The Journal of Biological Chemistry
Vol. 279, No. 16, Issue of April 16, pp. 16161–16169, 2004
Printed in U.S.A.
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Fe65 Is Not Involved in the Platelet-derived Growth Factor-induced Processing of Alzheimer's Amyloid Precursor Protein, Which Activates Its Caspase-directed Cleavage*

Received for publication, October 7, 2003, and in revised form, January 20, 2004
Published, JBC Papers in Press, February 6, 2004, DOI 10.1074/jbc.M311027200

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The proteolytic processing of the precursor of the β-amyloid peptides (APP) is believed to be a key event in the pathogenesis of Alzheimer's disease. This processing is activated through a pathway involving the PDGF receptor, Src, and Rac1. In this paper, we demonstrate that this pathway specifically acts on APP and requires the YENPTY motif present in the APP cytosolic domain. Considering that several results indicate that the adaptor proteins interacting with this domain affect the processing of APP, we examined their possible involvement in the PDGF-induced pathway. By using an APP-Gal4 reporter system, we observed that the overexpression of Fe65 activates APP-Gal4 cleavage, whereas X11 stabilizes APP. Although mDab1 and Jip1 have no effect, Shc induces a strong activation of APP cleavage, and the contemporary exposure of cells to PDGF causes a dramatic cooperative effect. The analysis of point mutations of the APP YENPTY motif indicates that Fe65 and PDGF function through different mechanisms. In fact, Fe65 requires the integrity of APP695 Tyr^{682} residue, whereas PDGF effect is dependent upon the integrity of Asn^{684}. Furthermore, the mutation of Asp^{664} of APP, which is the target site for the caspase-directed APP cleavage, strongly decreases the effect of Fe65. This suggests that Fe65 activates the cleavage of APP by caspases, and in fact, caspase inhibitor Z-VEVD decreases the APP cleavage induced by Fe65. On the contrary, the effects of Shc overexpression, like those of PDGF, are completely absent in the presence of compound X and require the integrity of the Asn^{684} residue of APP695. The involvement of Shc in the pathway regulating APP processing is confirmed by the effects of constitutively active and dominant negative mutants of Src and Rac1.

Numerous experimental results indicate that the β-amyloid precursor protein (APP)[1] plays a crucial role in the pathogenesis of Alzheimer's disease (AD). In fact, the main constituent of senile plaques, the β-amyloid peptide (Aβ), derives from the proteolytic processing of APP, and mutations of the APP gene are responsible for some rare cases of genetically inherited AD. Furthermore, presenilins are key factors of the molecular machinery catalyzing the cleavage of APP, and mutations of the two presenilin genes are responsible for most of the cases of familial AD (for reviews, see Refs. 1 and 2).

It is well demonstrated that APP is a type I membrane protein that is cleaved by two different proteolytic enzymes, named α- and β-secretases. These cleavages generate soluble extracellular fragments, named α- and β-APPs, and two forms of transmembrane stubs, Cys^{83} and Cys^{99}, as a consequence of the activity of α- and β-secretases, respectively. These stubs are then substrates of a third enzyme, the γ-secretase, which cleaves them within the transmembrane helix, thus generating from Cys^{99} the 40–42 amino acid-long peptide Aβ and from Cys^{83} a smaller fragment named p3. The cleavage of Cys^{83} and Cys^{99} by γ-secretase also results in the generation of a C-terminal peptide of 57 residues (APP intracellular domain, AICD) (3, 4). APP is also a substrate of caspase activities that cleave its cytosolic domain, 31 residues upstream from the C terminus (5).

Numerous results suggest that the short cytodomain of APP plays a key role in the regulation of these events. In fact, this cytodomain contains a YENPTY motif that interacts with several PTB-containing adaptor proteins, such as those belonging to the Fe65 protein family (6–8), X11 (9), mDab1 (10), Jip1 (11), Shc (12, 13), and Numb (14). Among these proteins, X11 stabilizes APP, preventing its cleavage by α- and β-secretases (15, 16), whereas Fe65 overexpression, at least in some cells, increases the generation of Aβ (17). Furthermore, the APP cytodomain interacts with other proteins, such as kinesin-I (18) and PAT1 (19) and is phosphorylated by Abl tyrosine kinase at the Tyr^{682} residue (20) and by various enzymes at Thr^{668} (21).

The functions of APP and its proteolytic processing are still unclear. Several results indicated that APP could be considered a cargo receptor for kinesin-I because of its interaction with the light chain subunit of this motor protein (18). Fast anterograde axonal transport of presenilin-1 is dependent upon APP, and kinesin-I and the latter are involved in the transport of the vesicle compartment where the secretase-directed processing of APP occurs (22). Additionally, the proteolytic processing of APP recalls that of another membrane protein named Notch, whose

* This work has been supported by grants from the Italian Ministry of Health-Regione Campania (Progetto Alzheimer), Miur-FIRB RBNE0IWY7B from Biogem-Italy, European Community Grant CT-2003-503330 (to T. R.), and MIUR-PRIN 2003 (to N. Z.). 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; AD, Alzheimer's disease; Aβ, beta-amyloid peptide; AICD, APP intracellular domain; CAT, chloramphenicol acetyl transferase; Z-D(or V)EVD-fmk, benzyloxycarbonyl-D(or V)EVD-fluoromethyl ketone; IC, intracellular; PTB, phosphotyrosine binding domain; PDGF, platelet derived growth factor; PDGF-R, PDGF receptor.

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intracellular domain is released from the membrane upon a γ-secretase cleavage and is translocated into the nucleus where it associates with the transcription machinery and regulates several genes, in association with other transcription factors (for a review, see Ref. 23). The similarity between APP and Notch is strengthened by the observations that AICD has been also found in the nucleus (24–25) and that a complex containing AICD, the adaptor protein Fe65, and the histone acetyl transferase Tip60 are able to activate the transcription of a reporter gene (26) and are found associated with active chromatin on the promoter of the KAI1 gene (27). The possibility that APP could regulate nuclear functions is further supported by results indicating that Fe65, one of the cytosolic ligands of APP, is a nuclear protein (28) interacting, further than with Tip60, with the late Simian virus 40 transcription factor (LSF) and regulating the expression of the thymidylate synthase and GS3Kβ genes (29–30). Furthermore, AICD also interacts with JIP1 and X11; in these cases, it could also be involved in gene regulation (31–32). Therefore, it has been hypothesized that the cleavage of APP by α- and/or β-secretases and the resulting cleavage of Cys^{83} or Cys^{99} by γ-secretase result in the release of AICD from the membrane anchor and in its translocation into the nucleus, where, in association with other proteins, it could regulate gene transcription.

