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Fe65 does not stabilize AICD during activation of transcription in a luciferase assay

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Abstract

The APP intracellular domain (AICD) could be involved in signaling via interaction with the adaptor protein Fe65, and with the histone acetyl transferase Tip60. However, the real function of AICD and Fe65 in regulation of transcription remains controversial. In this study, the human APPGal4 fusion protein was expressed in CHO cells and the transcriptional activity of AICDGal4 was measured in a luciferase-based reporter assay. AICDGal4 was stabilized by expression of Fe65 and levels of AICDGal4 controlled luciferase activity. On the contrary, when human APP was expressed in CHO cells, coexpression of Fe65 increased luciferase activity without affecting the amount of AICD fragment. AICD produced from APP was protected from degradation by orthophenanthroline, but not by lactacystine, indicating that AICD is not a substrate of the chymotryptic activity of the proteasome. It is concluded that Fe65 can control luciferase activity without stabilizing the labile AICD fragment.
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Keywords: Amyloid precursor protein; AICD; Fe65; Transcription; Luciferase

The amyloid precursor protein (APP) is a type I transmembrane protein processed by two catabolic pathways. In the non-amyloidogenic pathway, APP is cleaved by an α-secretase activity generating a soluble α-APP and an α-C-terminal fragment (α-CTF) anchored in the membrane [1,2]. In the amyloidogenic pathway, APP is cleaved by the β-APP-cleaving enzyme (BACE) generating a soluble β-APP and a transmembrane β-C-terminal fragment (β-CTF) (for a review see [3]). Both α- and β-CTFs are substrates of a γ-secretase activity which releases P3 and Aβ, respectively, with the concomitant production of APP intracellular domain (AICD) [4–7]. By homology with the Notch intracellular domain (NICD) released from Notch by the same secretase activities, AICD could be involved in the regulation of gene transcription [8–10]. The possible function of AICD in nuclear signaling was first tested by Cao and Südhof [11], using APP fused to the DNA binding domain of a yeast transcriptional factor Gal4, which can activate transcription of a luciferase reporter gene cloned downstream of a Gal4 responsive element sequence. This function of AICD could be mediated by its interaction with Fe65 [12,13], an adaptor protein, which binds to Tip60, an histone acetyl transferase [11,14]. Although AICD released from APP is a highly labile fragment [15], transfection of cDNA corresponding to the AICD sequence allowed to demonstrate that this fragment is stabilized by forming complexes with Fe65 [16,17]. Therefore, the key role of Fe65 in the transactivation process was proposed to result from the stabilization of AICD. Several genes were identified as possible targets of AICD-mediated regulation of transcription, including tetraspanin KAI1/CD82, APP, Glycogen Synthase Kinase-3β, and neprilysin [16,18–20]. However, reduction of AICD expression levels did not affect expression of these target genes, and Fe65 was dem-

Abbreviations: Aβ, β-amyloid peptide; APP, amyloid precursor protein; AICD, APP intracellular domain; sαAPP, soluble α-amyloid precursor protein; BACE, β-site APP-cleaving enzyme; CHO, Chinese hamster ovary; CTF, C-terminal fragment of APP; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine tert-butyl ester; NICD, Notch intracellular domain; PNT, 1-10 phenanthroline monohydrate.

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onstrated to play a dominant role in nuclear signaling [21,22]. We demonstrate here that Fe65 does not stabilize AICD released from APP, but activates transcription in a luciferase-based reporter assay.

Materials and methods

Antibodies and reagents. The human specific anti-APP WO-2 antibody was obtained from The Genetics Company (Schlieren, Switzerland). The anti-APP C-terminal antibody was kindly provided by N. Sergeant (INSERM U422, Lille, France). The monoclonal anti-Fe65 antibody was from Campro Scientific (Veenendal, The Netherlands). Secondary antibodies were from Amersham Bioscience (Uppsala, Sweden). All cell culture media and antibiotics were from Invitrogen (Carlsbad, CA). Inhibition of γ-secretase activity was achieved by an 8-h cell treatment with 250 nM DAPT (a generous gift from Luc Mercken, Aventis, Paris, France). Inhibition of metalloprotease was performed using 100 μM of 1-10 Phenanthroline monohydrate (PNT, Sigma–Aldrich) during 8 h.

Plasmids. The Gal4RE luciferase (pG5E1B-luc) reporter gene and the Fe65 expression vector (pCMV5-Fe65) have been previously described [11]. The *Renilla* luciferase reporter vector (phRG-TK) was from Promega (Madison, WI). All human APP695 and APP695Gal4 constructs were derived from the pSVK3-APP695 and pMst-APP (APPGal4) parental vectors [11].

Cell cultures and transfection. The culture of CHO cells has been previously described [23]. For transient transfection, cells were seeded at the density of 3×10^5 cells/cm^2 for 24 h prior to Lipofectamine™-based transfection with pSVK3-APP695 and/or pCMV-Fe65 (2 μg per well) according to manufacturer's instruction (Invitrogen, Carlsbad, CA).

