switching mode, selecting precursor ions based on their intensity and charge state, for sequencing by collision-induced fragmentation. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass/charge (m/z) and the charge state of the peptide and optimized for phosphorylated peptides.

The mass spectral data were processed into peak lists containing the m/z value of each precursor ion and the corresponding fragment ion m/z values and intensities. Data were searched against a custom built data base containing the full-length sequence of Fe65 using the Mascot searching algorithm (Matrix Science). Peptides and phosphopeptides of Fe65 were identified by matching the MS/MS data against mass values generated from the theoretical fragmentation of peptides based on the search criteria set (i.e., the cleavage enzyme used with up to 3 missed cleavages, carbamidomethyl modification of cysteine residues, oxidized methionine, deamidation of asparagine and glutamine residues, and N-acetylation of the protein). Phosphorylated peptides were identified by selecting for serine/threonine and tyrosine phosphorylation as a variable modification. The exact location of phosphorylation within each peptide was determined by the pattern of fragment ions produced.

Luciferase Assays—Luciferase assays were performed using a Dual-Glo luciferase assay system according to the manufacturer's instructions (Promega). Briefly, cells were harvested into Glo lysis buffer (Promega) 24 h post-transfection and the lysates then transferred to a 96-well luminometer plate (Wallac). An equal volume of Dual-Glo luminometer substrate was added and firefly luciferase activity was determined from transfected CHO cells using the GAL4UAS-dependent firefly luciferase reporter plasmid pFR-Luc measured using a Wallac TriLux luminometer. Renilla luciferase activities produced by the pHRL-TK transfection efficiency control plasmid were then assayed by adding an equal volume of Dual-Glo Stop&Glo substrate (comprising the stop solution for firefly luciferase and its substrate, Stop&Glo substrate, and firefly luciferase) and remeasuring in the luminometer. All luciferase transfections received the same number and amount of plasmids, which was achieved by transfection of vector pCNeo-CAT where appropriate; pCNeo is the vector used for expression of Fe65 in these assays. Firefly luciferase activities were standardized to the corresponding Renilla luciferase activities and statistical analyses performed using one-way analysis of variance tests. Results shown were obtained using CHO cells, but similar data were obtained using COS-7 cells.

In Vitro Phosphorylation of Fe65 by c-Abl—c-AblXXB was isolated from transfected CHO cells by immunoprecipitation using c-Abl antibody 24–11. For in vitro phosphorylation of recombinant GST fusion proteins, 1 μg of each substrate was incubated with immunoprecipitated kinase (prepared from 200 μg of precleared lysate) in 25 mM HEPES pH 7.5 containing 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM sodium orthovanadate, 2 mM diithothreitol, 0.185 mM Mg2+/F1ATP, and 20 μM ATP for 20 min at 30 °C in a final volume of 40 μL. Reactions were stopped by addition of SDS sample buffer and heating in a boiling water bath. Samples were separated on 10% (w/v) acrylamide SDS-PAGE gels, and the gels then subjected to autoradiography.

RESULTS

We initially confirmed that Fe65 interacts with c-Abl and a dominantly active mutant of c-Abl (c-AblΔXXB) using immunoprecipitation and GST pull-down assays. Fe65 from Fe65 + c-Abl or Fe65 + c-AblXXB-transfected CHO was immunoprecipitated using the Myc tag on Fe65 and bound c-Abl detected on
immunoblots. Fe65 interacted with both c-Abl and c-AbΔXB but the binding to c-AbΔXB was stronger (Fig. 1A). In complementory experiments, GST or GST-Fe65WW domain baits were used in pull-down assays from c-AbΔ- and c-AbΔXB-transfected CHO cells. Again, both c-AbΔ and c-AbΔXB bound Fe65 with the c-AbΔ interaction stronger (Fig. 1B). Mutation of the conserved aromatic residues Tyr-Tyr-Trp within the Fe65WW domain to Ala-Ala-Ala inhibited binding of c-Abl through its WW domain, that this interaction is stronger with active isosforms of c-Abl, and that a proportion of both Fe65 and c-Abl are present within the nucleus (27, 30).

