The c-Abl Tyrosine Kinase Phosphorylates the Fe65 Adaptor Protein to Stimulate Fe65amyloid Precursor Protein Nuclear Signaling*

Michael S. Perkinton‡, Claire L. Staden‡, Kwok-Fai Lau‡, Sashi Kesavapany‡, Helen L. Byers§, Malcolm Ward§, Declan M. McLoughlin‡, and Christopher C. J. Miller‡

From the ‡Department of Neuroscience, the §Section of Old Age Psychiatry, and ‡Proteome Sciences plc, The Institute of Psychiatry, King’s College London, De Crespigny Park, Denmark Hill, London SE5 8AF, United Kingdom

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 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 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 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 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 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.
TCCACAAAAACACCTG-3' and 5'-CAGGGTGTATTAGTTGTTGGAAAAGAAGACGAGCTGAGGATG-3'; Y467F, 5'-GAGAGGAGCTTCTTCTCTGCTAGTCCTGCTGATAAG-3' and 5'-CTTCTG-3' and 5'-CAGGTATACCTTCAGCTAGGAAAAGGACTGGAACTTCTGAG-3'; Y546F, 5'-CAGAAGCTTCTGCTAGTCCTGAGGATG-3' and 5'-CAGACGATGCTACGCTAGGAAAAGGACTGGAACTTCTGAG-3'; Y547F, 5'-GAGAGGAGCTTCTTCTCTGCTAGTCCTGCTGATAAG-3' and 5'-CTTCTG-3'. Tyrosine 682 in APP was mutated to phenylalanine in a similar fashion using oligonucleotides 5'-GAGAGGAGCTTCTTCTCTGCTAGTCCTGCTGATAAG-3' and 5'-CTTCTG-3' at IReL (Trinity College Dublin), on September 17, 2009.
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-GFP antibody was from BD Transduction Laboratories, and antibody DM1A to tubulin was from Sigma.

Immuno precipitation and GST Pull-down Assays—Immuno precipitation 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, 0.5% 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-AbLBX-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 NH4HCO3 and acetonitrile, lyophilized, and resuspended in 20 μl of 50 mM NH4HCO3.

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 database 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, carbamidomethylation 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 DualGlo 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 (Promega) was added to each well, and firefly and Renilla luciferase activities were measured using a Wallac TriLux luminometer. Renilla luciferase activities produced by the pRL-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 the stop and glow solution 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.

In Vitro Phosphorylation of Fe65 by c-Abl—c-AblLBX 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-nitrophenolphosphate, 0.1 mM sodium orthovanadate, 2 mM diithothreitol, 0.185 MBq [γ-32P]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-AblΔΔXB) using immunoprecipitation and GST pull-down assays. Fe65 from Fe65+c-Abl- or Fe65+c-AblΔΔXB-transfected CHO was immunoprecipitated using the Myc tag on Fe65 and bound c-Abl detected on
c-Abl Phosphorylates Fe65 to Enhance APP/Fe65 Transcription

Fig. 3. c-AbIΔXB phosphorylates APP and Fe65. A, phosphorylation of APP

immunoblots. Fe65 interacted with both c-AbI and c-AbIΔXB but the binding to c-AbIΔXB was stronger (Fig. 1A). In complementatory experiments, GST or GST-Fe65WW domain baits were used in pull-down assays from c-AbI- or c-AbIΔXB-transfected CHO cells. Again, both c-AbI and c-AbIΔXB bound Fe65 with the c-AbIΔXB 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-AbIΔXB (Fig. 1C). We also monitored c-AbI expression by immunostaining of Fe65+c-AbI- and Fe65+c-AbIΔXB-co-transfected CHO and COS-7 cells. Fe65, c-AbI, and c-AbIΔXB were all present in both the cytoplasm and nuclei of these cells, and Fe65 and c-AbI/c-AbIΔXB showed a marked overlap in their distributions (Fig. 1D). These results are in agreement with previous observations, which demonstrate that Fe65 binds to c-AbI through its WW domain, that this interaction is stronger with active isoforms of c-AbI, and that a proportion of both Fe65 and c-AbI are present within the nucleus (27, 30).

We next examined the effect of c-AbIΔXB 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-luciferase reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or the conserved aromatic residues Tyr-Tyr-Trp within the Fe65WW domain to Ala-Ala-Ala inhibited binding of c-AbIΔXB (Fig. 1C). We also monitored c-AbI expression by immunostaining of Fe65+c-AbI- and Fe65+c-AbIΔXB-co-transfected CHO and COS-7 cells. Fe65, c-AbI, and c-AbIΔXB were all present in both the cytoplasm and nuclei of these cells, and Fe65 and c-AbI/c-AbIΔXB showed a marked overlap in their distributions (Fig. 1D). These results are in agreement with previous observations, which demonstrate that Fe65 binds to c-AbI through its WW domain, that this interaction is stronger with active isoforms of c-AbI, and that a proportion of both Fe65 and c-AbI are present within the nucleus (27, 30).

We next examined the effect of c-AbIΔXB 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-luciferase reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or the conserved aromatic residues Tyr-Tyr-Trp within the Fe65WW domain to Ala-Ala-Ala inhibited binding of c-AbIΔXB (Fig. 1C). We also monitored c-AbI expression by immunostaining of Fe65+c-AbI- and Fe65+c-AbIΔXB-co-transfected CHO and COS-7 cells. Fe65, c-AbI, and c-AbIΔXB were all present in both the cytoplasm and nuclei of these cells, and Fe65 and c-AbI/c-AbIΔXB showed a marked overlap in their distributions (Fig. 1D). These results are in agreement with previous observations, which demonstrate that Fe65 binds to c-AbI through its WW domain, that this interaction is stronger with active isoforms of c-AbI, and that a proportion of both Fe65 and c-AbI are present within the nucleus (27, 30).