Protein analysis by Western blotting. Cells were lysed in 150 μl of sample buffer (Deoxycholate 1%, pH 11.3, 5 mM PNT) and sonicated for 5 s. Thirty micrograms of the sample was loaded onto NuPage™ 10–20% Tricine gels and transferred to nitrocellulose. Membranes were then saturated (5% skimmed milk in 0.05% Tween 20/PBS) for 30 min, washed, and incubated overnight at 4 °C with the primary antibody diluted in 0.05% Tween 20/PBS at the following concentrations: 0.5 μg/ml for the WO-2 antibody; 1:2000 for the anti-Fe65 antibody and 1:5000 for the anti-APP C-terminal antibody. Membranes were then washed and incubated at room temperature for 60 min with 1:10,000 of the appropriate horseradish peroxidase-conjugated secondary antibody. After washing, the immunoreactive bands were visualized by chemiluminescence. Signal ratios were quantified using the Quantity One software coupled to the Gel Doc 2000 device (Bio-Rad, Hercules, CA).

APPGal4 and Gal4Tip60 transactivation assays. APPGal4 transactivation assays were performed in CHO cells essentially as described [11]. Luciferase activity was corrected for transfection efficiency using a co-transfected phRL-TK *Renilla* luciferase vector (Promega). Cells (2×10^5) were transfected (see above) with 0.4 μg of APPGal4 or APP695 vectors, 0.2 μg of Gal4RE reporter gene, 0.2 μg of Fe65 or empty pSV2 vector, and 0.001 μg of phRL-TK. Luciferase activity was measured 48 h after transfection with the dual-luciferase assay system (Promega).

Statistical analysis. The number of samples (n) in each experimental condition is indicated in figure legends. When two experimental conditions were compared, statistical analysis was performed using an unpaired t test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-test.

Results

Release of AICDGal4 measured in a luciferase-based reporter assay

Human APP695 fused to the DNA binding domain of Gal4 (APPGal4) was expressed by transfection of CHO cells and cellular extracts were analyzed by Western blotting, using the anti-APP C-terminal antibody. Results from Fig. 1A indicate that this antibody detected endogenous APP from CHO cells. After transfection, an increased signal was observed at a higher molecular weight corresponding to the APPGal4 fusion protein. The same antibody detected C-terminal fragments of the APPGal4 fusion protein (CTFGal4). Following an 8 h treatment in the presence of 250 nM DAPT, an increase in CTFGal4 was observed together with an important decrease of a lower sharp band. Since DAPT very efficiently inhibits γ-secretase activity at this concentration [24], this band most probably corresponds to AICD fused to the DNA binding domain of Gal4 (AICDGal4).

When APPGal4 was coexpressed with the luciferase reporter gene in CHO cells, the transcription of the reporter gene could be turned on by the fusion protein (Fig. 1B). When coexpression occurred in the presence of DAPT, the γ-secretase inhibitor was able to significantly decrease the transcriptional activity, only when the fusion APPGal4 protein was expressed (Fig. 1B). Altogether, these results clearly demonstrate a correlation between transcriptional activity and the amount of AICDGal4 detected by Western blotting.

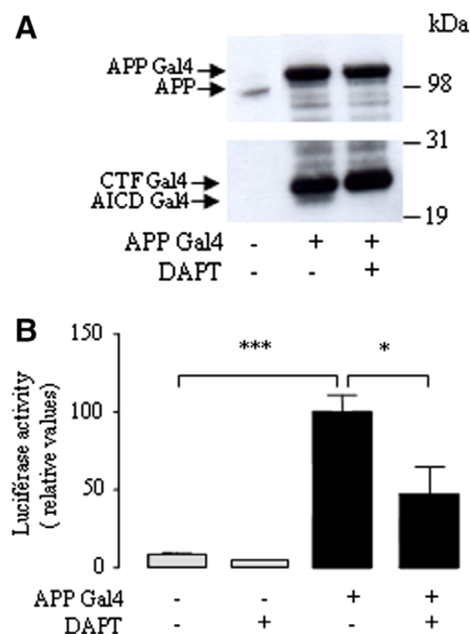

Fig. 1. Expression of human APPGal4 fusion protein in CHO cells and transactivation activity of AICDGal4. (A) Cellular extracts from CHO cells expressing (+) or not (−) human APPGal4 fusion protein (APPGal4) were analyzed by Western blotting using the anti-APP C-terminal polyclonal antibody, which detects APPGal4, APP, CTFGal4, and AICDGal4. Cells were treated (+) or not (−) for 8 h with 250 nM DAPT (DAPT). (B) A plasmid encoding the APPGal4 fusion protein (APP Gal4+) or an empty vector (APPGal4−) was co-transfected with the luciferase reporter plasmid in CHO cells, and cells were treated (+) or not (−) for 8 h with 250 nM DAPT (DAPT). Luciferase activities are given as relative values, the highest activity corresponding to 100 (mean ± SE, $n = 10$, $^{***}p < 0.001$, $^{*}p < 0.05$).

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