The attractiveness of this hypothesis would be increased if the cleavage of APP and the resulting gene regulation could be triggered by events, such as extracellular signals, thus assigning to APP a role in signal transduction. We have demonstrated recently that APP processing is induced by activated PDGF receptor through a pathway involving the activation of Src and Rac1 (33). In this paper, we demonstrate that some partners of the APP cytodomain play a key role in the regulation of APP processing. In particular, Fe65 and Shc increase the cleavage of APP by two different mechanisms: Fe65 induces the caspase-dependent cleavage of APP, whereas Shc cooperates with PDGF in the activation of the secretase-directed processing of APP.

MATERIALS AND METHODS

DNA Constructs—The APP-Gal4 construct and the CAT reporter construct controlled by Gal4 cis-elements (G5B-CAT) have been described (33). N-terminal mutants of APP-Gal4 were generated in a Gal4 expression cassette obtained by cloning a PCR fragment of the yeast Gal4 cDNA between the sites NotI and ApaI of the pRcCMV vector (Invitrogen). The primers used to amplify Gal4 were: 5′-AAGGAAAAAAGCGGCCGCTGGTGGTGGTGGTGGTGGTAAGCTACTGTCTTCTATCGAACAAGC and 5′-CATCGGGCCCTTACTCTTTTTTTGGGTTTGGTGGGG. To ensure the proper membrane location of the deleted proteins, the coding sequence of the APP signal peptide was added to the 5′ end of the vector polylinker by cloning (in the HindIII site) a double-stranded synthetic oligonucleotide with a modified 5′ Hind III sequence (the upper strand sequence was: 5′-AGCTAACTAAGGCCATGCTGCCCGGTTTGGCACTGCTCCTGCTGGCCGCCTGGACGGCTCGGGCGCTGA). Next, the PCR fragments encoding the N-terminal portions of the human APP695 cDNA used in this study were obtained with the forward primers D19–119, 5′-CCCAAGCTTGGTGAGTTTGTAAGTGATGCCCTTC; D19–260, 5′-CCCAAGCTTCCCTACGAAGAAGCCACAGAGAG; D19–467, 5′-CCCAAGCTTCAGTCTCTCTCCCTGCTCTACAAC; and D19–581, 5′-CCCAAGCTTGGGTTGACAAATATCAAGACGGAGG. The reverse primer, common to all N-terminal mutants, was 5′-AAGGAAAAAAGCGGCCGCGTTCTGCATCTGCTCAAAGAACTTG.

The C-terminal deletion mutants have been amplified by PCR with the common forward primer, 5′-CCCAAGCTTACTAAGGCCATGCTGCCCGGTTTGGCACTGC, and with the reverse primers DC07, 5′-AAGGAAAAAAGCGGCCGCCTTGTAGGTTGGATTTTCGTACGGC; DC14, 5′-AAGGAAAAAAGCGGCCGCGCCGTTCTGCTGCATCTTGGAC; and DC44, 5′-AAGGAAAAAAGCGGCCGCTTTCTTCTTCAGCATCACCAAGG.

The PCR products of the N- and C-terminal deletions, obtained from a human APP695 cDNA template, have been cloned in the Gal4/signal peptide cassette between the Hind III and NotI sites.

The point mutations were introduced in the wild-type APP-Gal4 construct through site-directed mutagenesis (Stratagene mutagenesis kit) with complementary primer pairs bearing the desired mutations. The sequences of the corresponding forward primers were Y682G, 5′-CCAAGATGCAGCAGAACGGCGGCGAAAATCCAACCTACAAGTTCTTTG; N684A, 5′-AAGATGCAGCAGAACGGCTACGAAGCTCCAACCTACAAGTTCTTTGAGCA; Y687A, 5′-CGGCTACGAAAATCCAACCGCCAAGTTCTTTGAGCAGATGC; D664N, 5′-GGTGTGGTGGAGGTTAACGCCGCTGTCACC. The underlined nucleotides represent the mutated positions.

Fe65, Fe65C655F, Fe65Δ-PTB (28), SrcYF, SrcYFKM, RacN17, and RacQL (33), X11 (9), Notch, Notch ΔE, cbf1-Luc (34), myc-tagged ShcA (35), and Jip-1 (11) vectors have been described. m-Dab1 construct was generated in the pRcCMV vector from the m-Dab1 cDNA.

Cell Culture Conditions, Transfections, and Treatments—HEK293, HeLa, and HeLaAG cells were grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Transfections were performed by calcium phosphate-DNA co-precipitation with equal amounts of plasmid DNA in 60-mm cell culture dishes. PDGF stimulation of transfected cells was started 24 h after transfection and allowed to proceed for 16–24 h in the presence of 40 ng/ml of recombinant human PDGF-BB (Sigma). Treatments of the cell cultures with the inhibitors were performed for the indicated times at the following concentrations: 10 μM PP2 (Calbiochem), 2 μM AG1296 (Calbiochem), 10 μM secretase inhibitor compound X (Calbiochem), 50 μM Z-DEVD-fmk (Alexis).

Preparation of Cell Extracts, Western Blotting, Reporter Analyses, and Aβ Assay—For Western blot analyses, cells were harvested in cold PBS, resuspended in lysis buffer (40 mM Tris-HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) in the presence of a protease inhibitor mixture (complete EDTA-free, Roche Applied Science) and kept on ice for 15 min. Then the extracts were clarified by centrifugation at 14,000 rpm at 4 °C. 20 μg of each extract were resolved on 4–12% SDS-polyacrylamide gradient gels (Invitrogen) under reducing conditions and transferred to Immobilon-P membranes (Millipore). Filters were blocked in 5% nonfat dry milk in T-PBS solution (phosphate-buffered saline and 0.05% Tween) and incubated overnight at 4 °C with appropriate dilutions of primary antibodies. The excess antibody was removed by sequential washing of the membranes in T-PBS, and then a 1:5000 dilution of the appropriate secondary antibody (conjugated horseradish peroxidase) was added to the filters for 30 min at room temperature. The excess was removed by sequential washing of the membranes in T-PBS, and the signals were detected by chemiluminescence, using the ECL system (Amersham Biosciences). The antibodies used and their dilutions were: anti-APP 6E10 (Sigma), 1:1000; anti-APP CT695 (Zymed Laboratories Inc., 1:250; anti-Fe65 (36), 1:2000; anti-caspase 3 (Upstate Biotechnology), 1:1000.

CAT expression was measured by using colorimetric CAT enzyme-linked immunoadsorbent assay (Roche Applied Science). Transiently transfected HeLa cells were harvested in cold TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl), lysed by freezing at −80 °C and rapid thawing at room temperature, and resuspended in lysis buffer (10 mM Hepes, pH 7.9, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 400 mM NaCl). Total extracts were clarified by centrifugation at 14,000 rpm at 4 °C, and their protein concentration was determined by the Bio-Rad protein assay. For CAT concentration measurement, 150 μg of each protein extract were used. Luciferase assays were carried out as described (29), using a Berthold tube luminometer.