We next examined the effect of c-AbΔXB on APP/Fe65-mediated transcription. To do so, we utilized a previously described GAL4-dependent reporter system that involves monitoring the transcriptional activity of APP-GAL4 DNA binding domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter system that involves monitoring the transcriptional activity of APP-GAL4 DNA binding domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or domain fusion genes using a GAL4UAS-luciferease reporter (19).
the absence of co-transfected Fe65 may well involve endoge-
nous Fe65, because Fe65 is expressed in CHO and COS-7 cells (42). Mutation of the conserved aromatic residues Tyr-Tyr-Trp
within the Fe65WW domain to Ala-Ala-Ala markedly inhibited
its stimulatory effect (Fig. 2). This finding is consistent with
the GST pull-down assays, which showed that this mutation
inhibited binding of the WW domain to c-AblΔXB (Fig. 1C).
Tyrosine 682 within the intracellular C-terminal domain of
APP has been shown to be phosphorylated by active c-Abl (27),
and so one possibility is that the effect of c-AblΔXB on APP/
Fe65-mediated transcription is due to phosphorylation of this
residue. We therefore confirmed that c-AblΔXB phosphorylated
PPY-Tyr682 by monitoring the reactivity of APP and a mutant of
APP in which tyrosine 682 was altered to phenylalanine with
antibody 4G10 that detects phosphorytrosines. APP immuno-
precipitated from APP+c-AblΔXB- but not APP-transfected
cells was reactive with antibody 4G10, and this reactivity
was abolished by mutation of APPY682 (Fig. 3A). Thus, we con-
figured that APPY682 is phosphorylated in APP+c-AblΔXB-
transfected cells as previously described (27).

An alternative possibility is that c-Abl also phosphorylates
Fe65, and the stimulatory effect of c-AblΔXB on transcription
is caused by Fe65 phosphorylation. We therefore isolated Fe65 by
immunoprecipitation from Fe65- or Fe65+c-AblΔXB-co-trans-
fected cells, and it was sequenced by mass spectrometry. We
obtained over 80% sequence coverage and detected one peptide
with a phosphorylated tyrosine residue (Fig. 3B). This peptide
contains two adjacent tyrosines (tyrosines 546/547), although
despite repeated sequence runs, we were unable to unambigu-
ously distinguish which tyrosine was phosphorylated. We
therefore prepared mutants of Fe65 in which either of these
tyrosines were mutated to phenylalanine to preclude phos-
phorylation and examined their reactivities with antibody
4G10 in Fe65+c-AblΔXB-co-transfected cells. Immunoprecipi-
tated Fe65Y546F but not Fe65Y547F was reactive with 4G10 in
these assays (Fig. 3C). To confirm that no other tyrosines in
Fe65 were phosphorylated in the Fe65+c-AblΔXB-co-trans-
fected cells, we mutated individually, the remaining seven ty-
rosines to phenylalanine, and in a similar fashion, monitored
the reactivities of these mutants with antibody 4G10. Mutation
of these other tyrosines had no effect on 4G10 labeling (Fig.
3C). Thus, Fe65 is phosphorylated on a single residue, tyrosine
547, in c-AblΔXB-transfected cells.

The above studies are consistent with a direct phosphoryla-
tion of Fe65 by c-AblΔXB but do not eliminate the possibility
that c-AblΔXB activates some other tyrosine kinase that then
phosphorylates Fe65. To determine whether c-AblΔXB can
directly phosphorylate Fe65, we therefore performed in vitro
phosphorylation assays using the second Fe65-PTB domain
(which contains tyrosine 547) as a GST fusion protein
substrate. These assays revealed that c-AblΔXB could directly
phosphorylate both APP (on tyrosine 682) and Fe65 (on tyrosine
547).

To examine whether the stimulatory effect of c-AblΔXB on
APP/Fe65-mediated transcription involves phosphorylation of
either APPY682 or Fe65Y547, we performed further GAL4-
APPc-dependent transcription assays using mutants in which
these residues were altered to phenylalanine to preclude phos-
phorylation. Fe65 and c-AblΔXB both stimulated transcription
of mutant GAL4-APPY682F, and this stimulation was greater
than that of wild-type GAL4-APPc (Fig. 4A). Others have also
shown that mutation of APPY682 to phenylalanine enhances
transcriptional activity of GAL4-APP fusions (43). The mecha-
nisms that underlie this effect are not known but mutation of
APPY682 to phenylalanine does not influence either APP/Fe65 or
APP/JIP-1 interactions (Ref. 43 and see below). Thus, the stim-
ulatory effect of c-AblΔXB on APP/Fe65 transcription cannot be
through phosphorylation of APP682.

