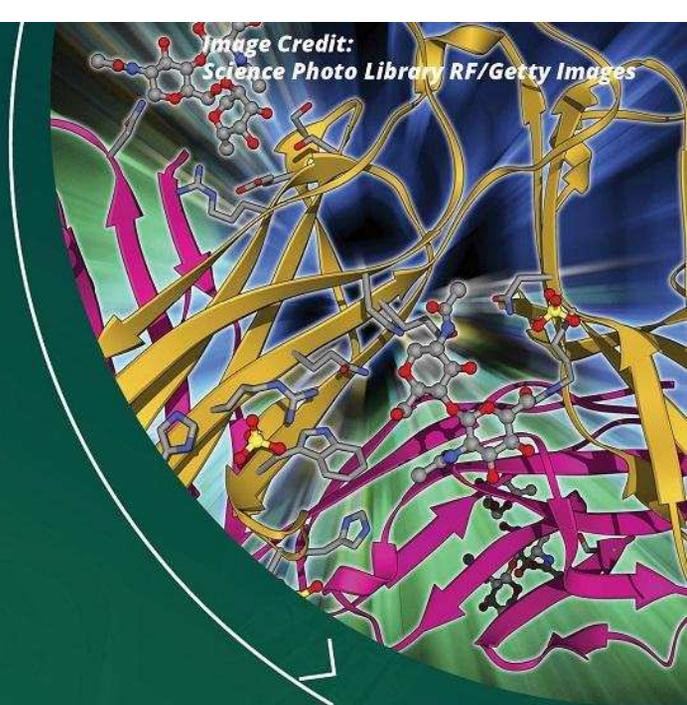
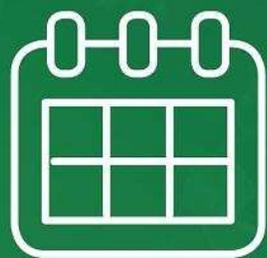


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c-Abl Modulates AICD Dependent Cellular Responses: Transcriptional Induction and Apoptosis

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APP intracellular domain (AICD) has been proposed as a transcriptional inductor that moves to the nucleus with the adaptor protein Fe65 and regulates transcription. The two proteins, APP and Fe65, can be phosphorylated by c-Abl kinase. Neprilysin has been proposed as a target gene for AICD. We found that AICD expression is decreased by treatment with STI-571, a c-Abl inhibitor, suggesting a modulation of AICD transcription by c-Abl kinase. We observed interaction between c-Abl kinase, the AICD fragment and the Fe65 adaptor protein. In addition, STI-571 reduces apoptosis in APPSw, and the apoptotic response induced by Fe65 over-expression was inhibited by with the expression of a kinase dead (KD) c-Abl and enhanced by over-expression of WT-c-Abl. However, in the APPSw cells, the ability of the KD-c-Abl to protect against Fe65 was reduced. Finally, in APPSw clone, we detected higher trans-activation of the pro-apoptotic p73 isoform, TAp73 promoter. Our results show that c-Abl modulates AICD dependent cellular responses, transcriptional induction as well as the apoptotic response, which could participate in the onset and progression of the neurodegenerative pathology, observed in Alzheimer's disease (AD).

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During the last 2 decades numerous efforts have been done trying to elucidate the function of the Amyloid Precursor Protein (APP), one of the main actors involved in Alzheimer's disease (AD) pathology (Hardy, 2006). Special attention was focused on the APP's proteolytic fragments. Among them, one of the most interesting fragments is the APP intracellular domain or AICD, which has been proposed as a transcriptional inductor (Cao and Sudhof, 2001; Kimberly et al., 2001; Leissring et al., 2003) based on the analogy between APP and Notch proteolytic processing. Indeed, it has been shown that AICD enters the nucleus when bound to the adaptor protein Fe65 (Kimberly et al., 2001; Kinoshita et al., 2002a). Several target genes are proposed for AICD, including KAI1 (Baek et al., 2002), APP itself (von Rotz et al., 2004), GSK3 β (Kim et al., 2003), and Neprilysin (Pardossi-Piquard et al., 2005) (an endopeptidase involved in A β peptide degradation) (Takaki et al., 2000; Carson and Turner, 2002). This latter gene is the only one with evidence for being as AICD target in vitro as well as in vivo (Belyaev et al., 2009).

There are many functions associated to AICD (Senechal et al., 2006; Zheng and Koo, 2006), such as cellular migration, synaptic remodeling, axonal transport, and apoptosis induction (Weidemann et al., 1999; Bertrand et al., 2001; Nakayama et al., 2008). The APP cleavage could be involved in neurodegeneration by the production of AICD (Hase et al., 2002). Then, it seems that AICD production and its cellular functions may be important for understanding APP biology. In addition, it has been recently suggested that AICD may be involved in the early pathological manifestations of AD (for a review see Müller et al., 2008).

