THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 21, Issue of May 21, pp. 22084–22091, 2004
Printed in U.S.A.

The c-Abl Tyrosine Kinase Phosphorylates the Fe65 Adaptor Protein to Stimulate Fe65/Amyloid Precursor Protein Nuclear Signaling*

Received for publication, October 20, 2003, and in revised form, February 16, 2004
Published, JBC Papers in Press, March 18, 2004, DOI 10.1074/jbc.M311479200

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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)[1] 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, mints), c-Jun N-terminal kinase (JNK)-interacting protein-1 (JIP-1), Numb, ShcA, and disabled (2–16).

The functions of APP are not properly understood, although recently its proteolytically processed C-terminal domain has been shown to translocate to the nucleus to regulate transcription (17–24). To do so, it complexes with a number of nuclear proteins, one of which is the adaptor protein Fe65. Fe65 contains a variety of protein-protein interaction domains including two C-terminal PTB domains (the second of which binds APP) and a WW domain that interacts with proline-rich ligands. The first Fe65 PTB domain binds to two transcription factors, the histone acetyltransferase Tip60 and CP2/LSF/LBP1, and Tip60 stimulates APP/Fe65 transcriptional activity (19, 25). The full complement of binding partners for the Fe65WW domain are not known, but include the mammalian homologue of *Drosophila* enabled (Mena) and the c-Abl tyrosine kinase (26, 27).

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 glutamine, 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 APP_{695} isoform were as 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′-GGCCTGATACACCTGTTCTCTGAGCTGGAGCTC-3′ and 5′-GAGCTCCAGCTCAGAGAACAGGTGTATCAGGCC-3′; Y234F, 5′-CAGGGCAGCCCCTCCTTTGGCTCCCCAGAGGAC-3′ and 5′-GTCCTCTGGGGAGCCAAAGGAGGGGCTGCCCTG-3′; Y269F, 5′-GGACACCTCAGGGACCTTTTACTGGCACATCCCAACAGGG-3′ and 5′-CCCTGTTGGGATGTGCCAGTAAAAGGTCCCTGAGGTGTCC-3′; Y270F, 5′-GGACACCTCAGGGACCTATTTCTGGCACATCCCAACAGGG-3′ and 5′-CCCTGTTGGGATGTGCCAGAAATAGGTCCCTGAGGTGTCC-3′; Y403F, 5′-CATCCGTCAGCTCTCTT-

* This work was supported by grants from the Medical Research Council, Wellcome Trust, European Union Vth Framework, and Research into Aging. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: APP, amyloid precursor protein; GSK3β, glycogen synthase kinase-3β; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; PTB, phosphotyrosine binding; APPc, the C-terminal domain of APP.

