Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes

Sébastien S. Hébert¹, Lutgarde Serneels¹, Alexandra Tolia¹, Katleen Craessaerts¹, Carmen Derks¹, Mikhail A. Filippov^{2,3}, Ulrike Müller^{2,3} & Bart De Strooper¹⁺

¹Neuronal Cell Biology and Gene Transfer, CME, Flanders Interuniversity Institute for Biotechnology (VIB4) and Katholieke Universiteit Leuven, Leuven, Belgium, ²Max-Planck Institute for Brain Research, Frankfurt, Germany, and ³Institute for Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany

γ -Secretase-dependent regulated intramembrane proteolysis of amyloid precursor protein (APP) releases the APP intracellular domain (AICD). The question of whether this domain, like the Notch intracellular domain, is involved in nuclear signalling is highly controversial. Although some reports suggest that AICD regulates the expression of KAI1, glycogen synthase kinase-3 β , Neprilysin and APP, we found no consistent effects of γ -secretase inhibitors or of genetic deficiencies in the γ -secretase complex or the APP family on the expression levels of these genes in cells and tissues. Finally, we demonstrate that Fe65, an important AICD-binding protein, transactivates a wide variety of different promoters, including the viral simian virus 40 promoter, independent of AICD coexpression. Overall, the four currently proposed target genes are at best indirectly and weakly influenced by APP processing. Therefore, inhibition of APP processing to decrease A β generation in Alzheimer's disease will not interfere significantly with the function of these genes.

Keywords: Alzheimer's disease; amyloid precursor protein; regulated intramembrane proteolysis; secretase

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INTRODUCTION

Alzheimer's disease is characterized by the accumulation of insoluble A β deposits in the brain (Selkoe, 2001). A β peptides are produced by proteolytic processing of the amyloid precursor

protein (APP) by β - and γ -secretases (Haass, 2004). Most problematic for therapy, but very interesting from a fundamental point of view, is the possibility that γ -secretase cleavage of APP activates a signalling pathway analogous to the Notch signalling pathway (Annaert & De Strooper, 1999). Indeed, proteolytic processing of APP by γ -secretase releases, concomitantly with the A β peptides, the APP intracellular domain (AICD), which has been proposed to function in gene transcription regulation (Cao & Südhof, 2001).

A series of candidate AICD target genes have been identified in the past few years, including tetraspanin KAI1/CD82, APP, glycogen synthase kinase-3 β (GSK-3 β) and Neprilysin (Baek *et al*, 2002; Kim *et al*, 2003; von Rotz *et al*, 2004; Pardossi-Piquard *et al*, 2005). In the present work, we raised the questions of whether γ -secretase inhibition would affect the expression of these putative AICD target genes and whether AICD indeed has a direct role in gene transcription regulation of these genes.

RESULTS

Pharmacological inhibition of AICD generation

To assess the risk of blocking AICD generation on gene transcription events, we probed the effects of inhibiting γ -secretase activity on the expression of the putative AICD target genes. Inhibition of γ -secretase activity with L-685,458 (γ -secretase inhibitor X) or DAPT (γ -secretase inhibitor IX) did not significantly affect endogenous (full-length) APP, KAI1, GSK-3 β or Neprilysin protein levels in any of the cell lines tested, including murine embryonic fibroblasts (MEFs), HeLa, COS and the neuronal cell type Neuro2A (Fig 1A). We confirmed the presence of endogenous AICD, migrating below endogenous APP carboxy-terminal fragments (CTFs) in MEFs (Fig 1B, left panel), and its disappearance in the presence of γ -secretase inhibitors (Fig 1B).