Aβ1–40 was assayed in triplicates from conditioned media of HEK293 cells transiently transfected with APP695 and with Shc p46, Fe65, or SrcYF. 48 h after transfection, 100 μl of culture media were harvested and assayed with an EIA kit (IBL, Gunma, Japan), according to the instructions of the manufacturer.

RESULTS

The Cytodomain of APP Is Required for the PDGF-induced Cleavage of APP-Gal4—To examine the structural constraints of APP that are necessary to allow PDGF to induce APP processing, we have generated a series of deletion mutants of the extracellular/intraluminal domain of APP, and these cDNAs have been cloned in frame with that encoding the whole Gal4 transcription factor. These constructs, under the control of the cytomegalovirus promoter, direct the expression of fusion proteins in which Gal4 is fused at the C terminus of APP deletion mutants, all possessing a signal peptide to obtain the proper

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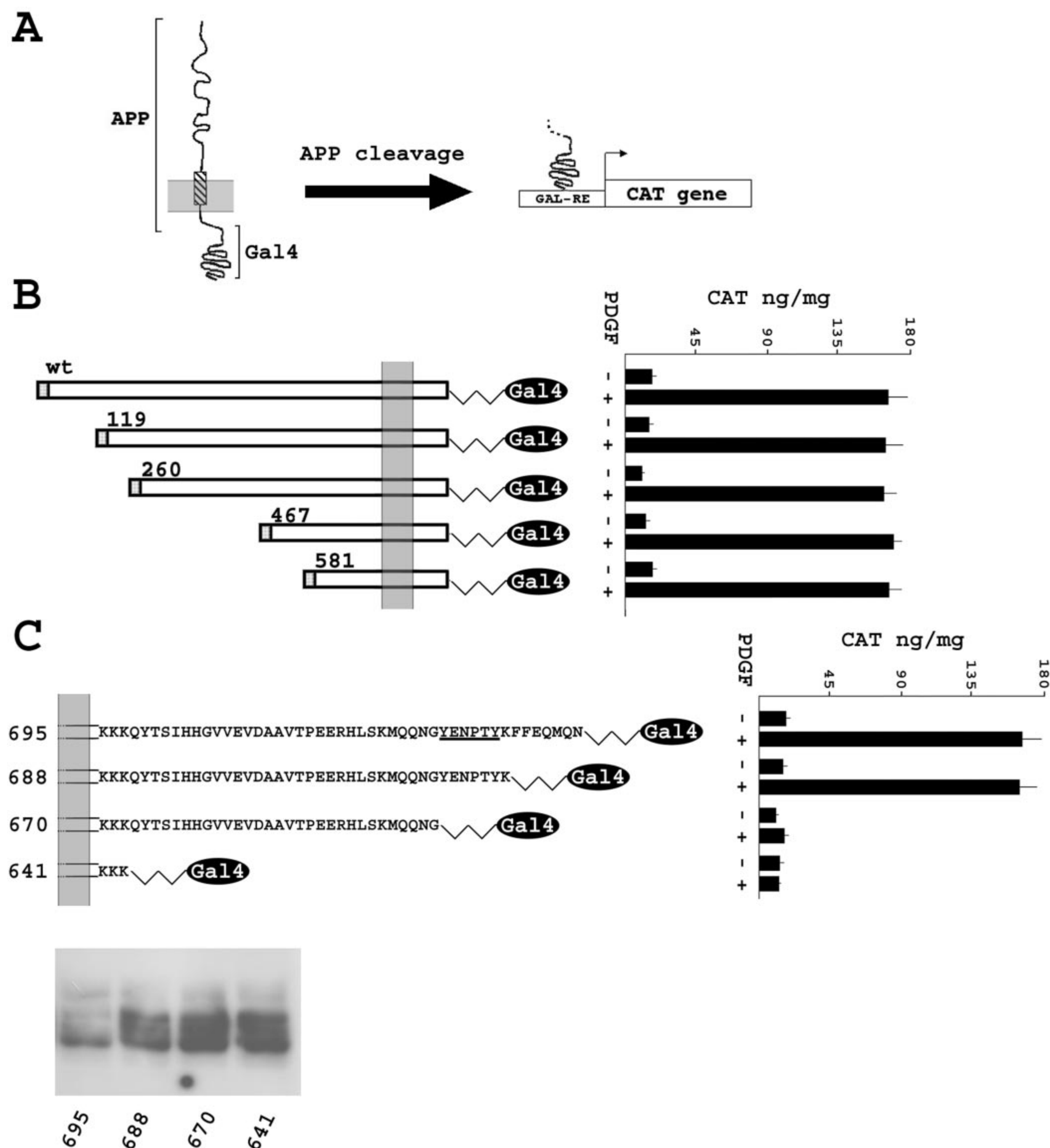

FIG. 1. **The cytodomain of APP is required for PDGF-induced cleavage of APP-Gal4.** *A*, schematic representation of the experimental system: upon the cleavage of APP-Gal4 fusion proteins, the Gal4 protein is released from the membrane and activates the transcription of the CAT gene cloned under the control of five Gal4 cis-elements. *B*, HeLa cells were transiently co-transfected with G5BCAT vector and expression vectors encoding APP-Gal4 fusion proteins bearing the indicated deletions of the APP EC/IL domain. 24 h after transfection, the cells were exposed or not for 24 h to 40 ng/ml recombinant human PDGF-BB. The sequence of the YENPTY motif is *underlined*. Standard deviations of triplicate CAT measurements are reported. *C*, HeLa cells were transfected and analyzed as in *B* to study the indicated deletion mutants of the cytodomain of APP. *Insert*, Western blot of the three mutant proteins (688, 670, 641) demonstrating that their expression is comparable with that of the wt APP-Gal4 (695).

membrane location. These expression vectors were transfected in HeLa cells together with G5B-CAT vector driving CAT expression under the control of five Gal4 responsive elements (see Fig. 1*A*); thus, the measurement of CAT functioning as a read-out of the cleavage of APP (33). 24 h after transfection, the cells were treated or not with PDGF to assess the ability of the growth factor to induce the processing of each construct. As shown in Fig. 1*B*, all of the deletion mutants of the extracellular/intraluminal domain are responsive to PDGF treatment to the same extent, thus indicating that this domain of APP is not involved in the response to PDGF. An additional deletion mutant, represented by Cys^{99} fused to Gal4, was not suitable for