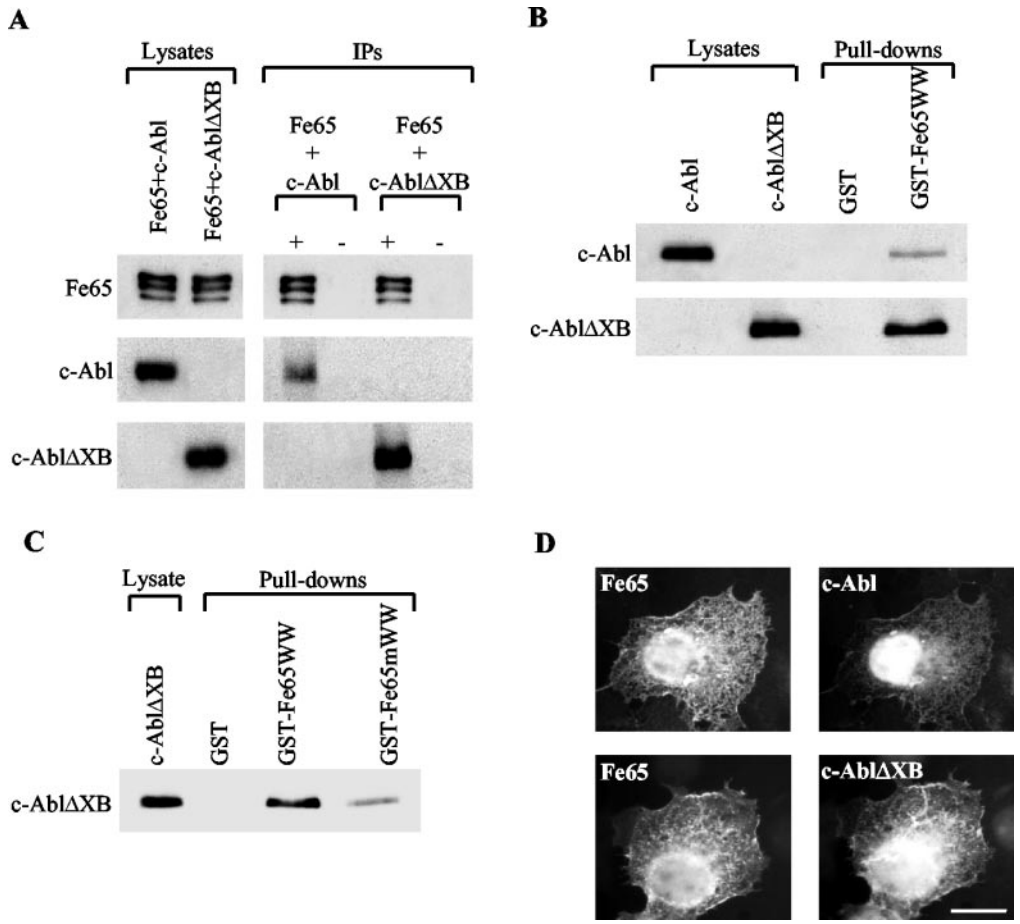

FIG. 1. **Fe65 binds to c-Abl and c-AblΔXB through its WW domain, and Fe65 and c-Abl are both present in the nucleus.** *A*, the presence of c-Abl and c-AblΔXB in Fe65 immunoprecipitates from Fe65+c-Abl- and Fe65+c-AblΔXB-co-transfected cells. Fe65 was immunoprecipitated using antibody 9B11 to the Myc 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 as baits equimolar amounts of GST, GST-Fe65WW domain, or a GST-Fe65WW mutant involving altering the Tyr-Tyr-Trp motif to Ala-Ala-Ala (GST-Fe65mWW). Captured proteins were probed for c-Abl on immunoblots. A sample of the lysate is also shown. GST-Fe65mWW 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.

TCCACAAAAACAACCTG-3′ and 5′-CAGGTTGTTTTTGTGGAAAGAGAGCTGACGGATG-3′; Y467F, 5′-GAGAGGGACTTTGCCTTCGTAGCTCGTGATAAG-3′ and 5′-CTTATCACGAGCTACGAAGGCAAAGTCCCTCTC-3′; Y546F, 5′-CAGAAGTTCCAAGTCTTTTACCTGGGGAATGTACCTG-3′ and 5′-CAGGTACATTCCCCAGGTAAAAGACTTGGAACTTCTG-3′; Y547F, 5′-CAGAAGTTCCAAGTCTATTTCCTGGGGAATGTACCTG-3′ and 5′-CAGGTACATTCCCCAGGAAATAGACTTGGAACTTCTG-3′; Y658F, 5′-GCGTGCATGCTTCGCTTCCAGAAGTGTCTGGATG-3′ and 5′-CATCCAGACACTTCTGGAAGCGAAGCATGCACGC-3′. Tyrosine 682 in APP was mutated to phenylalanine in a similar fashion using oligonucleotides 5′-GATGCAGCAGAACGGCTTCGAAAATCCAACCTACAAGTTC-3′ and 5′-GAACTTGTAGGTTGGATTTTCGAAGCCGTTCTGCTGCATC-3′. Expression plasmids for c-Abl and an active isoform of c-Abl in which the N-terminal autoinhibitory domain is deleted (c-AblΔXB) in pCDNA3 were as described (33).

GST fusion proteins containing the Fe65WW domain and the Fe65PTB2 domain were created by amplifying the appropriate sequences by PCR using *Pfu* polymerase and cloning the products into pGEX-5X-2 and pGEX-5X-1 as EcoR1-SalI and SalI fragments, respectively. Primers used were: 5′-GCGGAATTCAACCCCAACGCCTTCGAGACGGAT-3′ and 5-CGCGTCGACCTATGAGGGGGAGGCCCGGCCGGGGGGTTCCCACTGGGTGGTCCC-3′ (Fe65WW domain) and 5′-GCGGTCGACTCCAAGTGGAATTCCCAGCGC-3′ and 5-GCGGTCGACTCATGGGGTATGGGCCCCCAG-3′ (Fe65PTB2 domain). Residues Tyr-Tyr-Trp within the Fe65WW domain were mutated to Ala-Ala-Ala as described above using oligonucleotides 5′-GGACACCTCAGGGACCGCTGCCGCGCACATCCCAACAGGG-3′ and 5′-CCCTGTTGGGATGTGCGCGGCAGCGGTCCCTGAGGTGTCC-3′.

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 γ/ε cleavage (34–36) was created by subcloning APPc-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′-CTGTATCGATTGACTAGGCAGCTCATCATG-3′ and 5′-CATGATGAGCTGCCTAGTCAATCGATACAG-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 (APPab 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 DM1A 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 $\times$ *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-AblΔXB-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 extracted from the gel pieces with two wash cycles of 50 mM NH_4HCO_3 and acetonitrile, lyophilized, and resuspended in 20 μl of 50 mM NH_4HCO_3.

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 automated 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 luciferase substrate was added and firefly luciferase activities produced by 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 substrate for *Renilla* luciferase) and remeasuring in the luminometer. All luciferase transfections received the same number and amount of plasmids, which was achieved by transfection of vector pCIneo-CAT where appropriate; pCIneo 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.