The non-receptor tyrosine protein kinase c-Abl participates in neuronal development and morphogenesis (Moresco and Koleske, 2003). c-Abl controls the function and stabilization of p73 in response to genotoxic stress (Tsai and Yuan, 2003), and

in non-neuronal systems is a pathway that controls the choice between cell survival in an arrested state and apoptosis. We have shown that the kinase c-Abl plays a central role in neurodegeneration induced in vitro by A β fibrils (A β _f; Alvarez et al., 2004; Cancino et al., 2008). A β _f induce c-Abl levels and activity in rat hippocampal neurons. They also increase total and nuclear p73 protein levels as well as the formation of the p73/c-Abl complex (Alvarez et al., 2004).

The interaction between Fe65 and APP is modulated by APP phosphorylation and its subsequent nuclear translocation (Chang et al., 2006; Nakaya and Suzuki, 2006) could be affected

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by the same mechanism. c-Abl kinase phosphorylates Fe65 and also APP (Zambrano et al., 2001; Perkinson et al., 2004), this data suggests that phosphorylation by c-Abl could regulate AICD and Fe65 interaction.

We studied if c-Abl participates in the transcriptional activity of AICD and the potential functional significance of this regulation. We used over-expression of human APP carrying the Swedish mutation in a mouse neuroblastoma cell line as a model for AICD over-expression. The Swedish mutation (K670N/M671L), which favors the β -secretase cleavage (Citron et al., 1992), gives rise to an increase in production of the β -CTF fragment, which in turn is a substrate for γ -secretase, increasing the production of A β peptide and AICD (Passer et al., 2000; Kerr and Small, 2005). In the present study we found that c-Abl kinase modulates the cellular activity of the AICD fragment, inducing transcriptional activation of its target genes such as Nephrilysin, as well as the apoptotic response with the adaptor protein Fe65.

Materials and Methods

Cell culture and generation of APPSw cells

To generate APPSw expressing cells (APPSw), Neuro 2a cells (N2a) (Klebe and Ruddle, 1969) (ATCC, cat. number CCL-131) cells were transfected with APPSw construction inserted in pCDNA3.1 plasmid or its control plasmid (empty vector) using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions. For transient transfections, Control and APPSw clones were transfected by Lipofectamine method, with expression vectors encoding for Fe65-myc, GFP-c-Abl wild-type (WT), c-Abl WT; GFP-c-Abl (KD), c-Abl-kinase dead; those were a kind gift from Dr. Zhi-Min Yuan (Department of Genetics and Complex Diseases, Harvard School of Public Health) (Tsai and Yuan, 2003), and GFP alone as a Control, and 24 h post-transfection were analyzed by immunofluorescence.

Western blot analysis

Cells extracts were obtained in ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM NaF, and 1 mM NaVO₃) and analyzed by Western blot. The samples (50 μ g/lane) were resolved by 10% SDS-PAGE and Western blotting was carried out following standard procedures. The primary antibodies and their dilutions used were: 1/1,000; anti-Nephrilysin AB5458 (Calbiochem, San Diego, CA), 1/1,000; monoclonal anti-Tubulin (Sigma, St. Louis, MO), 1/20,000; anti-c-Abl K12 (Santa Cruz Biotechnologies, Santa Cruz, CA), 1/500; polyclonal anti Fe65 serum, 1/1,000; monoclonal anti-GAPDH, 1/10,000. The secondary HRP conjugated antibodies (1:5,000) were from Pierce, Rockford, IL, and the blot was finally revealed by ECL system.

Lysis for AICD detection

APPSw and Control cells were plated at 4.5×10^5 cell/cm² in DMEM supplemented with 5% FBS, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 100 μ g/ml geneticin. After 48 h were lysed with TBS-EDTA without detergents (Tris-HCl 50 mM pH 7.4; NaCl 150 mM; EDTA 1 mM) plus proteases inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 mM DTT, and 1 mM PMSF). The post-nuclear supernatant was centrifuged at 20,817g by 1 h at 4°C. Pellet and cytosol fractions were resolved in 16% Tris-Tricine gels (Schägger and von Jagow, 1987). For AICD fragment detection, the PVDF membrane was boiled 1 min in pre-warmed PBS 1 \times , for better epitope exposition and blocking in 10% FBS and TBS-Tween-20 0.2% solution. The anti-AICD (171610, Chemicon, Temecula, CA), 1/1,000 was incubated by 1 h at room temperature. Then the Western blotting was continued following standard procedures.