We next examined the effect of c-AbIΔXB 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-luciferase reporter (19). GAL4-APP fusions involved either the GAL4 DNA binding domain fused to the C-terminal domain of APP (GAL4-APPc) or the conserved aromatic residues Tyr-Tyr-Trp within the Fe65WW domain to Ala-Ala-Ala inhibited binding of c-AbIΔXB (Fig. 1C). We also monitored c-AbI expression by immunostaining of Fe65+c-AbI- and Fe65+c-AbIΔXB-co-transfected CHO and COS-7 cells. Fe65, c-AbI, and c-AbIΔXB were all present in both the cytoplasm and nuclei of these cells, and Fe65 and c-AbI/c-AbIΔXB showed a marked overlap in their distributions (Fig. 1D). These results are in agreement with previous observations, which demonstrate that Fe65 binds to c-AbI through its WW domain, that this interaction is stronger with active isoforms of c-AbI, and that a proportion of both Fe65 and c-AbI are present within the nucleus (27, 30).
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 Fe65 WW 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-AbiΔXB (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-AbiΔXB on APP/Fe65-mediated transcription is due to phosphorylation of this residue. We therefore confirmed that c-AbiΔXB phosphorylated APP Tyr-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-AbiΔXB but not APP-transfected cells was reactive with antibody 4G10, and this reactivity was abolished by mutation of APP Tyr-682 (Fig. 3A). Thus, we confirmed that APP Tyr-682 is phosphorylated in APP+c-AbiΔXB-transfected cells as previously described (27).

An alternative possibility is that c-Abl also phosphorylates Fe65, and the stimulatory effect of c-AbiΔXB on transcription is caused by Fe65 phosphorylation. We therefore isolated Fe65 by immunoprecipitation from Fe65- or Fe65+c-AbiΔXB-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. 3B). 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-AbiΔXB-co-transfected cells. Immunoprecipitated Fe65 Y546F but not Fe65 Y547F was reactive with 4G10 in these assays (Fig. 3C). To confirm that no other tyrosines in Fe65 were phosphorylated in the Fe65+c-AbiΔXB-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. 3C). Thus, Fe65 is phosphorylated on a single residue, tyrosine 547, in c-AbiΔXB-transfected cells.

The above studies are consistent with a direct phosphorylation of c-AbiΔXB on APP/Fe65 transcription cannot be through phosphorylation of APP Tyr-682.

We next tested whether the stimulatory effect of c-AbiΔXB on APP/Fe65 transcription was through phosphorylation of Fe65 Tyr-547. Mutation of this residue to phenylalanine to preclude phosphorylation (Fe65 Y547F) completely eliminated the effect of c-AbiΔXB (Fig. 4A). However, Fe65 Y547F was still capable 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 due to phosphorylation of this residue. Because Fe65 is expressed in CHO and COS-7 cells and so one possibility is that the effect of c-Abl on APP/Fe65-mediated transcription involves phosphorylation of either APP Tyr-682 or Fe65 Tyr-547, we performed further GAL4-APPc-dependent transcription assays using phosphorylated GAL4-APPc and wild-type GAL4-APPc, and this stimulation was greater than that of wild-type GAL4-APPc (Fig. 4A). Others have also shown that mutation of APP Tyr-682 to phenylalanine enhances transcriptional activity of GAL4-APP (43). The mechanisms that underlie this effect are not known but mutation of APP Tyr-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-AbiΔXB on APP/Fe65 transcription cannot be through phosphorylation of APP Tyr-682.
c-Abl Phosphorylates Fe65 to Enhance APP/Fe65 Transcription

of APP to endogenous Fe65, we immunoprecipitated APP from APP- or APP+c-AbΔXB-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-AbΔXB 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-AbΔXB stimulated the APPc-dependent expression of GSK3β by analyzing GSK3β protein levels in cells transfected with APPc either alone or with Fe65 and c-AbΔXB 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-AbΔXB 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-AbΔXB on GSK3β protein levels by immunostaining, and this revealed that transfection of APPc+Fe65+c-AbΔXB 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-Abl (25). c-Abl phospho-lysylates 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 Fe65Y547 but not APPY682.

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 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 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-AbΔXB. As such, phosphorylation of APPY682 or Fe65Y547 by active c-Abl appears not to influence APP/Fe65 interactions in any overt manner. Thus, while the stimulatory effect of c-AbΔXB on APP/Fe65 transcription is mediated by Fe65Y547 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...
GSK-3β  

Fe65  
c-AblΔXB

**FIG. 6.** c-AblΔXB stimulates APPc-dependent expression of GSKβ in transfected CHO cells. **A,** immunoblots of cells transfected with empty vector or APPc either alone or with Fe65, c-Abl ΔXB, or Fe65+c-Abl ΔXB as indicated. The blots were also probed for tubulin to demonstrate equal loading. **B,** immunofluorescent labeling of CHO cells aimed at understanding the mechanisms regulating APP cleavage, and the APP C-terminal domain is thus  

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). Indeed, 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), X11a, X11b, 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-regulation 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**

22. Kinoshita, A., Whelan, C. M., Smith, C. J., Berezovska, O., and Hyman, B. T.
c-Abl Phosphorylates Fe65 to Enhance APP/Fe65 Transcription

22091