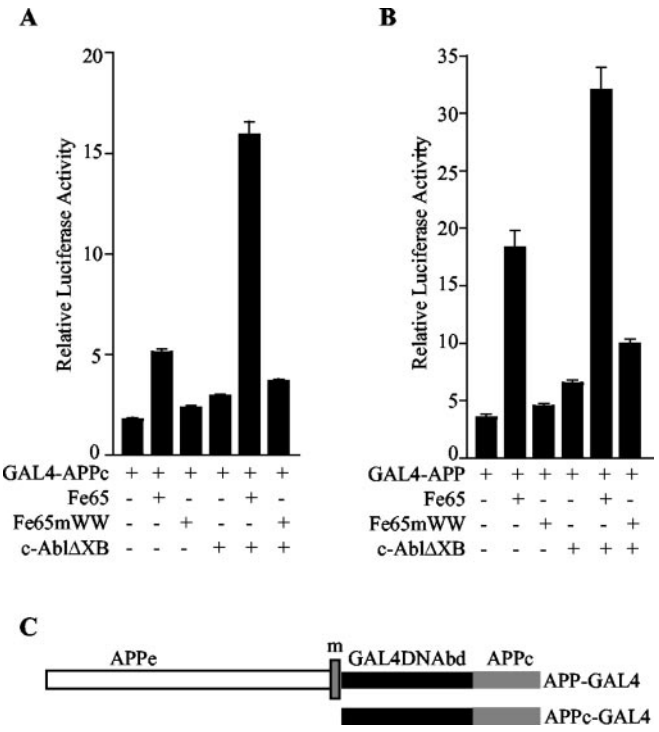

FIG. 2. **Fe65 and c-AblΔXB stimulate transcription of APP proteins fused to the GAL4 DNA binding domain.** *A*, transcription induced by a fusion gene comprising the GAL4 DNA binding domain fused to the C-terminal domain of APP (*GAL4-APPc*) is stimulated by Fe65, c-AblΔXB, and Fe65+c-AblΔXB. Fe65 stimulates transcription ($p < 0.001$), although this is significantly inhibited by mutation of the Fe65WW domain (*Fe65mWW*) (Fe65 *versus* Fe65mWW, $p < 0.001$). Transcription is mildly stimulated by c-AblΔXB alone ($p < 0.001$) and potently stimulated by Fe65+c-AblΔXB ($p < 0.001$). Mutation of the Fe65WW domain again markedly inhibits this effect (Fe65+c-AblΔXB *versus* Fe65mWW+c-AblΔXB, $p < 0.001$). *B*, a similar experiment using a full-length APP-GAL4 DNA binding domain fusion gene to drive reporter gene expression (*GAL4-APP*). Again, Fe65 stimulates transcription ($p < 0.001$), but this is significantly inhibited by mutation of the Fe656WW domain (Fe65 *versus* Fe65mWW, $p < 0.001$). Transcription is mildly stimulated by c-AblΔXB alone ($p < 0.001$) and potently stimulated by Fe65+c-AblΔXB ($p < 0.001$). Mutation of the Fe65WW domain markedly inhibits this effect (Fe65+c-AblΔXB *versus* Fe65mWW+c-AblΔXB, $p < 0.001$). Results shown are from 12 (*A*) and 16 (*B*) transfections; *error bars* are S.E. *C*, schematic of GAL4-APP fusion proteins used in this study with APP ectoplasmic and cytoplasmic domains (*APPe*, *APPc*), GAL4 DNA binding domain (*GAL4DNAbd*) and membrane (*m*) are highlighted.

In Vitro Phosphorylation of Fe65 by c-AblΔXB—c-AblΔXB 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 $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.185 MBq [γ-^{32}P]ATP, 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-AblΔXB) using immunoprecipitation and GST pull-down assays. Fe65 from Fe65+c-Abl- or Fe65+c-AblΔXB-transfected CHO was immunoprecipitated using the Myc tag on Fe65 and bound c-Abl detected on