AICD target genes in γ -secretase-deficient models

We next investigated whether genetic (continuous) inactivation of γ -secretase activity would affect the expression of any of the AICD

¹Neuronal Cell Biology and Gene Transfer, CME, Flanders Interuniversity Institute for Biotechnology (VIB4) and Katholieke Universiteit Leuven, Herestraat 49, Leuven 3000, Belgium

²Max-Planck Institute for Brain Research, Deutschordenstrasse 46, 60598 Frankfurt, Germany

³Institute for Pharmacy and Molecular Biotechnology, University of Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany

+Corresponding author. Tel: +32 163 46227; Fax: +32 163 47181; E-mail: bart.destrooper@med.kuleuven.ac.be

Fig 1 | Pharmacological inhibition of γ -secretase and effects on the expression of AICD target genes protein. (A) MEF, HeLa, COS and Neuro2A cells were treated with DAPT or L-685,458 for 16–18 h. Levels of endogenous proteins are shown. APP CTF accumulation demonstrates the activity of the inhibitors. (B) Control western blot analysis of MEFs showing endogenous AICD migrating below APP CTFs, downregulated in the presence of γ -secretase inhibitors (left panel). The presence of endogenous AICD is dependent on catalytically active PS (right panel). AICD, amyloid precursor protein (APP) intracellular domain; APP CTFs, APP carboxy-terminal fragments; APP FL, APP full-length; DMSO, dimethylsulphoxide; GSK-3 α/β , glycogen synthase kinase-3 α/β ; MEFs, murine embryonic fibroblasts; NEP, Neprilysin; WT, wild type.

candidate target genes. The steady-state protein levels of APP, KAI1, GSK-3 β and Neprilysin were essentially unaffected in γ -secretase-deficient presenilin (PS) double-knockout (dKO) fibroblasts (Fig 2A, compare lanes 1 and 2). Likewise, the expression levels of these genes were not significantly affected in other γ -secretase-deficient cells such as Aph-1A KO fibroblasts (Serneels *et al*, 2005; Fig 2C). To further assess the functional relationship between endogenous AICD levels and the expression of the target genes, we reconstituted the PS dKO cells with various PS1 constructs. We chose three types of PS1 molecule: (i) functionally active (that is, γ -secretase protein and complex maturation rescue)

and catalytically active (wild type (WT), Δ 1–81 and Δ 303–372); (ii) functionally but not catalytically active (P433L); or (iii) non-functionally and non-catalytically active (Δ 451–467). As shown in Fig 2A, endogenous AICD levels did not correlate with the target genes (compare, for example, lanes 3 and 7). For APP, GSK-3 β and Neprilysin, these observations were confirmed *in vivo* in PS1 KO embryo brain (Fig 2B) and in Aph-1A KO whole embryos (Fig 2D; Serneels *et al*, 2005). KAI1 was below detection levels in western blots of these samples (Fig 2B,D).

AICD target genes in APP-deficient models

We also investigated different MEF cell lines deficient for APP, the APP family member APLP2 or both (Fig 3A; supplementary Fig 1A online). As APLP1 is not expressed in fibroblasts (Herms *et al*, 2004), APP/APLP2 dKO MEFs provide a cellular model in which all APP family members are lacking. We reconstituted, in addition, APP expression in APP/APLP2 dKO MEFs using human APP isoform 695 (see supplementary information online). As observed in Fig 3A, GSK-3 β and Neprilysin expressions were not significantly affected by the presence or absence of APP. However, we found, in the different MEF cell lines, large variations in KAI1 expression that were not APP genotype dependent (Fig 3A, compare lanes 3 and 4). No significant changes in expression of KAI1 were detected at the mRNA level by quantitative reverse transcription-PCR (RT-PCR; Fig 3B). Deficiency of APP/APLP2 in the embryo brain did not significantly affect GSK-3 β or Neprilysin protein levels (Fig 3C). Likewise, we could not observe any significant change in Neprilysin mRNA levels in APP/APLP2 dKO and APP/APLP1/APLP2 triple-knockout (tKO) embryonic cortices when compared with WT controls (Fig 3D). By quantitative RT-PCR, we found a very slight upregulation of KAI1 mRNA (1.5- to 1.9-fold) in APP/APLP2 dKO and APP/APLP1/APLP2 tKO embryonic cortices when compared with WT controls (Fig 3D). However, we observed no significant difference in KAI1 protein levels in the brain of APP-deficient adult mice (supplementary Fig 1B online).