Cell fractionation

Cytoplasmic and nuclear fractions were obtained as described (Andrews and Faller, 1991). Briefly, 4.5×10^5 cells/cm² cells were washed in ice-cold PBS, scraped, and then homogenized in ice-cold hypotonic buffer A (10 mM Hepes pH 7.4; 10 mM KCl; 1.5 mM MgCl₂; 1 mM EDTA; 1 mM DTT, and 10% glycerol) containing protease inhibitors (100 μ g/ml PMSF, 2 μ g/ml aprotinin, 2 μ M leupeptin, and 1 μ g/ml pepstatin). The suspension was mechanically homogenized and centrifuged at 4,000g by 15 min at 4°C. The pellet obtained was resuspended in buffer B (20 mM Hepes pH 7.9; 0.4 mM NaCl; 1.5 mM MgCl₂; 0.4 mM EDTA; 0.5 mM DTT, and 25% glycerol) containing protease inhibitors (100 μ g/ml PMSF, 2 μ g/ml aprotinin, 2 μ M leupeptin, and 1 μ g/ml pepstatin) and incubated 30 min on ice and finally centrifuged at 13,000g by 20 min at 4°C to obtain the soluble nuclear fraction. The supernatant of the prior centrifugation was ultracentrifuged at 20,000g by 45 min at 4°C to obtain the cytosol fraction and the membrane enriched pellet. The protein concentration of the extracts was measured with the Bradford reagent (BioRad Labs, Hercules, CA).

Immunoprecipitation assay

After 24 h treatment with or without STI-571 10 mM by 24 h in serum-free media cells were scrapped in RIPA buffer plus protease inhibitors. Total homogenates were centrifuged at 17,500g by 15 min at 4°C and the supernatants (Input) were immunoprecipitated with monoclonal antibody against c-Abl overnight at 4°C, using 2 μ g of antibody for 1 mg of homogenate. The immunocomplexes were spun down by incubation with protein-G agarose for 3 h.

Nephrilysin mRNA levels

Total RNA was extracted from N2a Control and APPSw cultures with 1×10^6 cells using the Chomczynski-Fenol method. The amount of RNA obtained was determined by spectrophotometric measurements at 260 nm. RT-PCR was carried out with 4 μ g of total RNA, to which 100 ng of the random primers were added in a volume of 12 μ l. The sample was incubated for 10 min at 70°C followed by addition of 4 μ l of 5 \times transcription buffer, 2 μ l of 0.1 M DTT, 1 μ l of 10 mM dNTPs, and 200 U of Superscript II (Gibco Invitrogen Corporation). The mixture was incubated for 50 min at 42°C. The final volume of the reaction was 20 μ l. For PCR amplification, different amounts of the synthesized cDNA (diluted 1:10 in water) were analyzed to evaluate the linearity of the reaction. Then, polymerase reaction buffer was added. This solution contained 1.5 mM MgCl₂, 200 μ M of each nucleotide in PCR buffer, 10 pmol of the primers, and 0.25 U of *Platinum Taq* DNA polymerase (Gibco Invitrogen Corporation). The primers and annealing temperatures are described in Supplemental Material Table 1.

Cytotoxicity assays

The cells were seeded in 96-well plates at 6×10^3 cell/100 μ l per well and maintained in DMEM medium supplemented with geneticin and after 24 h were serum deprived for 1 h. Then, the cells were treated with H₂O₂. After incubation for 24 h, cell viability was measured by the modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTS) assay (Mossman, 1983), using DMEM medium without phenol red (Sigma).

TUNEL assay

The TUNEL staining was performed using the apoptosis detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, the coverslips were incubated in a blocking solution containing 3% H₂O₂ in methanol. Afterward, slices were incubated in 0.1% Triton X-100 in 0.1% sodium citrate overnight at 4°C. Then the sections were immersed in the TUNEL reaction mixture for 60 min at 37°C and washed twice in PBS, pH 7.4.

Luciferase reporter assay

The transient co-transfections of TAp73 or Δ N p73 promoters coupled to luciferase with β -galactosidase construction were performed using the Luciferase Assay Kit (Stratagene, Cedar Creek, TX) according to manufacturer's instructions. The luciferase activity relative units were normalized by β -galactosidase activity.

Statistical analysis

A mean \pm standard error (SE) for the number of experiments is indicated in the figure legend. To obtain the "P" value, no-paired Student's *t*-analysis of the data was performed using Sigma Plot 10.0.