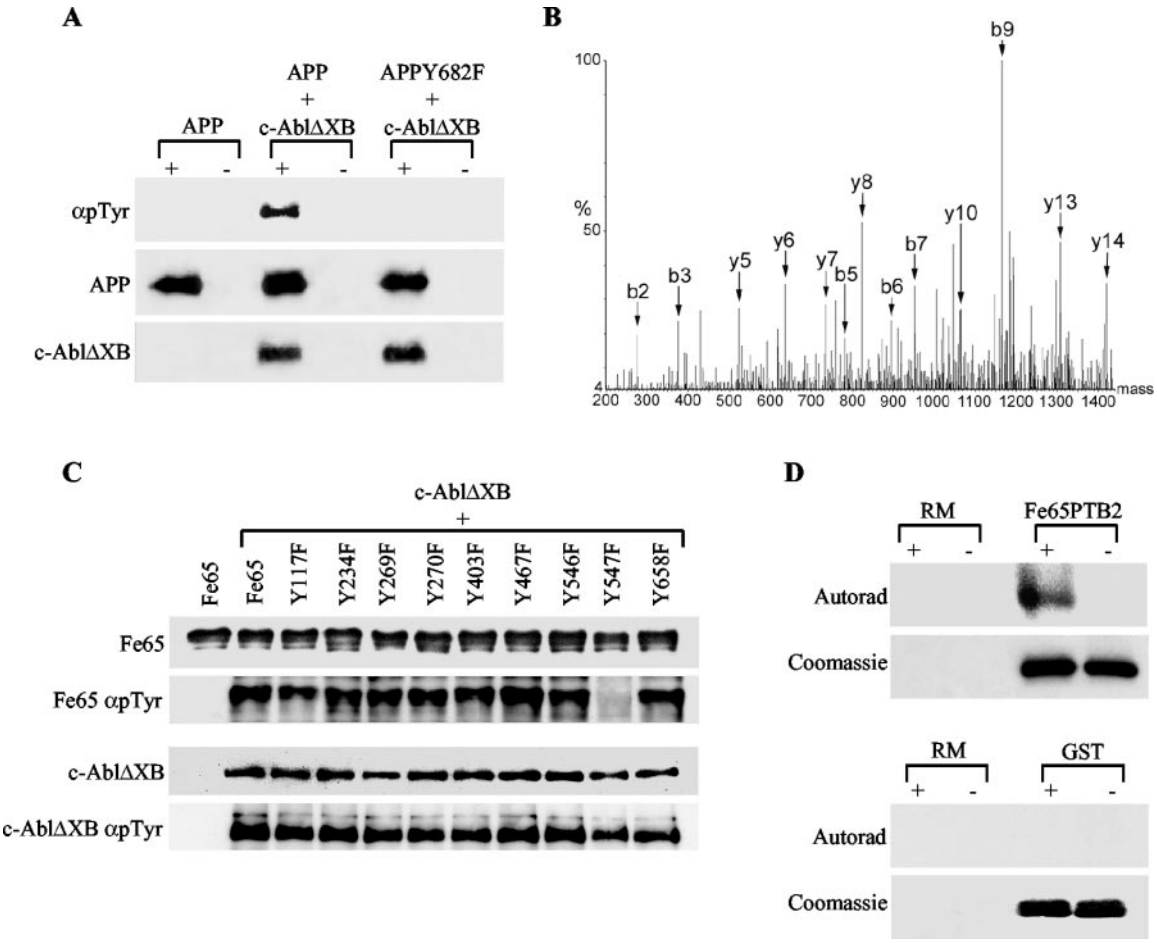

FIG. 3. **c-AblΔXB phosphorylates APP and Fe65.** *A*, phosphorylation of APP$^{Tyr\text{-}682}$ in cells transfected with APP and c-AblΔXB. APP or mutant APP^{Y682F} was immunoprecipitated from either APP-only- or APP+c-AblΔXB-transfected cells. The samples were then probed on immunoblots with antibody 4G10 (*αpTyr*) that detects phosphotyrosines, antibody 22C11 to APP (*APP*), and antibody 24-11 to c-Abl. Wild-type but not APP^{Y682F} reacts with 4G10. (+) and (−) refer to the presence or absence of antibody APPab in the immunoprecipitations. *B*, tandem MS/MS spectra for Fe65 phosphopeptide FQVYYLGNVPVAKPVGVDVINGALESVLSSSSR (M_r 3544.35) (residues 543–575). The triply charged precursor ion of the phosphorylated peptide, with an *m/z* of 1182.46^{3+}, was sequenced by collision-induced fragmentation. The *m/z* of the fragment ions is plotted against intensity. The peptide mass and the *m/z* of the fragment ions in the *b* ion series (nomenclature as described by Ref. 50) show a tyrosine-phosphorylated peptide with the phosphate located on either Tyr-546 or Tyr-547. The absence of the *b4* ion within the MS/MS fragmentation spectrum prevents the assignment of the phosphorylation to a specific tyrosine residue. *C*, tyrosine phosphorylation of Fe65 in cells transfected with Fe65 and c-AblΔXB. Fe65 was immunoprecipitated from cells transfected with wild-type Fe65 (*Fe65*) or mutants of Fe65 in which each tyrosine was mutated to phenylalanine (Y117F, Y234F, Y269F, Y270F, Y403F, Y467F, Y546F, Y547F, Y658F) either alone or with c-AblΔXB as indicated. The samples were then probed on immunoblots for both Fe65 and co-immunoprecipitating c-AblΔXB and for tyrosine phosphorylation of these using antibody 4G10 (*αpTyr*). Fe65 tyrosine phosphorylation is detected only in the presence of c-AblΔXB and only mutation of Fe65$^{Tyr\text{-}547}$ (*Y547F*) abrogates this phosphorylation. Tyrosine autophosphorylation of c-AblΔXB (which has previously been described (30) is seen in all samples. *D*, *in vitro* phosphorylation of GST-Fe65PTB2 domain (that harbors tyrosine 547) by c-AblΔXB. GST or GST-Fe65PTB2 domain were phosphorylated with [γ-^{32}P]ATP and c-AblΔXB. *RM* is reaction mix only with no substrate; GST-Fe65PTB2 and GST substrates are as indicated. (+) and (−) refer to the presence or absence of c-Abl antibody in the kinase immunoprecipitations. The *upper panels* show the autoradiographs, the *lower panels* are the corresponding Coomassie-stained gels.