AICD is a poor gene transcription stimulator

To answer the question of whether AICD could directly modulate gene transcription, we tested the transactivation properties of AICD in a luciferase-based reporter assay using the endogenous promoter regions of *KAI* and *APP*. We overexpressed two non-tagged AICD constructs on the basis of the ϵ - and γ -secretase cleavage sites of human APP (C50 and C60, respectively). In contrast with the approach of other authors who used artificial chimaeric AICD/DNA binding domain constructs (Cao & Südhof, 2001), this strategy allowed a direct and quantitative measurement of the intrinsic transactivation properties of AICD. Hes1, an established target gene of the Notch intracellular domain (NICD) signalling pathway, was analysed in parallel to benchmark our reporter assays. Overexpression of C50, C60 and APP C99 (the product of APP generated by β -secretase cleavage) resulted in a weak (maximal $\sim$ 2-fold) activation of the KAI1 promoter (Fig 4A).

As expected, NICD expression resulted in a marked stimulation ($\sim$ 20-fold) of Hes1 promoter-mediated luciferase expression (Fig 4C). Although AICD overexpression *per se* can apparently weakly activate a subset of gene promoters, this effect is minor when compared with the effect of a protein fragment that is really involved in gene transcription regulation.

Fig 2 | Expression levels of AICD target genes in various biological models lacking γ -secretase activity. (A) Western blot analysis of PS1 and PS2 dKO MEF cells. PS dKO MEFs were reconstituted with human PS1 or with PS1 functional mutants. (B) Effects on protein expression levels of target genes in the absence of PS1 *in vivo*. No KAI1 expression is observed at this embryonic stage. (C) Western blot analysis of Aph-1A KO MEFs. In the third lane, Aph-1A KO MEFs were reconstituted with mouse WT Aph-1A. (D) Effects on protein expression levels of target genes in the absence of Aph-1A *in vivo*. Note that KAI1 protein levels are below detection at this embryonic stage. AICD, amyloid precursor protein (APP) intracellular domain; APP CTFs, APP carboxy-terminal fragments; APP FL, APP full-length; dKO, double knockout; E, embryonic day; GSK-3 α/β , glycogen synthase kinase-3 α/β ; MEFs, murine embryonic fibroblasts; NEP, Neprilysin; PS, presenilin; PS1 CTF, PS1 C-terminal fragment; PS1 NTF, PS1 amino-terminal fragment; WT, wild type.

We next tested the transactivation properties of AICD in the presence of the putative scaffolding protein Fe65. Fe65 can effectively bind to APP and APLP intracellular domains and is necessary to drive the expression of reporter genes by the APP-DNA binding domain fusion proteins (Cao & Südhof, 2001). Coexpression of Fe65 with C50 moderately stimulated KAI1 and APP expression (~ 5 - to ~ 8 -fold, respectively; Fig 4A,B). Interestingly, coexpression of Fe65 with NICD resulted also in a similar ~ 4 -fold increase of Hes1 expression (from ~ 20 - to ~ 80 -fold; Fig 4C). In addition and surprisingly, C50 could also stimulate Hes1 promoter activity when Fe65 was present (~ 4 -fold; Fig 4C), and coexpression of NICD with Fe65 resulted in a significant increase in APP and KAI1 promoter activity (comparable to C50/Fe65 induction of activity; Fig 4D,E). Finally, and consistent with a general role of Fe65 in gene transcription activation (Duilio *et al*, 1991; Yang *et al*, 2006), coexpression of Fe65 with AICD or NICD stimulated the expression of a control simian virus 40 (SV40) promoter (Fig 4F).