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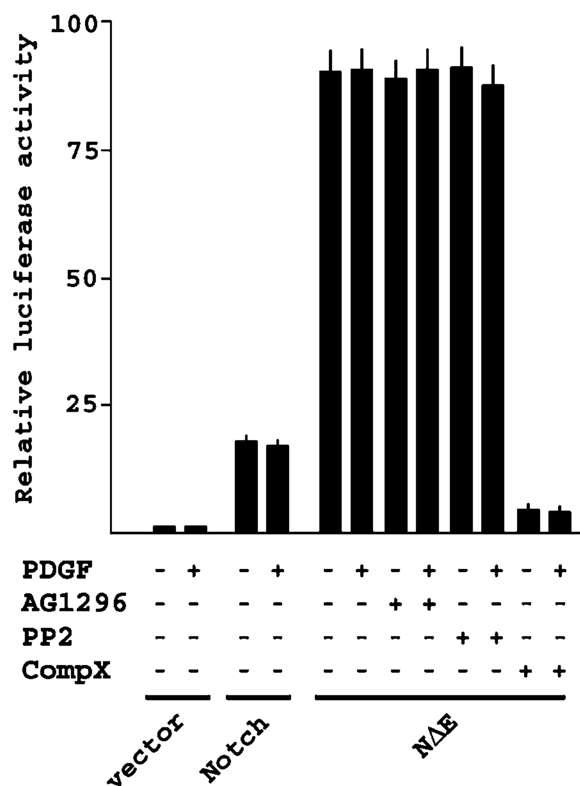

FIG. 2. **PDGF treatment has no effect upon Notch processing.** HeLa cells were transiently co-transfected with cbf1-luc vector and with expression vectors encoding Notch or its deletion mutant NotchΔE (NΔE). 24 h after transfection, the cells were exposed or not for 24 h to 40 ng/ml recombinant human PDGF-BB and, as indicated, to 2 μM PDGF-R inhibitor AG1296, 10 μM Src inhibitor PP2, or 10 μM γ-secretase inhibitor compound X. Standard deviations of triplicate luciferase measurements are reported.

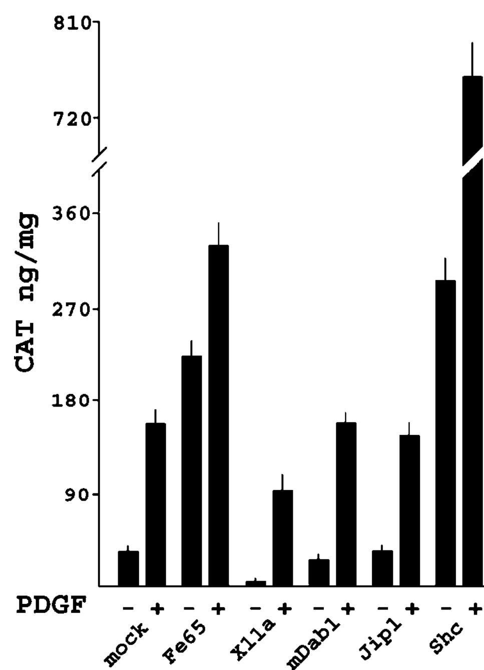

FIG. 3. **Fe65, X11, and Shc overexpression affects the cleavage of APP-Gal4.** HeLaAG cells, stably expressing APP-Gal4 protein, were transiently co-transfected with G5B-CAT plasmid and with vectors driving the expression of Fe65, X11, mDab, JIP1, or Shc or with empty vector. 24 h after transfection, the cells were exposed or not to recombinant PDGF-BB for an additional 24 h. Standard deviations of triplicate CAT measurements are reported.

this experiment, given the high basal level of CAT protein observed upon transfection, regardless of the treatment with PDGF. Similarly, three deletion mutants of the intracellular (IC) domain of APP have been generated and cloned in-frame with the whole Gal4 protein. The analysis of these deletion mutants allowed us to observe that the removal of the C-terminal 14 residues of APP results in the abolition of the responsiveness to PDGF (see Fig. 1*C*). This result is in agreement with those demonstrating that the deletion of the YENPTY motif present in this region abolishes the β-secretase-mediated processing of APP and strongly decreases Aβ production (37).

PDGF Specifically Activates the Secretase-directed Processing of APP—The abolition of the PDGF effect on APP-Gal4 processing observed in APP mutants lacking part of the cytodomain suggests that the PDGF-Src-Rac pathway could specifically activate the processing of APP and not that of other γ-secretase substrates lacking specific signals present in APP. To address this point, we examined the effects of the PDGF-activated pathway on Notch, another substrate of the secretase machinery. To do this, we transfected HeLa cells with Notch or with its deletion mutant, NotchΔE, together with a plasmid in which the luciferase gene is transcribed under the control of cbf-1 cis-elements. As shown in Fig. 2, in cells transfected with the entire Notch protein, the expression of the reporter gene was not activated by the PDGF treatment, and in cells transfected with NotchΔE, which is a substrate of γ-secretase (34), the treatments with the PDGF-R inhibitor AG1296 or with the Src inhibitor PP2 had no effect. As expected, however, the γ-secretase inhibitor, compound X, completely inhibited luciferase gene expression. These results support the hypothesis of a specific effect of the PDGF on APP processing. Considering the results reported in Fig. 1*C*, it is possible that one or more specific ligands of the APP cytodomain could play a role in the induction of APP cleavage by PDGF.

Adaptor Proteins Interacting with the Cytodomain of APP Affect the PDGF-induced Cleavage of APP-Gal4—The region of the APP cytodomain, whose deletion causes the abolition of the response to PDGF, contains the YENPTY motif that is known to interact with several different PTB-containing proteins. To evaluate the role of these molecules on APP-Gal4 cleavage induced by PDGF, HeLaAG cells, stably expressing APP-Gal4 (33), were transfected with vectors driving the expression of Fe65, X11α, mDab1, JIP1, or Shc and then treated or not with PDGF-BB. As shown in Fig. 3, some of these proteins have a significant effect on CAT accumulation, either in the presence or absence of PDGF treatment. The overexpression of Fe65 results in an accumulation of CAT that is higher than that previously observed after PDGF treatment. The exposure to PDGF of Fe65-transfected cells let us observe even higher levels of CAT, but lower than those expected if the CAT accumulation due to PDGF treatment is simply summed to that because of Fe65 overexpression alone. On the contrary, CAT expression upon PDGF treatment in X11α-transfected HeLaAG cells was significantly lower than that observed in cells not overexpressing X11. Finally, although mDab1 and JIP1 have no evident effect upon PDGF-induced APP-Gal4 cleavage, the consequences of Shc transfection were dramatic. In fact, the levels of CAT reached in cells transfected with Shc vector were two times higher than those observed in mock-transfected cells exposed to PDGF. More importantly, PDGF and Shc seem to act cooperatively, given the very high levels of CAT measured when Shc-transfected cells were exposed to PDGF.