immunoblots. Fe65 interacted with both c-Abl and c-AblΔXB but the binding to c-AblΔXB was stronger (Fig. 1*A*). In complementary experiments, GST or GST-Fe65WW domain baits were used in pull-down assays from c-Abl- or c-AblΔXB-transfected CHO cells. Again, both c-Abl and c-AblΔXB bound Fe65 with the c-AblΔXB interaction stronger (Fig. 1*B*). Mutation of the conserved aromatic residues Tyr-Tyr-Trp within the Fe65WW domain to Ala-Ala-Ala inhibited binding of c-AblΔXB (Fig. 1*C*). We also monitored c-Abl expression by immunostaining of Fe65+c-Abl- and Fe65+c-AblΔXB-co-transfected CHO and COS-7 cells. Fe65, c-Abl, and c-AblΔXB were all present in both the cytoplasm and nuclei of these cells, and Fe65 and c-Abl/c-AblΔXB showed a marked overlap in their distributions (Fig. 1*D*). These results are in agreement with previous observations, which demonstrate that Fe65 binds to c-Abl through its WW domain, that this interaction is stronger with active isoforms 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-AblΔXB 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 full-length APP into which the GAL4 DNA binding domain had been engineered (GAL4-APP) (19). Both GAL4-APPc and GAL4-APP transcription was stimulated by transfection of Fe65, and this stimulation was enhanced further by co-transfection with c-AblΔXB (Fig. 2). c-AblΔXB also stimulated transcription of the reporter in the absence of co-transfected Fe65, although to a lesser extent (Fig. 2). This effect of c-AblΔXB in

the absence of co-transfected Fe65 may well involve endogenous 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. 1*C*).

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 $APP^{Tyr\text{-}682}$ by monitoring the reactivity of APP and a mutant of APP in which tyrosine 682 was altered to phenylalanine with antibody 4G10 that detects phosphotyrosines. APP immunoprecipitated from APP+c-AblΔXB- but not APP-transfected cells was reactive with antibody 4G10, and this reactivity was abolished by mutation of $APP^{Tyr\text{-}682}$ (Fig. 3*A*). Thus, we confirmed that $APP^{Tyr\text{-}682}$ 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-transfected cells, and it was sequenced by mass spectrometry. We obtained over 80% sequence coverage and detected one peptide with a phosphorylated tyrosine residue (Fig. 3*B*). This peptide contains two adjacent tyrosines (tyrosines 546/547), although despite repeated sequence runs, we were unable to unambiguously distinguish which tyrosine was phosphorylated. We therefore prepared mutants of Fe65 in which either of these tyrosines were mutated to phenylalanine to preclude phosphorylation and examined their reactivities with antibody 4G10 in Fe65+c-AblΔXB-co-transfected cells. Immunoprecipitated $Fe65^{Y546F}$ but not $Fe65^{Y547F}$ was reactive with 4G10 in these assays (Fig. 3*C*). To confirm that no other tyrosines in Fe65 were phosphorylated in the Fe65+c-AblΔXB-co-transfected cells, we mutated individually, the remaining seven tyrosines 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. 3*C*). Thus, Fe65 is phosphorylated on a single residue, tyrosine 547, in c-AblΔXB-transfected cells.

The above studies are consistent with a direct phosphorylation 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) prepared as a GST fusion protein substrate. These assays revealed that c-AblΔXB could directly phosphorylate Fe65 (Fig. 3*D*). Thus c-Abl can 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 $APP^{Tyr\text{-}682}$ or $Fe65^{Tyr\text{-}547}$, we performed further GAL4-APPc-dependent transcription assays using mutants in which these residues were altered to phenylalanine to preclude phosphorylation. Fe65 and c-AblΔXB both stimulated transcription of mutant GAL4-$APPc^{Y682F}$, and this stimulation was greater than that of wild-type GAL4-APPc (Fig. 4*A*). Others have also shown that mutation of APP^{682} to phenylalanine enhances transcriptional activity of GAL4-APP fusions (43). The mechanisms that underlie this effect are not known but mutation of APP^{682} to phenylalanine does not influence either APP/Fe65 or APP/JIP-1 interactions (Ref. 43 and see below). Thus, the stimulatory effect of c-AblΔXB on APP/Fe65 transcription cannot be through phosphorylation of APP^{682}.