DISCUSSION

Since the demonstration that APP and Notch are proteolytically processed by PS to release their intracellular domain, it has been speculated, by analogy with the Notch signalling pathway, that AICD could function directly in gene transcription regulation (Annaert & De Strooper, 1999). Since then, many conflicting data and models concerning the role of AICD in promoting gene transcription have emerged in which AICD could function inside (Cao & Südhof, 2001; Cupers *et al*, 2001; Kimberly *et al*, 2001) or outside (Cao & Südhof, 2004; Hass & Yankner, 2005) the nucleus. In addition, several authors have described changes in gene transcription or protein expression of KAI, APP, GSK-3 β , Neprilysin and other genes (von Rotz *et al*, 2004; Ryan & Pimplikar, 2005) due to APP or AICD overexpression or γ -secretase inhibition. However, the overall reported changes have always been weak, and in many cases additional controls to benchmark the reported observations were lacking. Obviously, it is very important to know whether the reported effects of AICD on gene transcription are biologically significant, as inhibition of

Fig 3 | Expression levels of AICD target genes in various biological models lacking APP and its family members. (A) Western blot analysis of MEFs deficient for APP and APLP2 (two independent cell lines are shown). The APP/APLP dKO MEF (1.4) cell line was reconstituted with human N-Myc-tagged APP695 WT using the retroviral vector FUW (see supplementary information online). Clonal cell lines resistant to puromycin selection were categorized as ‘APP rescued’ or ‘APP non-rescued’ cell lines. Western blot analysis of four independent cell lines positive or negative for APP expression was performed. Here, KAI1, GSK-3 β and Neprilysin protein levels are shown. Considerable variations in KAI1 expression in the different cell lines were observed but were not dependent on the presence or absence of APP expression. (B) Quantitative RT-PCR analysis showed no significant difference in KAI1 expression between ‘APP rescued’ cell lines ($n=4$) and APP/APLP2-deficient cell lines (APP^{-/-}, APLP2^{-/-}; $n=2$, line 1.1 and 1.4 in A). Likewise, quantitative RT-PCR analysis of ‘APP rescued’ ($n=4$) versus ‘APP non-rescued’ ($n=4$) cell lines showed no significant difference in KAI1 messenger RNA expression. Bars represent standard errors; P -values were calculated by pairwise fixed reallocation randomization test. (C) Western blot analysis of AICD candidate target genes in APP/APLP2 dKO embryos (lethal combination). GSK-3 β and Neprilysin protein levels from two independent age-matched (E15.5) controls and knockout mice are shown. KAI1 was below detection levels (data not shown). (D) Quantitative RT-PCR analysis of KAI1 and Neprilysin expression in embryonic brain. mRNA expression of KAI1 or Neprilysin was analysed in the cortices of E15.5 dKO (APP^{-/-} APLP2^{-/-}, open boxes, $n=4$) or tKO (APP^{-/-} APLP1^{-/-} APLP2^{-/-}, filled boxes, $n=4$) mice relative to WT controls ($n=4$). Note that no significant difference in expression was found for Neprilysin, whereas KAI1 was slightly upregulated in dKO and tKO brains. Bars represent standard errors; P -values (shown above SE bars) were calculated by pairwise fixed reallocation randomization test. AICD, amyloid precursor protein (APP) intracellular domain; APP FL, APP full-length; dKO, double knockout; E, embryonic day; GSK-3 α/β , glycogen synthase kinase-3 α/β ; MEFs, murine embryonic fibroblasts; NEP, Neprilysin; RT-PCR, reverse transcription-PCR; tKO, triple knockout; WT, wild type.

A β generation by γ -secretase inhibitors would affect such a putative signalling pathway.