Fe65-induced Cleavage of APP-Gal4 Is Independent from the Pathway Activated by PDGF—We first explored the effects of Fe65 and asked whether these effects are due to the interaction of Fe65 with APP. To this aim, we transfected HeLaAG cells

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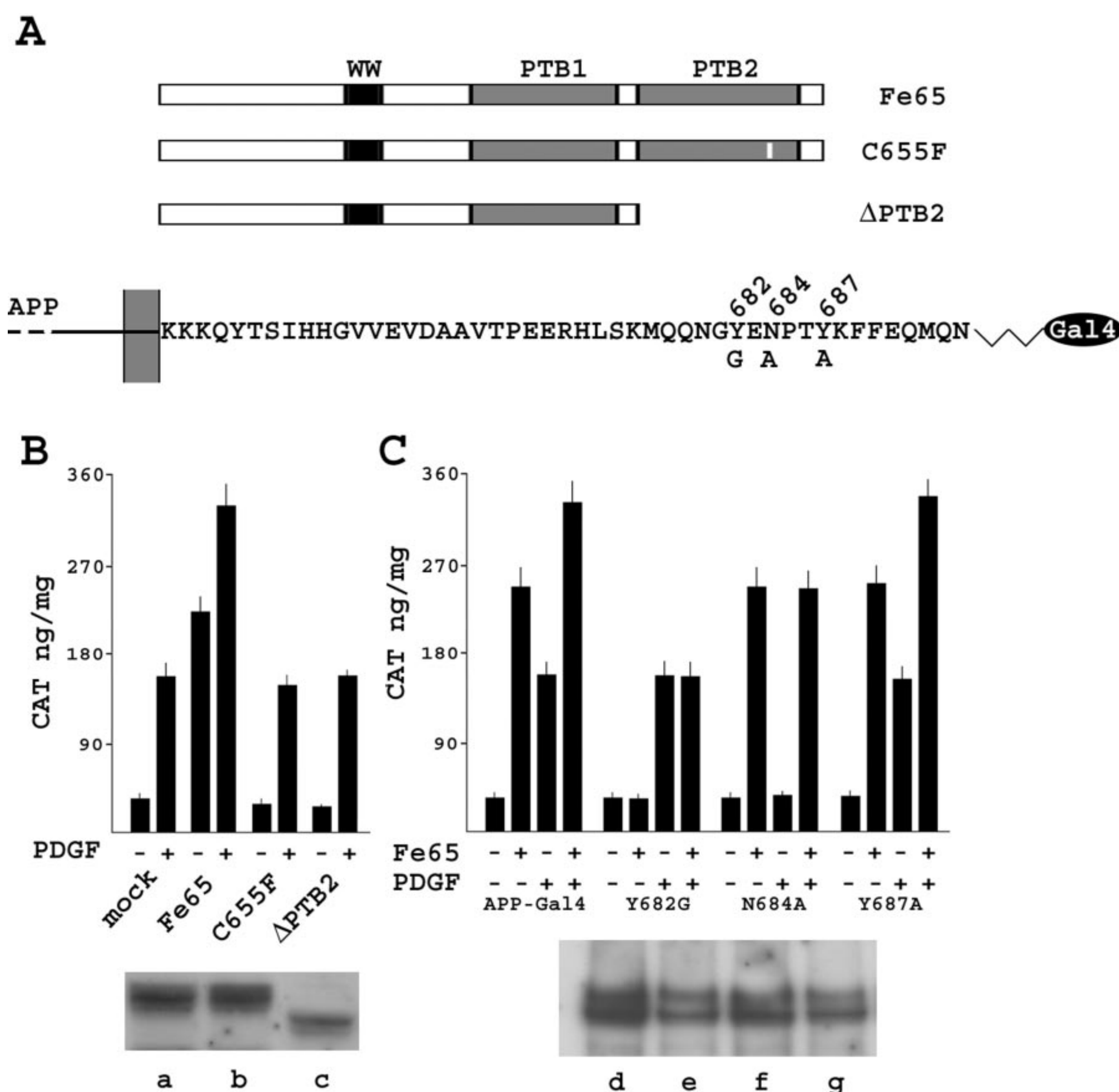

FIG. 4. **Fe65 induces the cleavage of APP-Gal4 through a pathway different from that activated by PDGF.** *A*, the constructs used in these experiments were two Fe65 mutants, C655F and ΔPTB2, and three APP-Gal4 mutants, Y682G, N684A, and Y687A. The first two mutants bear the point mutation of the cysteine 655 into phenylalanine and the deletion of the C-terminal PTB domain, respectively, whereas in the APP-Gal4 mutants, tyrosine 682 is changed into glycine, and asparagine 684 and tyrosine 687 are changed into alanine. *B*, HeLaAG cells, stably expressing APP-Gal4 protein, were transiently co-transfected with G5B-CAT plasmid and with vectors driving the expression of wild-type rat Fe65 or mutant Fe65, which is unable to interact with APP. 24 h after transfection, the cells were exposed or not to recombinant PDGF-BB for an additional 24 h. *Inset*, Western blot of the two Fe65 mutant proteins used, demonstrating that their expression is comparable with that of the wt Fe65 (*a*, wt Fe65; *b*, C655F; *c*, ΔPTB2). *C*, HeLa cells were transfected with G5B-CAT and APP-Gal4 or mutant APP-Gal4 Y682G, N684A, and Y687A. Cells were also transfected with Fe65 and/or exposed to PDGF where indicated. *Inset*, Western blot of the three APP-Gal4 mutant proteins used, demonstrating that their expression is comparable with that of the wt APP-Gal4 construct (*d*, wt APP-Gal4; *e*, Y682G; *f*, N684A; *g*, Y687A). Standard deviations of triplicate CAT measurements are reported in all of the panels.

with mutant forms of Fe65 lacking the PTB2 domain, which is involved in the interaction with APP, or bearing a point mutation in the PTB2 domain (C655F), which is known to prevent the formation of the Fe65-APP complex (8, 27). These two mutant proteins were completely devoid of activity, as demonstrated by the fact that CAT does not accumulate upon their transfection. Furthermore, PDGF treatment has the same effect on mock-transfected HeLaAG cells and in cells transfected with Fe65 mutants, thus suggesting no dominant-negative effect of these mutant proteins on PDGF-induced pathway (see Fig. 4*B*). The observation that the effects of Fe65 and those of PDGF are not cooperative suggests that Fe65 could induce the cleavage of APP-Gal4 through a pathway different from that activated by PDGF. This possibility is supported by the analysis of the effects of PDGF treatment or of the Fe65 overexpression on the cleavage of APP-Gal4 proteins, bearing point mutations of the YENPTY motif. In fact, as shown in Fig. 4*C*, the mutation of the Tyr[682] residue, which is known to prevent the binding of Fe65 (8), completely abolishes the effect of Fe65 overexpression, whereas the mutations of Asn[684] and Tyr[687] residues, which are compatible with the formation of Fe65-APP complex (8), have no effect on the cleavage induced by Fe65. On the contrary, the mutation of Tyr[682] does not affect the cleavage of APP-Gal4 induced by PDGF, whereas the mutation of Asn[684] completely abolishes the PDGF-induced cleavage.