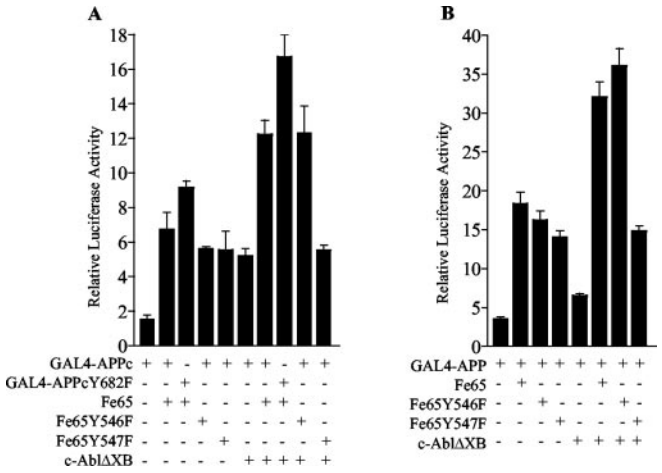

FIG. 4. **Phosphorylation of $Fe65^{Tyr\text{-}547}$ but not $APP^{Tyr\text{-}682}$ by c-AblΔXB stimulates APP/Fe65 transcription.** *A*, transcription induced by GAL4-APPc/Fe65 is stimulated by c-AblΔXB, but mutation of $Fe65^{Tyr\text{-}547}$ but not $APP^{Tyr\text{-}682}$ (both of which are phosphorylated by c-AblΔXB) to preclude phosphorylation blocks the effect of c-AblΔXB. Mutants $Fe65^{Y546F}$ and $Fe65^{Y547F}$ do not significantly alter Fe65 stimulation. Mutant $Fe65^{Y547F}$ but not $Fe65^{Y546F}$ blocks the stimulatory effect of c-AblΔXB (GAL4-APPc+Fe65+c-AblΔXB *versus* GAL4-APPc+Fe65Y546F+c-AblΔXB, no significant difference; GAL4-APPc+Fe65+c-AblΔXB *versus* GAL4-APPc+Fe65Y547F+c-AblΔXB, $p < 0.001$). Mutant GAL4-$APPc^{Y682F}$ stimulates GAL4-APPc/Fe65 transcription in both the presence or absence of c-AblΔXB ($p < 0.05$, absence of c-AblΔXB; $p < 0.05$, presence of c-AblΔXB). *B*, similar experiment using full-length APP into which the GAL4 DNA binding domain has been engineered (*GAL4-APP*). c-AblΔXB again stimulates GAL4-APP/Fe65 transcription, and this effect is abolished by mutant $Fe65^{Y547F}$ but not $Fe65^{Y546F}$ (GAL4-APP+Fe65+c-AblΔXB *versus* GAL4-APP+Fe65Y546F+c-AblΔXB, no significant difference; GAL4-APP+Fe65+c-AblΔXB *versus* GAL4-APP+Fe65Y547F+c-AblΔXB, $p < 0.001$). Results shown are from 12 (*A*) or 16 (*B*) transfections; *error bars* are S.E.

We next tested whether the stimulatory effect of c-AblΔXB on APP/Fe65 transcription was through phosphorylation of $Fe65^{Tyr\text{-}547}$. Mutation of this residue to phenylalanine to preclude phosphorylation ($Fe65^{Y547F}$) completely eliminated the effect of c-AblΔXB (Fig. 4*A*). However, $Fe65^{Y547F}$ was still capable of stimulating GAL4-APPc-dependent transcription (Fig. 4*A*). This latter observation suggests that a component of the Fe65 stimulatory effect on GAL4-APPc-mediated transcription is not dependent upon phosphorylation of $Fe65^{Tyr\text{-}547}$. It also suggests that mutation of $Fe65^{Tyr\text{-}547}$ to phenylalanine does not induce some conformational change to this domain of the protein that precludes the ability of Fe65 to stimulate transcription from GAL4-APPc. This was further confirmed by monitoring the effect of mutating the adjacent tyrosine ($Fe65^{Tyr\text{-}546}$) to phenylalanine ($Fe65^{Y546F}$). This mutant behaved in a similar fashion to wild-type Fe65 (Fig. 4*A*). We also tested whether mutation of $Fe65^{Tyr\text{-}547}$ inhibited transcription from full-length GAL4-APP and obtained similar results to GAL4-APPc (Fig. 4*B*). Thus, the stimulatory effect of c-AblΔXB on APP/Fe65 transcription is through phosphorylation of $Fe65^{Tyr\text{-}547}$.