Results

The APPSw cells express higher levels of AICD

We generated stable clones for the human APP Swedish variant and the Control empty vector in N2a cells. Then, we analyzed AICD fragment levels in APPSw and Control cells. Since it has been shown that AICD is more frequently found associated to membrane components (Ryan and Pimplikar, 2005) we performed a partial fractionation by lysis in TBS-EDTA buffer without detergents and Western blot analysis of high-speed pellet [membrane components rich fraction] to detect it. We detected the 6 kDa band corresponding to the AICD fragment and the bands corresponding to α - and β -CTFs [\sim 10–12 kDa bands] in the pellet fraction for both clones (Fig. 1A). However, it was not possible to detect the 6 kDa fragment (AICD) or its precursors in the cytosolic fraction. Figure 1A also shows the effect of DAPT, a γ -secretase inhibitor, over AICD levels in both clones. It clearly shows that 24 h treatment at 10 μ M diminishes AICD fragment levels and, at the same time, induces the accumulation of the precursor fragments (α - and β -CTFs), confirming it is the APP derived fragment AICD. More important, the levels of AICD in APPSw cells were higher than in Control cells. The graph in Figure 1B shows the densitometry analysis of three independent assays, indicating that AICD levels in the APPSw clone are significantly higher than the Control.

The APPSw cells show higher expression of Nprilysin

We then analyzed the AICD dependent transcription in APPSw cells. We detected by RT-PCR, a 614 bp band corresponding to the fragment amplification of the Nprilysin mRNA. The APPSw cells showed higher Nprilysin mRNA levels than the Control cells (Fig. 1C). The densitometry analysis (Fig. 1D) shows an increase of Nprilysin mRNA levels, of about 50%, in APPSw clone. Also, the protein levels of Nprilysin were increased in APPSw (Fig. 1E,F). In Control cells, the band corresponding to Nprilysin molecular weight (94 kDa), was almost undetectable whereas in the APPSw the Nprilysin signal detected is quite significant (Fig. 1E).

Treatment with the c-Abl kinase inhibitor, STI571, decreases Nprilysin levels in APPSw cells

Next we evaluated the effect of c-Abl inhibition on Nprilysin expression. APPSw cells treated with STI571 10 μ M showed lower levels of Nprilysin mRNA than APPSw Control cells (Fig. 2A). The quantification of three independent assays (Fig. 2B) shows that this decrease is over 30%. Moreover, the Nprilysin expression quantified by Western blot analysis exhibited an equivalent decrease in APPSw cells when treated with the c-Abl inhibitor STI571 (Fig. 2C). We observed (Fig. 2D) a dose-dependent effect of STI571 on Nprilysin protein levels, and also a drastic decrease on Nprilysin levels after

γ -secretase inhibitor treatment. The basal Nprilysin levels in APPSw clone are significantly higher than in Control.

c-Abl interaction with Fe65 and AICD in APPSw cells

In the APPSw cells we detected an interaction between c-Abl kinase, AICD fragment, and the Fe65 adaptor protein. In Figure 3A, we show that monoclonal antibodies against c-Abl efficiently immunoprecipitate the kinase, according to the mayor band in the immunoprecipitate, whereas we can see just a slight mark in the supernatant (SN) of the immunoprecipitation, for both clones. More important, the AICD fragment appears in the immunoprecipitates with the c-Abl and Fe65, whereas AICD is undetectable in the homogenates (Input) as well as in the supernatants. Also, the protein Fe65 appears in the immunoprecipitates with the c-Abl kinase, this protein has a high-expression level and is possibly to detect it in the supernatants. The interaction between c-Abl, AICD, and Fe65 was also observed in the presence of the STI571.

Next, we performed a sub-cellular fractionation (Fig. 3B) in order to analyze the localization of c-Abl and Fe65 in APPSw and Control cells. The Western blot analysis of the fractions shows that, for both clones, c-Abl is mainly present in the cytosolic fraction (Cyt), however, the APPSw cells show higher levels of c-Abl at the membrane (Mb) than the Control cells. Also, we can observe an increase in c-Abl nuclear levels. It is important to highlight that c-Abl is fairly undetectable in the nuclear fraction of Control clone, but its presence becomes evident in this fraction in the APPSw clone. On the other hand, the Fe65 expression pattern in the different fractions showed that Fe65 is present at high levels in all fractions (Cyt, Mb, and Nuc) in both the APPSw and Control cells, and the levels are similar between them. In summary, the APPSw clone showed an increase in nuclear c-Abl as well as in membrane bound c-Abl.

Higher levels of AICD correlate with decrease viability after pro-apoptotic injury

It has been described that AICD expression itself induces apoptosis even to a greater extent than the full length APP does (Nakayama et al., 2008). Then we evaluated the viability of APPSw cells exposed to damage. By MTS assay, APPSw cells show decrease viability compared to Control cells after 24 h treatment with H₂O₂ (Fig. 4A). This decrease is significantly higher at a concentration of 1 mM. This result suggests that AICD over-expression, after APPSw stable transfection, induces increased sensibility after this kind of injury.