We next tested whether the stimulatory effect of c-AblΔXB
on APP/Fe65 transcription was through phosphorylation of
Fe65Y547. Mutation of this residue to phenylalanine to pre-
clude phosphorylation (Fe65Y547F) completely eliminated the
effect of c-AblΔXB (Fig. 4A). However, Fe65Y547F was still capa-
cble of stimulating GAL4-APPc-dependent transcription (Fig.
4A). This latter observation suggests that a component of the
Fe65 stimulatory effect on GAL4-APPc-mediated transcription
is not dependent upon phosphorylation of Fe65Y547. It also
suggests that mutation of Fe65Y547 to phenylalanine does not
induce some conformational change to this domain of the pro-
tein that precludes the ability of Fe65 to stimulate transcrip-
tion from GAL4-APPc. This was further confirmed by monitor-
ing the effect of mutating the adjacent tyrosine (Fe65Y546F)
to phenylalanine (Fe65Y546F). This mutant behaved in a similar
fashion to wild-type Fe65 (Fig. 4F). We also tested whether
mutation of Fe65Y547F inhibited transcription from full-length
GAL4-APP and obtained similar results to GAL4-APPc (Fig.
4B). Thus, the stimulatory effect of c-AblΔXB on APP/Fe65
transcription is through phosphorylation of Fe65Y547.

Tyrosine 547 resides within the second PTB domain of
Fe65 and because this domain binds APP, it is possible that
its phosphorylation influences Fe65-APP interactions in
some manner. We therefore performed immunoprecipitation
assays to determine whether c-AblΔXB altered binding of
APP and GAL4-APPc to Fe65. Fe65 isolated from Fe65 +APP-
or Fe65 +APP+c-AblΔXB-transfected cells bound identical
amounts of APP (Fig. 5A). Likewise, the presence of c-AblΔXB
did not influence the amounts of GAL4-APPc bound to Fe65
(Fig. 5B). To determine whether c-AblΔXB influenced binding
of APP to endogenous Fe65, we immunoprecipitated APP from APP- or APP+c-Ab1ΔXB-transfected cells and probed for bound Fe65. However, we again could detect no differences in the amounts of co-immunoprecipitating Fe65 (Fig. 5C). We also probed these samples with antibody 4G10, and this demonstrated that at least a proportion of Fe65 that was bound to APP was tyrosine-phosphorylated.

We next tested whether the Fe65Y547F and APPY682F mutations altered binding of APP and Fe65, respectively, in immunoprecipitation experiments. However, neither of these mutants had altered binding properties (Fig. 5D). Mutant APPY682F has previously been shown to bind Fe65 (43). Thus, c-Ab1ΔXB phosphorylates Fe65 within its second PTB domain to stimulate APP/Fe65 transcriptional activity, but this stimulation does not appear to be through an overt effect on Fe65/APP interactions.

Recently, APPc has been shown to induce expression of the GSK3β gene (44). We therefore asked whether c-Ab1ΔXB stimulated the APPc-dependent expression of GSK3β by analyzing GSK3β protein levels in cells transfected with APPc either alone or with Fe65 and c-Ab1ΔXB using immunoblotting techniques. Because we obtain 30–40% transfection efficiencies, any changes observed in these pooled samples of transfected and non-transfected cells is likely to be less than that seen in individual transfected cells. Nevertheless, although we observed little change to GSK3β protein levels following co-transfection of APPc with Fe65 or c-Ab1ΔXB alone, we detected a marked increase in GSK3β signal in cells transfected with all three plasmids (Fig. 6A). We also studied the effect of c-Ab1ΔXB on GSK3β protein levels by immunostaining, and this revealed that transfection of APPc+Fe65+c-Ab1ΔXB increased the GSK3β signal compared with non-transfected cells (Fig. 6B).