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. 5*A*). Likewise, the presence of c-AblΔXB did not influence the amounts of GAL4-APPc bound to Fe65 (Fig. 5*B*). To determine whether c-AblΔXB influenced binding

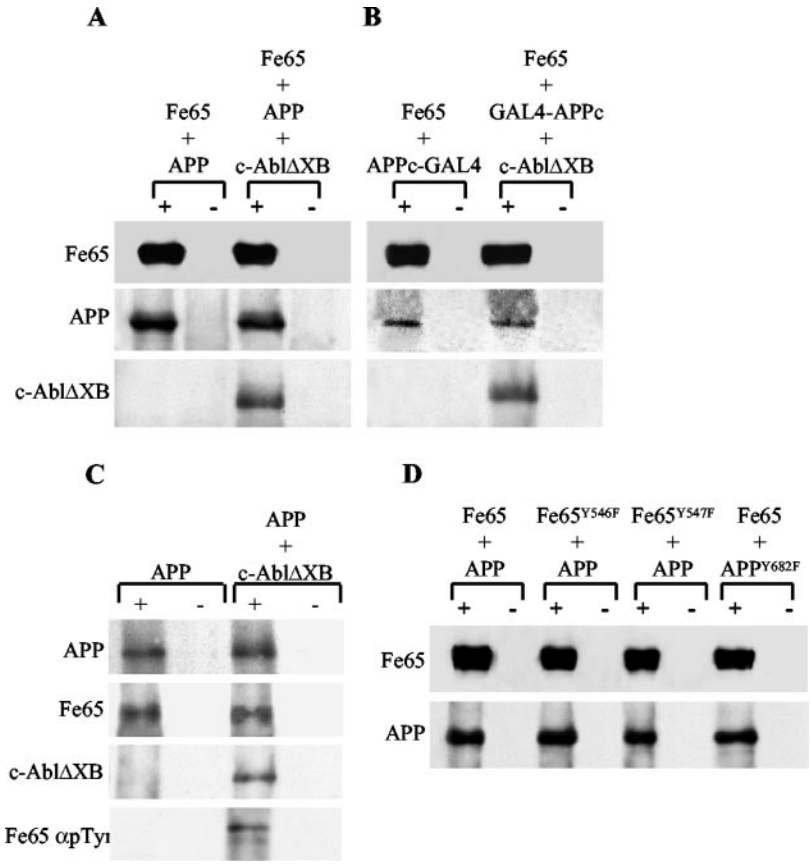

FIG. 5. **Neither c-AblΔXB nor mutation of either Fe65^{Y547F} or APP^{Y682F} alters APP-Fe65 interactions.** *A*, equal amounts of APP in Fe65 immunoprecipitates from Fe65+APP- and Fe65+APP+c-AblΔXB-transfected cells. *B*, similar experiment from Fe65+GAL4-APPc- and Fe65+GAL4-APPc+c-AblΔXB-transfected cells. In both *A* and *B*, Fe65 was immunoprecipitated using the Myc tag and co-immunoprecipitated APP/GAL4-APPc and c-AblΔXB were detected with APPab and c-Abl antibodies, respectively. Fe65 was detected using a polyclonal antibody. *C*, equal amounts of endogenous Fe65 bound to APP in APP- and APP+c-AblΔXB-transfected CHO cells. APP was immunoprecipitated and detected with antibody 22C11 and co-immunoprecipitated Fe65 and c-AblΔXB detected as above; tyrosine-phosphorylated Fe65 in the APP immunoprecipitates was detected with antibody 4G10. *D*, equal amounts of APP in Fe65 immunoprecipitates from Fe65+APP-, Fe65^{Y546F}+APP (*Fe65Y546F+APP*)-, Fe65^{Y547F}+APP (*Fe65Y547F+APP*)-, and Fe65+APP^{Y682F} (*Fe65+APPY682F*)-transfected cells. Fe65 and APP were immunoprecipitated and detected as in *A* and *B*. (+) and (−) refer to the presence or absence of immunoprecipitating antibodies in the assays.

of APP to endogenous Fe65, we immunoprecipitated APP from APP- or APP+c-AblΔXB-transfected cells and probed for bound Fe65. However, we again could detect no differences in the amounts of co-immunoprecipitating Fe65 (Fig. 5*C*). 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 Fe65^{Y547F} and APP^{Y682F} mutations altered binding of APP and Fe65, respectively, in immunoprecipitation experiments. However, neither of these mutants had altered binding properties (Fig. 5*D*). Mutant APP^{Y682F} has previously been shown to bind Fe65 (43). Thus, c-AblΔXB 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-AblΔXB stimulated the APPc-dependent expression of GSK3β by analyzing GSK3β protein levels in cells transfected with APPc either alone or with Fe65 and c-AblΔXB using immunoblotting techniques. Because we obtain 30–40% transfection efficiencies, any changes observed in these pooled samples of transfected and non-transfected cells are 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-AblΔXB alone, we detected a marked increase in GSK3β signal in cells transfected with all three plasmids (Fig. 6*A*). We also studied the effect of c-AblΔXB on GSK3β protein levels by immunostaining, and this revealed that transfection of APPc+Fe65+c-AblΔXB increased the GSK3β signal compared with non-transfected cells (Fig. 6*B*).