We evaluated the apoptotic response after 4 h H₂O₂ treatment by TUNEL assay. Apoptosis, expressed as TUNEL positive nuclei, increases in a dose-dependent manner (Fig. 4B) in both clones. For the APPSw clone the sensibility after this pro-apoptotic injury is significantly higher than in Control clone, along all the H₂O₂ concentrations analyzed (see Supplemental Material, Fig. S2). Also, we observed that serum deprivation alone induces a larger basal apoptotic response in the APPSw clone than in the Control clone (see Supplemental Material, Fig. S1).

Role of c-Abl, Fe65, and AICD in induction of the apoptotic response

We co-transfected N2a cells with Fe65 alone and with WT-c-Abl or KD-c-Abl and evaluated the induction of apoptosis. By microscopy analysis we observed that transfection of WT-c-Abl or Fe65 alone induces apoptosis, whereas transfection with the KD-c-Abl construction confers protection to apoptosis (Supplemental Material, Fig. S3). Moreover, in double transfections with WT-c-Abl/Fe65A

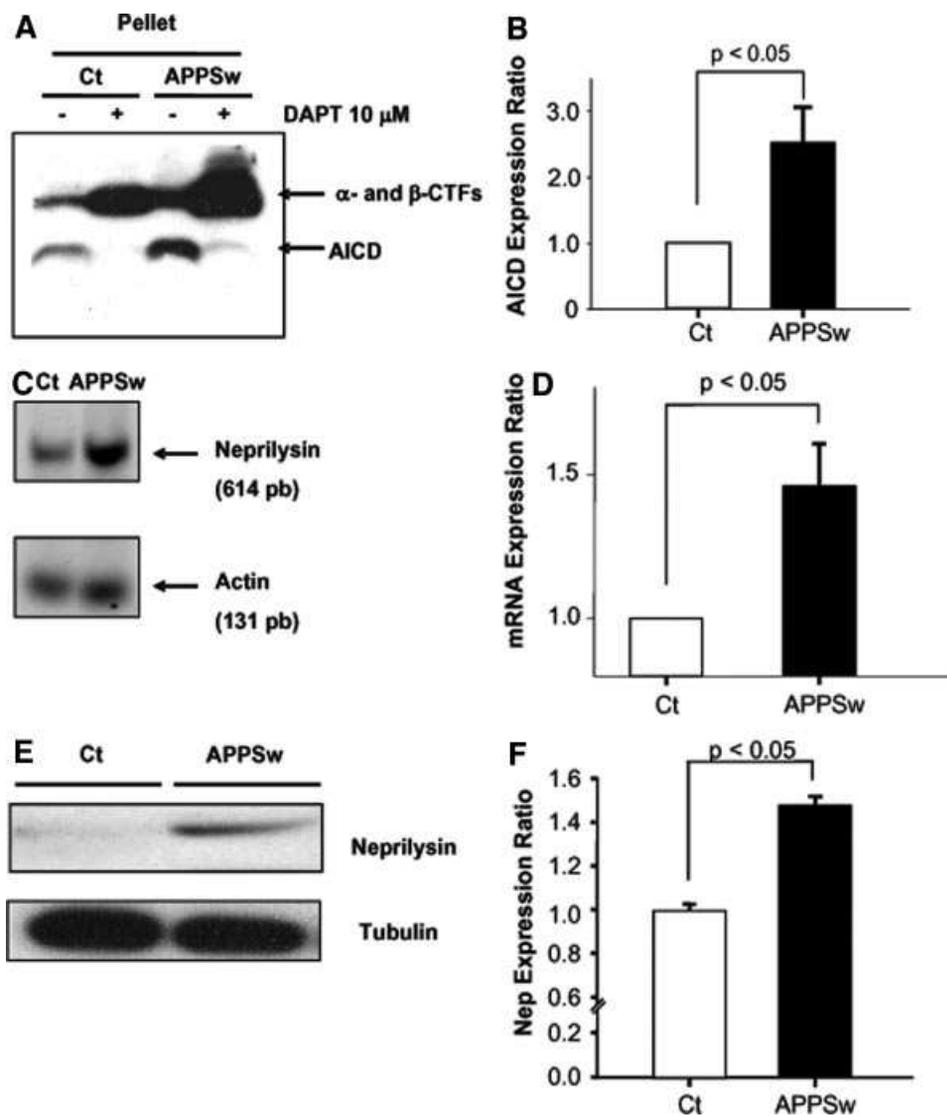


Fig. 1. AICD and Neprilysin expression in Neuro 2A clones. **A:** Western blot analysis of pellet fractions in Neuro 2A clones. Representative Western blot assay of the pellet fractions from both clones. The pattern corresponds to α - and β -CTFs, the AICD precursors, and AICD itself. The latter one is increased in APPSw cells. DAPT 10 mM (γ -secretase inhibitor) treatment dramatically decreases AICD production and leads to the accumulation of AICD precursors. **B:** Densitometry analysis of the AICD band from three independent assays, normalized by Tubulin levels. There is a significant increase on AICD levels in the APPSw clone ($P < 0.05$; unpaired t-test). **C:** RT-PCR analysis of Neprilysin mRNA. Representative RT-PCR assay for Neprilysin mRNA (614 pb) expression in Control (Ct) and APP Swedish (APPSw) clones. Actin (131 pb) levels are shown as Control. **D:** Densitometry analysis of six independent assays, normalized by actin