Fe65 Induces the Cleavage of the APP C-terminal Domain by Caspase 3—The cleavage of APP-Gal4 induced by PDGF treatment is completely prevented by the γ-secretase inhibitor compound X (33). In HeLa cells transiently transfected with APP-Gal4 and Fe65, compound X treatment only decreases the cleavage of APP-Gal4 induced by Fe65 (see Fig. 5*A*), and no significant effect was seen in HeLaAG cells (data not shown).

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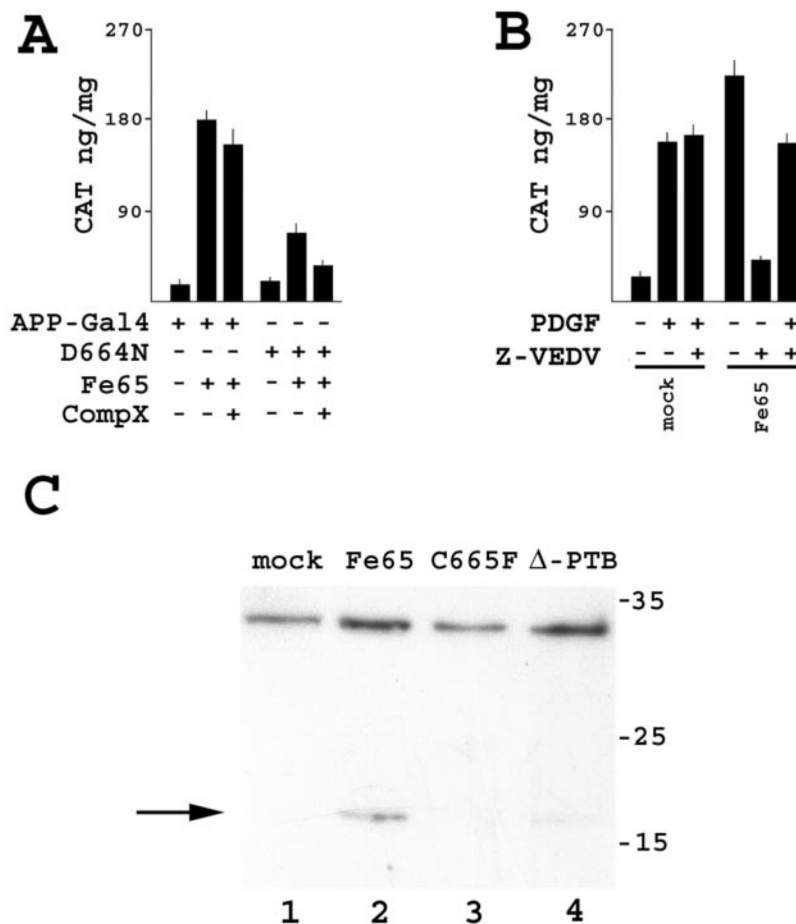

FIG. 5. **Fe65-induced cleavage of APP-Gal4 is dependent upon caspase activity.** *A*, HeLa cells were transfected with G5B-CAT and APP-Gal4 or mutant APP-Gal4 D664N, with or without Fe65 expression vector, in the presence or in the absence of γ-secretase inhibitor compound X (as indicated). *B*, HeLaAG cells stably expressing APP-Gal4 protein were transiently co-transfected with G5B-CAT plasmid, with or without the vector driving the expression of wild-type rat Fe65. 24 h after transfection, the cells were exposed or not to recombinant PDGF-BB for an additional 24 h in the presence or absence of the caspase inhibitor Z-VEDV-fmk. Standard deviations of triplicate CAT measurements are reported in both the panels. *C*, Western blot with caspase3 antibody (Upstate Biotechnology) of extracts from HeLa cells transfected with APP-Gal4 and with empty vector (*lane 1*), wt Fe65 (*lane 2*), mutant Fe65 C655F (*lane 3*), or Δ-PTB2 (*lane 4*). The *arrow* indicates the cleaved form of caspase 3.

These results suggest that the main mechanism responsible for the effects of Fe65 overexpression is not the γ-secretase-directed cleavage. Another possible mechanism through which Fe65 could induce the release of AID-Gal4 from the membrane is the cleavage of APP cytodomain by caspases. It was, in fact, demonstrated that several caspases are able to cleave APP between Asp^{664} and Ala^{665} (5). On this basis, we generated an APP-Gal4 mutant in which the Asp^{664} is mutated into an asparagine residue and demonstrated that this mutation significantly inhibits the cleavage observed as a consequence of Fe65 overexpression (Fig. 5*A*). The residual ability of Fe65 to induce the cleavage of the APP-D664 mutant is counteracted by the γ-secretase inhibitor compound X, thus indicating that Fe65 may also induce the APP cleavage by this enzyme when the caspase-directed cleavage is prevented. In accord with these results, we observed that a caspase-3 inhibitor, Z-DEVD-fmk, inhibits the Fe65-induced CAT accumulation and APP-Gal4 cleavage, whereas it has no effect on the PDGF-induced phenomenon (see Fig. 5*B*).

There are several results indicating that caspase-mediated cleavage of APP cytodomain takes place in various experimental conditions, and the accumulation of APP itself induces caspase-3 activation in neuronal cells (38). In HeLaAG cells expressing APP-Gal4 protein, no induction of caspase-3 was observed, whereas the contemporary expression of APP-Gal4 and Fe65 is accompanied by the activation of caspase-3 (see Fig. 5*C*). FACS analysis excluded the possibility that Fe65 overexpression induces any HeLaAG cell apoptosis, and this is in agreement with the absence in these cells of any poly(ADP-ribose) polymerase cleavage (not shown).

Shc and PDGF-BB Cooperatively Induce APP Processing—As shown in Fig. 3, Shc overexpression in HeLaAG cells causes a great increase of CAT gene transcription, and the contemporary treatment of transfected cells with PDGF results in a cooperative effect leading to a massive CAT accumulation. This observation suggests that Shc and PDGF act through the same mechanism, and this possibility is strongly supported by the results of the co-transfection of Shc with the mutant APP-Gal4 constructs shown in Fig. 6*A*. In fact, only the mutant APP-Gal4, in which asparagine 684 is changed into alanine, failed to be sensitive to both Shc transfection with or without PDGF treatment, thus showing the identical behavior observed in the experiment of Fig. 4*C*. Furthermore, the effects of Shc are prevented by the treatment of HeLaAG cells with the γ-secretase inhibitor compound X (see Fig. 6*B*), as observed in the same cells treated with PDGF, and not by the caspase inhibitor Z-DEVD-fmk, which inhibits the Fe65-induced effects (see Fig. 5*B*).