We therefore checked the expression levels of APP, GSK-3 β , KAI1 or Neprilysin, four putative AICD-regulated genes, in cell lines treated with γ -secretase inhibitors or in biological models genetically deficient for AICD generation. The significant decrease

or even absence of endogenous AICD production was not reflected in systematic changes in the levels of expression of three out of four putative AICD target genes, namely APP itself and GSK-3 β and Neprilysin. For KAI1, the situation is slightly more complex. On the one hand, in all AICD-deficient cell lines tested (that is, PS KO, Aph-1A KO and APP/APLP2 dKO) and in adult

Fig 4 | Non-specific activation of gene transcription by exogenous Fe65 in luciferase-based reporter assays. Transactivation assays performed with the endogenous promoter regions of KAI1 (A,D), APP (B,E), Hes1 (C) or SV40 (F) coupled to luciferase. The different APP constructs (C50, C60, C99 or full-length 695 isoform) or NICD were expressed alone or in combination with Fe65 in HeLa cells. Control experiments were performed with pGL3-luciferase empty vector (that is, no promoter; indicated with an asterisk). All assays were performed at least three times in duplicate. Similar results were obtained in COS cells (data not shown). APP, amyloid precursor protein; NICD, Notch intracellular domain; SV40, simian virus 40.

mice haploinsufficient for APP (that is, APP^{-/-}, APLP1^{-/-}, APLP2^{+/-} and APP^{-/-}, APLP1^{+/-}, APLP2^{+/-}), KAI1 expression was never correlated with the presence or absence of AICD (thus, in some cell lines, high AICD was correlated with low KAI1 expression, and vice versa in other experiments). Only a slight increase (1.4- to 1.9-fold) in KAI1 message was demonstrated in embryos deficient for APP and its family members (Fig 3D), but the significance of this observation and whether this really supports the role of AICD in KAI1 regulation is at least doubtful, the more so because APP or AICD was suggested to cause upregulation of KAI1 (Baek *et al*, 2002; Ryan & Pimplikar, 2005). Taken together, our data show that the role of AICD in KAI1 expression cannot be considered as established.

We finally moved to an overexpression paradigm, analysing the effects of transfected AICD on the activity of the promoter regions of KAI1 (and APP) in a luciferase-based reporter assay. Although AICD was able to stimulate (less than twofold) KAI1 and APP gene expression, these effects turned out to be minimal when compared with the positive control experiment with NICD, which induced in a similar assay the activity of the Hes1 promoter more than 20-fold (Fig 4C). As AICD has a very relaxed tertiary conformation and is probably undergoing an 'induced fit' when binding its various binding partners (a phenomenon called 'binding promiscuity'; discussed by Reinhard *et al*, 2005), overexpression of AICD could lead to rather unspecific protein-protein interactions. Therefore, the subtle alterations of gene transcription induced by C50 (and C60) overexpression have to be interpreted with caution. Interestingly, in the same luciferase reporter assays, coexpression of Fe65 with AICD led consistently to a more pronounced increase in gene transcription, in line with

previously published observations (Cao & Südhof, 2001). However, and importantly, we show here that this stimulating effect is not specific for AICD, as Fe65 when coexpressed with NICD resulted in a similar activation of the APP and KAI1 promoter (Fig 4D,E) and also of the Hes1 promoter (Fig 4C). This suggested that Fe65 has a general, not AICD-dependent, weak stimulating effect on different promoters, enhancing their activity with a factor of ~3–4. This conclusion is strikingly confirmed with the viral SV40 promoter, on which FE65 has a similar effect.

Regulated intramembrane proteolysis is a novel type of cell signalling mechanism (Annaert & De Strooper, 1999; Brown *et al*, 2000). Apart from Notch family members, most evidence regarding the role of AICD or other intracellular domains in gene transcription regulation was deduced from experiments *in vitro* and relies in most cases on artificial overexpression approaches. The results presented here suggest, for APP at least, that the intracellular fragment generated by γ -secretase cleavage is not necessarily directly involved in a nuclear signalling pathway. We suggest that in the case of APP, the main role of γ -secretase cleavage is the removal of the membrane-bound CTF after ectodomain shedding (Kopan & Ilagan, 2004).