mRNA levels. The APPSw clone (black bar) presents higher levels of Neprilysin mRNA compared to the Control clone (white bar). There is a 50% statistically significant ($P < 0.05$; unpaired t-test) difference. **E:** Western blot analysis of Neprilysin protein expression in both clones. Representative Western blot assay of Neprilysin in Control (Ct) and APP Swedish (APPSw) clones. **F:** Densitometry analysis of three independent assays, normalized by Tubulin levels. We observe an increase in Neprilysin protein level in the APPSw clone (black bar) (over 40%), which correlates with the increase in mRNA levels. This difference was statistically significant ($P < 0.05$; unpaired t-test).

(see Supplemental Material, Fig. S4A) both clones showed apoptotic induction. Double transfections with KD-c-Abl/Fe65 (see Supplemental Material, Fig. S4B), prevents apoptotic response only in Control cells and not in APPSw cells where, even in the presence of KD-c-Abl, still develop a high apoptotic response, slightly reduced compared to WT-c-Abl transfection. In Figure 4D we see the graph corresponding to HOECHST quantification for transfections, expressed as percentage of HOECHST positive nuclei. Analyzing Control (white bars) and APPSw clones (black bars), we see that transfection of WT-c-Abl, KD-c-Abl, or Fe65 alone does not change

significantly the apoptotic nuclei number in one clone versus the other. But, if we compared the apoptotic response in WT-c-Abl or Fe65 transfections with KD-c-Abl transfection, there is an increase. This was expected according to the pro-apoptotic function previously described for c-Abl kinase. Furthermore, double transfection with Fe65 and KD-c-Abl induces a drastic decrease in the percentage of HOECHST positive nuclei in the Control clone, reaching a level equivalent to KD-c-Abl transfection alone, highlighting the relevance of c-Abl in the apoptotic response. However, in the APPSw clone we observed that KD-c-Abl transfection, in the presence of Fe65,