We demonstrated previously that PDGF induces the cleavage of APP through a pathway that includes Src and Rac1 (33). To ascertain whether Shc also takes part in the same pathway, we co-transfected HeLaAG cells with Shc and with constitutively active (SrcYF and RacQL) or dominant-negative mutants (SrcYFKM and RacN17) of Src and Rac1. As shown in Fig. 6*C*, SrcYF and RacQL act cooperatively with Shc, mimicking the behavior of PDGF, whereas the co-transfection with Shc of the two dominant-negative mutants of Src and Rac1 results in a strong inhibition of the effects of Shc. A similar inhibition was also observed in HeLaAG cells transfected with Shc and treated with the PDGF-R inhibitor AG1296 and the Src inhibitor PP2.

We demonstrated previously that PDGF and Src activate the processing of APP through the consecutive action of β- and γ-secretases (33). To support the finding that Shc activates the

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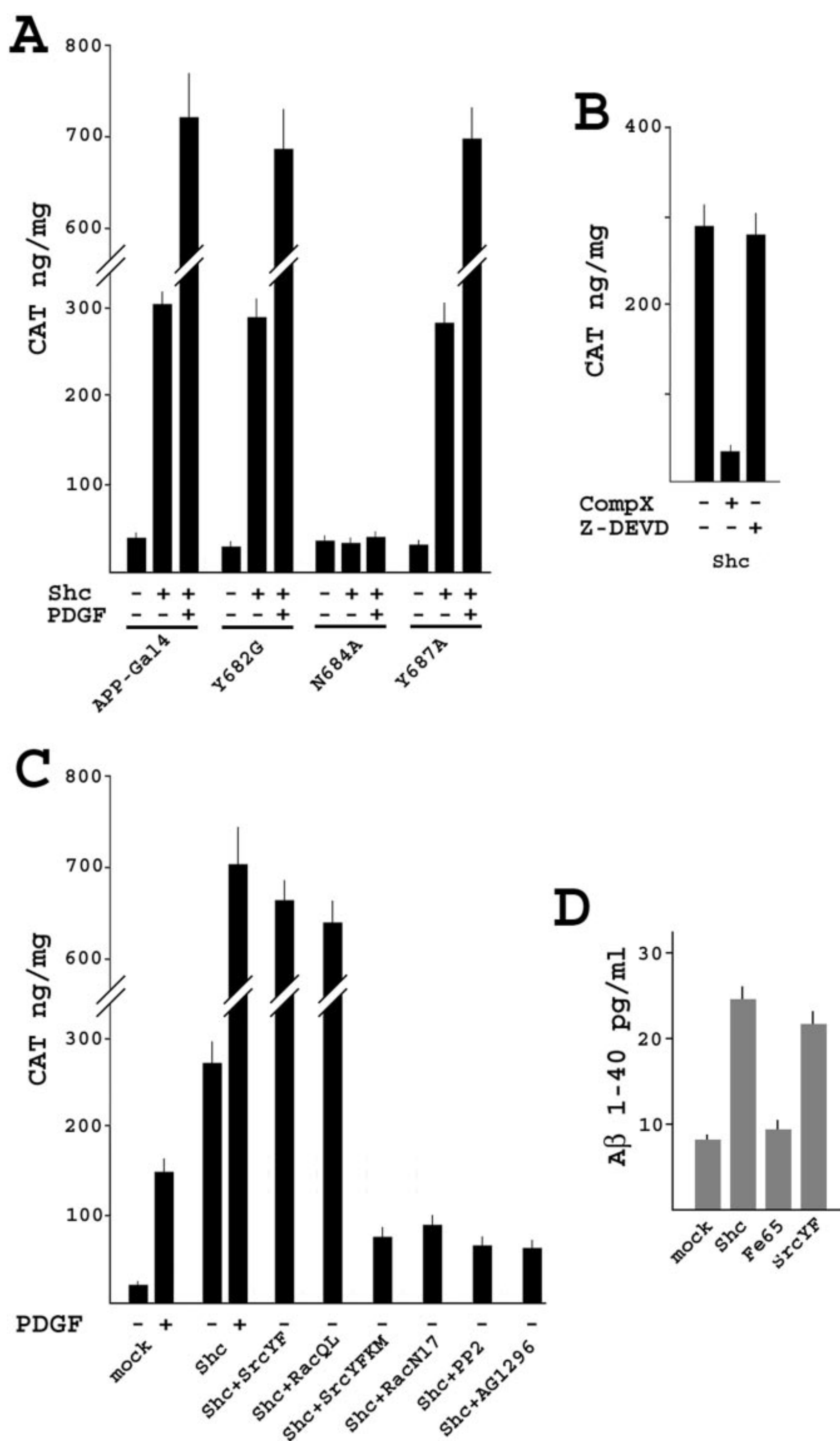

FIG. 6. **Shc acts in the same pathway activated by PDGF.** *A*, HeLa cells were transfected with G5B-CAT and APP-Gal4 or mutant APP-Gal4 Y682G, N684A, and Y687A. Cells were also transfected with Shc p46 vector and/or exposed to PDGF where indicated. *B*, HeLa cells were transfected with G5B-CAT, APP-Gal4, and Shc and treated or not with γ-secretase inhibitor compound X or caspase inhibitor Z-VEDV-fmk. *C*, HeLa cells were transfected with G5B-CAT, APP-Gal4, and/or Shc and, where indicated, with vectors directing the expression of constitutively active forms of Src (SrcYF) and Rac1 (RacQL) or dominant-negative mutants of these two proteins (SrcYFKM and RacN17). The *last two bars* indicate the amount of CAT in cells transfected with APP-Gal4 and Shc and treated with Src tyrosine kinase inhibitor PP2 or PDGF-R tyrosine kinase inhibitor AG1296. Standard deviations of triplicate CAT measurements are reported in all of the panels. *D*, HEK293 cells were transiently transfected with human APP695 and vectors encoding the indicated proteins. Aβ1–40 was measured as reported under "Materials and Methods." Standard deviations of triplicate experiments are reported. *, $p < 0.01$, indicates that the mean value is significantly different from that of the mock-transfected cells.

processing of APP through the same pathway of PDGF and Src, we measured the levels of Aβ peptides in HEK293 cells transfected with APP695 and/or Shc or Fe65 or the constitutively active form of Src (SrcYF). As shown in Fig. 6*D*, Shc transfection induced a significant accumulation of Aβ, similar to that observed in cells expressing SrcYF, whereas the modest increase observed after Fe65 expression was not significant.