**DISCUSSION**

The functions of APP are not properly understood. However, several recent studies have demonstrated that the C-terminal domain of APP, produced by γ-secretase activity, can translocate to the nucleus to regulate transcriptional events (19–24, 43, 45, 46). One APP binding partner that is involved in this process is the adaptor protein Fe65 (19, 21–24, 43). Fe65 is present within the nucleus and, aside from APP, binds to at least two transcription factors, CP2/LSF/LBP1 and Tip60 (19, 25). The Fe65WW domain is required for its stimulatory effect on APP-mediated transcription (19) and also for nuclear translocation of Fe65 (28). This suggests that WW domain ligands are required for the nuclear functions of Fe65. One Fe65WW domain ligand is the tyrosine kinase c-Ab1 (25). c-Ab1 phosphorylates APP on tyrosine 682 (25) and here, we demonstrate that it additionally phosphorylates Fe65 on tyrosine 547. We also show that active c-Ab1 stimulates APP/Fe65-mediated transcription and that this is through phosphorylation of Fe65Y547F but not APPY682F.

Tyrosine phosphorylation of the C-terminal domain of APP does not influence APP binding to Fe65, although it can modulate interactions with ShcA and disabled, two other PTB domain proteins (5, 10, 14, 47). The residue within Fe65 that is phosphorylated by active c-Ab1 (tyrosine 547) resides within the second PTB domain, and this domain binds to APP. However, Fe65 and Fe65Y547F (which cannot be phosphorylated) both bound APP equally well in immunoprecipitation experiments, and we could detect no changes in Fe65/APP or Fe65/APPc-GAL4 interactions in cells co-transfected with c-Ab1ΔXB. As such, phosphorylation of APPY682F or Fe65Y547F by active c-Ab1 appears not to influence APP/Fe65 interactions in any overt manner. Thus, while the stimulatory effect of c-Ab1ΔXB on APP/Fe65 transcription is mediated by Fe65Y547F phosphorylation, this does not involve any marked changes in binding of APP to Fe65.

Platelet-derived growth factor has recently been shown to induce β,γ-secretase cleavage of APP and this was characterized by monitoring the transcriptional activity of an APP-GAL4
fusional evidence to support this notion. Nevertheless, it remains likely that phosphorylation of Fe65 (and perhaps APP) by c-Abl somehow alters the various protein–protein interactions of the Fe65/APP transcriptional complex. One way to gain insight into this would be to solve the structure of non-phosphorylated and phosphorylated Fe65 bound to the C terminus of APP.

Tyrosine 547 is located toward the N terminus of the Fe65 PTB domain and falls within the motif Tyr-Leu-Gly. This motif is conserved in a number of other PTB-bearing proteins including Fe65like-1 and like-2 (Fe65like-2 contains the motif Tyr-Met-Gly), X11α, X11β, She, and Numb. We are unaware of any report describing phosphorylation of the homologous tyrosine in these other PTB domain proteins. However, our finding that the Fe65 PTB domain is phosphorylated raises the possibility that these other proteins may also be tyrosine-phosphorylated on their PTB domains. Tyrosine phosphorylation of PTB domains might therefore be a novel mechanism for regulating the function of this class of protein.

Mis-metabolism of APP is believed to be central to the pathogenesis of Alzheimer's disease. Altered APP processing leading to increased production of Aβ is one favored pathogenic event but such changes are also likely to influence APP/Fe65 nuclear signaling, and this too may contribute to the neurodegenerative process (48). Indeed, familial Alzheimer's disease mutant presenilin-1 has recently been shown to have altered nuclear signaling function and this has been causally related to familial forms of Alzheimer's disease (49). Thus, defective phosphorylation of Fe65 by active c-Abl may alter APP/Fe65 nuclear signaling, and this might also contribute to Alzheimer's disease.

Acknowledgments—We thank Thomas Sudhof (University of Texas, Dallas) and Richard Van Etten (Harvard University, Boston) for GAL4-APP and c-Abl plasmids, respectively.