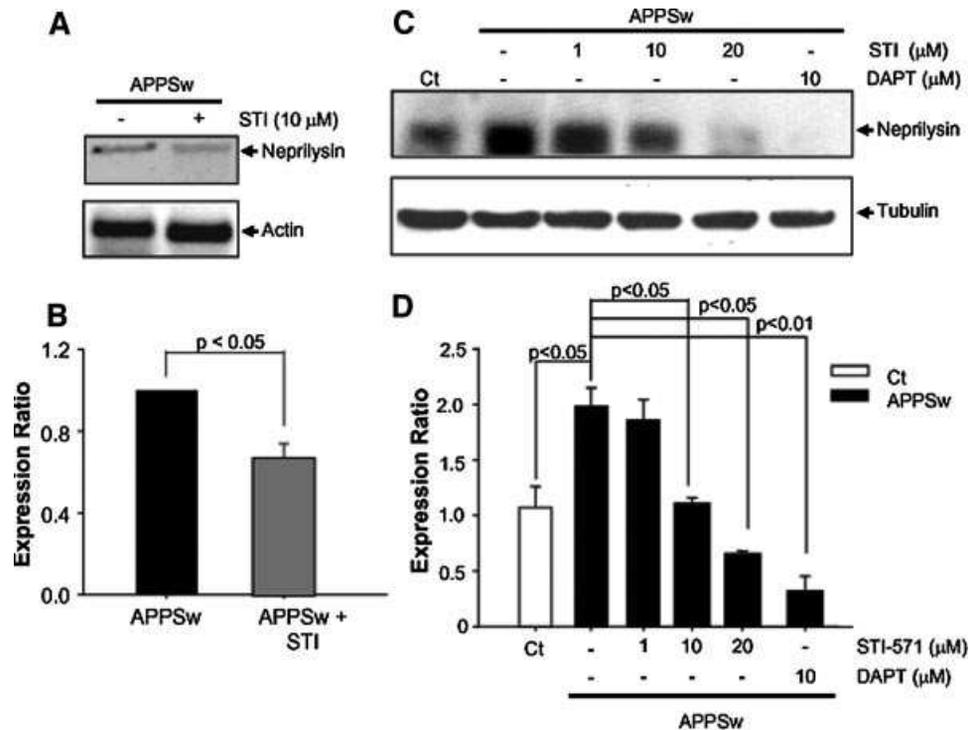


Fig. 2. Modulation of Nprilysin expression by the c-Abl kinase inhibitor, STI-571. **A:** RT-PCR analysis of Nprilysin mRNA in the APPSw clone treated with STI-571 10 μ M or untreated. We observe a decrease in the 614 bp band corresponding to Nprilysin mRNA, in the presence of the inhibitor. **B:** Densitometry analysis of three independent experiments, normalized by actin mRNA expression. We observe about 40% decrease in Nprilysin mRNA levels in the presence of the inhibitor (gray bar), and this difference is statistically significant ($P < 0.05$; unpaired t-test). **C:** Western blot analysis of Nprilysin protein expression in the Control clone without treatment and in the APPSw clone treated with several STI-571 concentrations (1, 10, and 20 μ M) and also with DAPT 10 μ M, for 24 h. **D:** Densitometry analysis corresponding to the Western blot assay, normalized by Tubulin expression. As was previously shown, the APPSw clone presents higher levels of the Nprilysin band compared to the Control clone ($P < 0.05$; unpaired t-test), in the absence of treatment. In the presence of the c-Abl kinase inhibitor STI-571, we observe a dose-dependent decrease in Nprilysin protein levels ($P < 0.05$; unpaired t-test; for STI-571 10 and 20 μ M) and this is even more pronounced in the presence of the γ -secretase complex inhibitor, DAPT, 10 μ M ($P < 0.01$; unpaired t-test).

does not restore basal apoptotic levels. Instead, it maintains an apoptotic response similar to that observed when Fe65 is transfected alone.

APPSw cells have higher expression levels of the pro-apoptotic protein TAp73

Finally, we perform a luciferase reporter assay by transient co-transfection of TAp73 or Δ Np73 promoters coupled to luciferase with β -galactosidase constructions in both clones. Figure 5A shows the relative luciferase activity for TAp73 and Δ Np73 promoters in Control (white bars) and APPSw (black bars) clones. The activity of both promoters is increased in APPSw clone compared to the Control. However, the ratio of increase of APPSw/Control (Fig. 5B) clearly shows that TAp73 promoter induction is higher than for Δ Np73 in the APPSw clone. This result is in agreement with prior Western blot results for p73 and its isoforms.

Discussion

In this work we show that AICD cellular activity is modulated by c-Abl kinase, inducing transcription of its target genes such as Nprilysin, and inducing apoptotic response along with the Fe65 adaptor protein.

Taking into account that c-Abl kinase phosphorylates APP as well as Fe65, that phosphorylation can modulate the interaction between both proteins (Zambrano et al., 2001; Perkinson et al.,

2004), and that c-Abl has nuclear localization and functions, we proposed that this kinase could modulate AICD dependent gene transcription in the presence of Fe65.

In order to test our hypothesis we used stable transfectants of the Neuro2A cell line that over-express the human APP Swedish mutant (APPSw), which allowed us to obtain high levels of the AICD fragment. In these cells we observed an increase in Nprilysin expression, a proposed target gene for AICD. This increase, measured at the mRNA and protein levels, was negatively modulated by the selective c-Abl kinase inhibitor, STI571, suggesting that c-Abl activity could be required in the mechanism of Nprilysin expression induction by AICD. It is important to remark that these results correspond to the expression analysis of an endogenous gene instead of the trans-activation of a reporter construct, which has been the most widely used strategy so far to study AICD mediated transcription and its interacting proteins.

Following the analysis of AICD, Fe65 and c-Abl interaction by sub-cellular fractionation assays, we detected an increase of c-Abl in the nuclear fraction of APPSw cells. From the results of these experiments we propose that after their interaction at the plasma membrane, c-Abl could lead the nuclear translocation of the complex formed with AICD and Fe65. Finally, we demonstrated the interaction between c-Abl, Fe65, and the AICD fragment by co-immunoprecipitation assays. We also showed that treatment with the c-Abl kinase inhibitor, STI571, does not alter formation and/or stability of this putative complex. However, we cannot discard the possibility that there