DISCUSSION

The regulation of APP processing and Aβ generation is still not fully understood. Knowing the mechanisms underlying this issue would give new information useful to solving the puzzling problem of the functions of APP and which, on the other hand, could suggest new targets for the pharmacological manipulation of the system. By searching for extracellular signals affecting APP processing, we found that PDGF-BB is a strong activator of the secretase-driven cleavage of APP and Aβ production (33). To identify the mechanisms bridging the PDGF-signaling pathway to APP, we searched for the molecular constraints of APP necessary for the PDGF-induced cleavage. Although the extracellular domain of APP seems to have no roles, the YENPTY motif present in the cytodomain of APP is absolutely necessary to allow APP to be sensitive to PDGF, in agreement with the well demonstrated role of this amino acid stretch that is involved in APP intracellular trafficking. It is, in turn, probably responsible for the targeting of APP toward the compartments where APP is cleaved (37). This YENPTY motif

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is known to bind several PTB-containing molecules; thus, we asked whether the PDGF-induced cleavage of APP is affected by the overexpression of these APP ligands. The results reported in this paper indicate that two of these molecules, X11α and ShcA, clearly affect the regulation of APP processing by PDGF. In fact, while in the presence of X11α overexpression, the PDGF-induced cleavage of APP-Gal4 was significantly decreased, the overexpression of Shc increased *per se* the cleavage of APP-Gal4 and dramatically strengthened the effects of PDGF, suggesting a cooperation between these two molecules.

The analysis of APP-Gal4 molecules bearing point mutations in the YENPTY motif led to some interesting findings. This analysis demonstrated, in fact, that the mutation of the Asn[684] residue resulted in a complete abolition of the PDGF-induced cleavage of APP-Gal4. Similarly, this mutation rendered APP-Gal4 no longer sensitive to the Shc-induced cleavage. This result confirms that Shc is acting through the same pathway activated by PDGF, but the analysis of the behavior of the Y682G mutant raises some doubts about the possibility that Shc is working as an APP ligand. In fact, it was reported that Shc interacts with APP cytodomain phosphorylated at Tyr[682] (12), but the mutation of this amino acid has no effect on both PDGF- and Shc-induced cleavage of APP-Gal4. Furthermore, although both Abl and Src are able to phosphorylate Tyr[682] of APP (Ref. 20 and unpublished results), our results suggest that this event is not required to induce APP-Gal4 cleavage. On this basis, it is possible to speculate that a further ligand of the YENPTY motif, whose binding is dependent upon the Asn[684] residue, is, in the main, responsible for the targeting of APP to the secretase pathway. The observation that X11 inhibits the PDGF-induced cleavage of APP-Gal4 (see Fig. 3) is in agreement with previous results indicating that this adaptor molecule stabilizes APP by decreasing its cleavage by secretases (15–16). Considering that X11 proteins do not interact with APP bearing the mutation of Asn[684] (9), one possibility that could be explored is that X11s compete for the binding to APP with the hypothesized new ligand of the YENPTY motif, sharing with it the conformational requirement of the APP cytodomain. One possible reason why the identification of this partner is still elusive could be that the experimental system used to isolate all of the known ligands of APP cytodomain, *i.e.* the two-hybrid interaction trap, fails to reveal the interactions that are dependent upon posttranscriptional modifications that do not take place in yeast.

We have also shown that the release of Gal4 from the membrane-bound APP-Gal4 and the consequent activation of the reporter gene observed in cells overexpressing Fe65 are mostly due to the cleavage of the APP cytodomain at the Asp[664] caspase site. This cleavage causes the removal of the YENPTY motif of the APP cytodomain; thus, it is expected to prevent part of the APP molecules from being targeted to the endocytic compartment where Aβ is supposed to be generated (37). Several studies have tried to address the effect of the binding of Fe65 to APP upon the processing fate of this molecule, but the results obtained so far are contradictory. In fact, in Madin-Darby canine kidney cells, it was observed that Fe65 overexpression induces the accumulation of APP on the cell surface and, at the same time, increases Aβ generation (17), whereas in HEK293 cells, it induces a significant decrease of Aβ 40 and 42 production (39). In another study (40), the expression of Fe65L1 protein in H4 neuroglioma cells resulted in the increased secretion of soluble αAPP without a measurable accumulation of Aβ. On the other hand, it was suggested that caspase-cleaved APP be directed to a more amyloidogenic pathway, thus increasing Aβ generation (41), but these findings were subsequently questioned by demonstrating that caspase activation *per se* did not increase Aβ generation and that, more importantly, caspase-cleaved APP molecules show decreased internalization and Aβ production (42). The levels of Aβ generated in our experimental system are not significantly changed in the presence or in the absence of Fe65 overexpression. However, the observation that the contemporary exposure of HeLaAG cells to both Fe65 overexpression and to PDGF treatment resulted in a CAT accumulation lower than the sum of the two separate effects suggests that Fe65 overexpression subtracts APP-Gal4 molecules from the PDGF-activated pathway. On the other hand, the D664N mutation does not completely abolish the Fe65-induced CAT accumulation, thus suggesting a slight stimulatory effect of Fe65 upon secretase-dependent cleavage of APP when caspase-directed cleavage is prevented. According to this hypothesis, the residual cleavage of APP-Gal4, seen in cells expressing the APP-D664N mutant and Fe65, is blocked by the γ-secretase inhibitor.

The results presented in this paper are in agreement with recent findings (43) demonstrating that the tyrosine kinase inhibitor Gleevec prevents Aβ production both in cultured cells and in animals. We also report experiments demonstrating that the inhibition of the PDGF-induced pathway by Src or PDGF-R inhibitors, PP2 and AG1296, has no effects upon Notch processing, similar to that observed for Gleevec (43). Taken together, all of these results support the possibility that new therapeutic strategies could be developed based upon the inhibition of the tyrosine kinase-directed regulation of APP processing and Aβ generation.

Acknowledgments—We thank Massimo Santoro for Shc-expressing vector and Raphael Kopan and Brian Howell for Notch and mDab1 constructs, respectively.

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Fe65 Is Not Involved in the Platelet-derived Growth Factor-induced Processing of Alzheimer's Amyloid Precursor Protein, Which Activates Its Caspase-directed Cleavage

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J. Biol. Chem. 2004, 279:16161-16169.
doi: 10.1074/jbc.M311027200 originally published online February 6, 2004

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 27, Issue of July 2, p. 28826, 2004
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Printed in U.S.A.

Addition and Correction

Vol. 279 (2004) 16161–16169

Fe65 is not involved in the platelet-derived growth factor-induced processing of Alzheimer's amyloid precursor protein, which activates its caspase-directed cleavage.

Nicola Zambrano, Davide Gianni, Paola Bruni, Fabiana Passaro, Francesca Telese, and Tommaso Russo

Pages 16161, 16162, and 16163: Throughout these pages, Cys^{83} and Cys^{99} should be changed to C83 and C99, since the latter are the standard designations for the intermediate products of the proteolytic cleavage of Alzheimer's β-amyloid precursor protein by α- and β-secretases, respectively. Therefore, there is no relation to the amino acid cysteine (Cys).

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