In conclusion, and from a more practical perspective, we demonstrate here that the selective inhibition of γ -secretase and A β production does not cause significant effects on the protein levels of genes that were suggested to be regulated by AICD. Although this work cannot exclude the possibility that other yet unidentified genes are controlled by AICD-mediated signalling, we dare to conclude that deregulation of the proposed AICD target

genes is not an important issue when contemplating γ -secretase inhibitors as a therapy in Alzheimer's disease.

METHODS

Chemicals and antibodies. L-685,458 and DAPT were from Calbiochem (VWR, Leuven, Belgium). The PS1 CTF, Aph-1A and APP (C-terminal) antibodies (Hébert et al, 2004), and the PS1 amino-terminal fragment (SB129) and Nicastrin antibodies were described elsewhere (De Jonghe et al, 1999; Esselens et al, 2004). The Neprilysin/CD10 (sc-9149), KAI1/CD82 (sc-1087) and GSK-3 α/β (sc-7291) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cells and cell culture. MEF, HeLa, COS and Neuro2A cells were cultured in Dulbecco's modified Eagle's medium supplemented with serum and antibiotics. The generation of the PS1/2 dKO MEFs reconstituted with human PS1 WT, the catalytically inactive PS1 D257A, was described elsewhere (Nyabi et al, 2003). The construction of the PS1 functional mutants will be described elsewhere, and details are available on request. The Aph-1A KO MEF cells were described previously (Serneels et al, 2005).

Mice. PS1 (De Strooper et al, 1998), Aph-1A (Serneels et al, 2005), APP/APLPs (Herms et al, 2004) KO mice were described before. We used embryonic day (E)9.5 (Aph-1A), E14.5 (PS1), E15.5 (APP/APLP1/APLP2) or 12- to 16-month-old (APP/APLPs) mice in our experiments. Tissue samples were extracted in 1% Triton X-100 lysis buffer and subjected to western blot analysis.

Complementary DNA cloning and plasmids. The regulatory promoter region of APP (bp $\sim$ -2,100 to $\sim$ +100; Quitschke & Goldgaber, 1992) was amplified by PCR from purified human chromosomal DNA and cloned into the pGL3-luciferase vector (Promega, Leiden, The Netherlands). The KAI1 promoter (Baek et al, 2002) was subcloned into the pGL3-luciferase vector (Promega). The murine HES-1 promoter (bp 7-251; Takebayashi et al, 1994) was generated by PCR from the pGL2-Hes1-Luc template (Jarriault et al, 1995). The coding sequence of the luciferase gene from the pGL2 basic vector (Promega) was subcloned downstream of the HES-1 promoter giving rise to the pHes1-Luc construct. The APP-C50 (ϵ 49 position) and APP-C60 (γ 59 position) constructs were generated by PCR. These fragments start at Leu 49 and Ile 41, respectively, amino acid 1 being the methionine of the A β sequence. Both constructs are preceded by an ATG codon. The sequences of all PCR primers are available on request. The APP pCS2-C99-6Myc-tagged construct was a generous gift from A.M. Goate (St Louis, MO, USA).

Preparation of cell protein lysates and immunoblotting. Cells were lysed in cell lysis buffer (1% Triton X-100, 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA and complete protease inhibitors). Crude protein lysates (20 or 60 μ g for AICD detection) were immunoblotted and detected using the Renaissance chemiluminescence detection system (Perkin-Elmer, Massachusetts, USA).

RNA preparation and quantitative PCR. Preparation of MEFs and brain samples for quantitative PCR as well as RT-PCR procedures including primer sequences are available as supplementary information online.

Luciferase assays and transfections. HeLa cells (60–80% confluent) were transfected with 0.5 μ g of the appropriate cDNAs (pGL3-Luc (that is, no promoter), pGL3-SV40-Luc, pGL3-APP-Luc, pGL3-KAI1-Luc, pHES1-Luc, pSG5-APP C50, pSG5-APP C60, pSG5-APP full-length, pCS2-APP C99-6Myc-tagged, pSG5-NICD)

using Fugene (Roche, Mannheim, Germany). At 26–28 h after transfection, the luciferase assays were performed (Promega).

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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