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-Abl (25). c-Abl phosphorylates APP on tyrosine 682 (25) and here, we demonstrate that it additionally phosphorylates Fe65 on tyrosine 547. We also show that active c-Abl stimulates APP/Fe65-mediated transcription and that this is through phosphorylation of Fe65$^{Tyr\text{-}547}$ but not APP$^{Tyr\text{-}682}$.

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-Abl (tyrosine 547) resides within the second PTB domain, and this domain binds to APP. However, Fe65 and Fe65^{Y547F} (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-AblΔXB. As such, phosphorylation of APP$^{Tyr\text{-}682}$ or Fe65$^{Tyr\text{-}547}$ by active c-Abl appears not to influence APP/Fe65 interactions in any overt manner. Thus, while the stimulatory effect of c-AblΔXB on APP/Fe65 transcription is mediated by Fe65$^{Tyr\text{-}547}$ 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

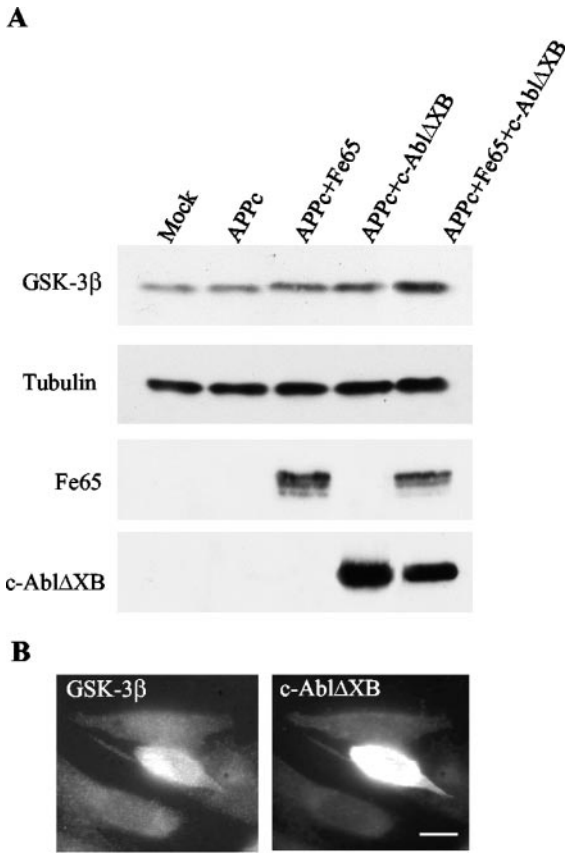

FIG. 6. **c-AblΔXB stimulates APPc-dependent expression of GSK3β in transfected CHO cells.** *A*, immunoblots of cells transfected with empty vector or APPc either alone or with Fe65, c-AblΔXB, or Fe65+c-AblΔXB as indicated. The blots were also probed for tubulin to demonstrate equal loading. *B*, immunofluorescent labeling of CHO cells transfected with APPc+Fe65+c-AblΔXB and double-labeled for GSK3β and c-Abl as indicated. *Scale bar* is 10 μm.

fusion gene similar to the ones we used here (38). In the course of this study, active c-Abl was shown to have no effect on APP-GAL4-mediated transcription (38). However, the APP-GAL4 fusion used in this earlier work comprised full-length GAL4 (containing both its DNA binding and transactivation domains) such that while transcription of the reporter gene required APP cleavage, it did not require the transactivation capability of the APP C-terminal domain. In contrast, our GAL4-APP and GAL4-APPc fusions involve only the GAL4 DNA binding domain, and the APP C-terminal domain is thus essential for transcriptional activity (see also Ref. 19). Thus, these different findings are most probably the result of the different GAL4-APP fusions used in these two studies. Indeed, the earlier work of Gianni *et al.* (38) was an elegant study aimed at understanding the mechanisms regulating APP cleavage and not APP/Fe65-mediated transcription as in our work. Nevertheless, we tested whether c-AblΔXB also stimulated reporter gene activity that was driven by an APP-GAL4 DNA binding domain fusion in which GAL4 DNA binding domain sequences were fused to the C terminus of APP (APP-GAL4). c-AblΔXB also stimulated transcription from this fusion protein (APP-GAL4 *versus* APP-GAL4+c-AblΔXB transcription, 1:1.65; $p < 0.001$).

The precise mechanisms by which phosphorylation of Fe65 by c-Abl stimulates its transcriptional activity are not clear. Because the phosphorylated residue (tyrosine 547) is within the second Fe65 PTB domain, we initially anticipated that it might influence Fe65/APP interactions in some manner. However, our immunoprecipitation assays have not provided experimental 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, Shc, 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.

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