REFERENCES

1. De Strooper, B., and Annenat, W. (2000) J. Cell Sci. 113, 1857–1870
2. Fiore, F., Zambrano, N., Minopoli, G., Donini, V., Duilio, A., and Russo, T. (1995) J. Biol. Chem. 270, 30853–30856
3. McLaughlin, D. M., and Miller, C. C. J. (1996) FEBS Lett. 397, 197–200
4. Bessler, S. L., Gray, M. D., Sopher, B. L., Hu, Q. B., Hearn, M. G., Pham, D. G., Dunois, M. B., Fukushima, K. I., Sisodias, S. S., Miller, M. A., Dieste, C. M., and Martin, G. M. (1996) Hum. Mol. Genet. 5, 1589–1598
5. Borg, J.-P., Ooi, J., Levy, E., and Margolis, B. (1996) Mol. Cell. Biol. 16, 6229–6241
6. Guenette, S. Y., Chen, J., Jondro, P. D., and Tonz, R. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10832–10837
7. Duilio, A., Faraco, R., Minopoli, G., Zambrano, N., and Russo, T. (1998) Biochem. J. 330, 513–519
8. Trommsdorff, M., Berg, J.-P., Margolis, B., and Herz, J. (1998) J. Biol. Chem. 273, 33556–33560
9. Tomita, S., Ozaki, T., Taru, H., Oguchi, S., Takeda, S., Yagi, Y., Sakiyama, S., Kirino, Y., and Suzuki, T. (1999) J. Biol. Chem. 274, 2243–2254
10. Howell, B. W., Lanier, L. M., Frank, R., Gertler, F. B., and Cooper, J. A. (1999) Mol. Cell. Biol. 19, 5179–5188
11. Matsuda, S., Yasakiwa, T., Homma, Y., Ito, Y., Niikura, T., Hiraki, T., Hira, S., Ohno, S., Kita, Y., Kawasumi, M., Koyama, K., Yanamato, T., Kyriakis, J. M., and Nishimoto, I. (2001) J. Neurosci. 21, 6597–6607
12. Roncarati, R., Sestan, N., Scheinfeld, M. H., Berechid, B. E., Lopez, P. A., Meucci, O., McGlade, J. C., Rakic, P., and D'Adamo, L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7102–7107
13. Scheinfeld, M. H., Roncarati, R., Vito, P., Lopez, P. A., Abdallah, M., and D'Adamo, L. (2002) J. Biol. Chem. 277, 3767–3775
14. Tarr, P. E., Roncarati, R., Pelcic, G., Pelcic, P. G., and D'Adamo, L. (2002) J. Biol. Chem. 277, 16798–16804
15. Taru, H., Iijima, K., Hase, M., Kirino, Y., Yagi, Y., and Suzuki, T. (2002) J. Biol. Chem. 277, 20070–20078
16. Homayouni, R., Rice, D. S., Sheldon, M., and Curran, T. (1999) J. Neurosci. 19, 7507–7515
17. Back, S. H., Ohgi, K. A., Rose, D. W., Koo, E. H., Glass, C. K., and Rosenfeld, M. G. (2002) Cell 110, 55–67
18. Gao, Y., and Pimplikar, S. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14979–14984
19. Cao, X., and Sudhof, T. C. (2001) Science 293, 115–120
20. Cuperus, P., Orlans, I., Craessaerts, K., Annaert, W., and De Strooper, B. (2001) J. Neurochem. 78, 1168–1178
21. Kinoshita, A., Zheng, J. B., Guenette, S. Y., and Selmke, D. J. (2001) J. Biol. Chem. 276, 40268–40292
22. Kinoshita, A., Whelan, C. M., Smith, J. C., Berezovska, O., and Hyman, B. T.
23. Scheinfeld, M. H., Gherzi, E., Laky, K., Fowlkes, B. J., and D'Adamio, L. (2002) *J. Biol. Chem.* 277, 44195–44201
24. Walsh, D. M., Fadewa, J. V., LaVoe, M. J., Paliga, K., Eggert, S., Kimberly, W. T., Wasko, W., and Selkoe, D. J. (2003) *Biochemistry* 42, 6664–6673
25. Zambrano, N., Minopoli, G., De Candia, P., and Russo, T. (1998) *J. Biol. Chem.* 273, 20128–20133
26. Ermekova, K. S., Zambrano, N., Linn, H., Minopoli, G., Gertler, F., Russo, T., and Sudo, M. (1997) *J. Biol. Chem.* 272, 32869–32877
27. Zambrano, N., Bruni, P., Minopoli, G., Mosca, R., Molino, D., Russo, C., Schettini, G., Sudo, M., and Russo, T. (2001) *J. Biol. Chem.* 276, 19787–19792
28. Minopoli, G., De Candia, P., Benetti, A., Farasino, R., Zambrano, N., and Russo, T. (2001) *J. Biol. Chem.* 276, 6545–6550
29. Shaul, Y. (2000) *Cell Death Differ.* 7, 10–16
30. Van Etten, R. A. (1999) *Trends Cell Biol.* 9, 179–186
31. McLoughlin, D. M., Irving, N. G., Brownlee, J., Brion, J.-P., Leroy, K., and Miller, C. C. J. (1999) *Eur. J. Neurosci.* 11, 1988–1994
32. Lau, K.-F., McLoughlin, D. M., Standen, C. L., Irving, N. G., and Miller, C. C. J. (2000) *NeuroReports* 16, 555–563
33. Zukerberg, L. R., Patrick, G. N., Nikolic, M., Humbert, S., Wu, C. L., Lanier, L. M., Gertler, F. B., Vidal, M., Van Etten, R. A., and Tsai, L.-H. (2000) *Neuron* 26, 633–646
34. Gu, Y. J., Misonou, H., Sato, T., Dohmae, N., Takio, K., and Ihara, Y. (2001) *J. Biol. Chem.* 276, 35235–35238
35. Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M. M., Teplow, D. B., and Haase, C. (2001) *EMBO Rep.* 2, 835–841
36. Weidemann, A., Eggert, S., Reinhardt, F. B., Vogel, M., Paliga, K., Baier, G., Masters, C. L., Beyreuther, K., and Evin, G. (2002) *Biochemistry* 41, 2825–2828
37. Sadowski, I., Bell, B., Broad, P., and Hollis, M. (1992) *Gene (Amst.)* 118, 137–141
38. Giann, D., Zambrano, N., Bimonte, M., Minopoli, G., Mercken, L., Talamo, F., Scalsini, A., and Russo, T. (2003) *J. Biol. Chem.* 278, 9280–9287
39. Lau, K.-F., McLoughlin, D. M., Standen, C., and Miller, C. C. J. (2000) *Mol. Cell. Neurosci.* 16, 555–563
40. Kesavapany, S., Banner, S. J., Lau, K. F., Shaw, C. E., Miller, C. C., Cooper, J. D., and McLoughlin, D. M. (2002) *Neuroscience* 115, 951–960
41. McLoughlin, D. M., Standen, C. L., Lau, K.-F., Ackerley, S., Bartnikas, T. P., Gitlin, J. D., and Miller, C. C. J. (2001) *J. Biol. Chem.* 276, 5903–5907
42. Standen, C. L., Perkinton, M. S., Byers, H. L., Kesavapany, S., Lau, K. F., Ward, M., McLoughlin, D., and Miller, C. C. (2003) *Mol. Cell Neurosci.* 24, 851–857
43. Scheinfeld, M. H., Matsuda, S., and D'Adamio, L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 1729–1734
44. Kim, H. S., Kim, E. M., Lee, J. P., Park, C. H., Kim, S., Seo, J. H., Chang, K. A., Yu, E., Jeong, S. J., Chong, Y. H., and Suk, Y. H. (2003) *FASEB J.* 17, 1951–1953
45. Kinoshita, A., Whelan, C. M., Berezovska, O., and Hyman, B. T. (2002) *J. Biol. Chem.* 277, 28530–28536
46. Biedermann, T., Cao, X., Sudhof, T. C., and Liu, X. (2002) *J. Neurosci.* 22, 7340–7351
47. Russo, C., Dolfini, V., Salis, S., Venezia, V., Violani, E., Carlo, P., Zambrano, N., Russo, T., and Schettini, G. (2002) *Ann. N. Y. Acad. Sci.* 973, 323–333
48. Robakis, N. K. (2003) *Amyloid* 10, 80–85
49. Maramboud, P., Wen, P. H., Dutt, A., Shini, J., Takashima, A., Siman, R., and Robakis, N. K. (2003) *Cell* 114, 635–645
50. Hoepstreff, P., and Fehlman, J. (1984) *Biomed. Mass Spectrom.* 11, 601