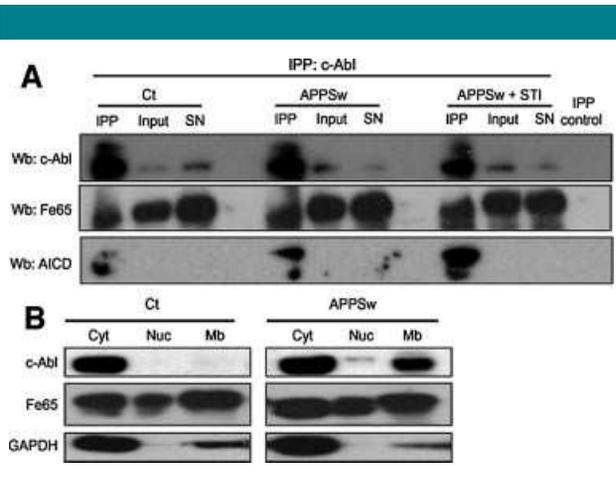


Fig. 3. The AICD fragment and the Fe65 adaptor protein interact and co-localize with c-Abl tyrosine kinase in Neuro 2A clones. **A:** Co-immunoprecipitation assay in the Control clone and in the APPSw clone with or without STI-571 treatment. Total homogenates were immunoprecipitated with monoclonal antibody against c-Abl kinase. An aliquot of the homogenates (Input), the immunocomplexes (Ipp) and an aliquot of the supernatant were resolved in polyacrilamide gels and blotted against c-Abl, Fe65, and AICD with polyclonal antibodies. The immunoprecipitates (Ipp) are positive for c-Abl, Fe65, and AICD in all cases, indicating that both Fe65 and AICD are interacting with c-Abl. This interaction was not inhibited by STI-571 treatment. **B:** Sub-cellular fractionation assay. We obtained cytosolic (Cyt), nuclear (Nuc) and membrane (Mb) enriched fractions of both clones and blotted them against c-Abl, Fe65, and also GAPDH as a Control. We observed the presence of Fe65 in the three fractions in both clones and, in the case of c-Abl we can detect it only in the cytosolic fraction of the Control clone; whereas in the APPSw clone there is a marked increase in the membrane and also in the nuclear fraction.

could be some other components involved in modulating the complex or being part of it.

It has been proposed that APP and Fe65 phosphorylation by c-Abl could modulate the interaction between these two proteins (Zambrano et al., 2001; Perkinson et al., 2004). Nevertheless, there is some controversy about this modulation. Some authors propose that APP phosphorylation facilitates or prevents Fe65 nuclear translocation and the subsequent transcriptional induction mediated by AICD. Other groups propose that membrane APP phosphorylation or AICD fragment phosphorylation is important in membranous intracellular compartments, probably in the recycling pathway, prior to its nuclear translocation (Kimberly et al., 2001; Chang et al., 2006; Nakaya and Suzuki, 2006; Nakaya et al., 2008). Considering our results obtained from APPSw cells treated with STI571, we propose that c-Abl kinase activity would be dispensable for a complex formation between AICD, Fe65, and c-Abl itself. However, c-Abl could have some unknown function in the transcriptional induction mechanism, after the complex is translocated to the nucleus and binds to the promoter region of target genes such as Nephrilysin. It is also important to mention that the data opposing AICD as an inducer of gene expression come from models not necessarily related to AD pathophysiology where AICD as well as other APP's fragments are actually relevant.

Another line of investigation in the search for APP functions and, particularly, its AICD fragment, points toward the role of AICD in triggering the apoptotic response (Kinoshita et al., 2002b; Ozaki et al., 2006; Nakayama et al., 2008). This is especially relevant in the neurodegenerative process associated to AD pathophysiology. Therefore, we evaluated cellular viability of APPSw cells, by MTS reduction assay, after pro-apoptotic injury such as hydrogen peroxide. We observed that APPSw cells

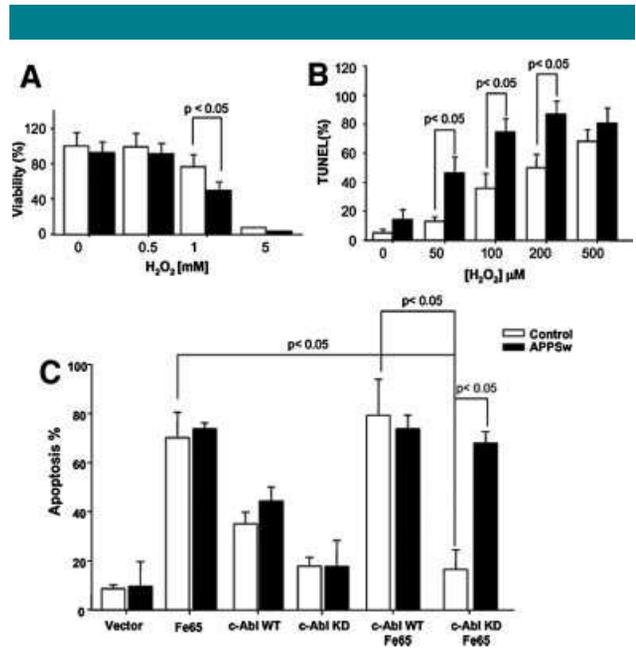


Fig. 4. Apoptotic response to stress stimuli. **A:** Viability assay against hydrogen peroxide treatment. Viability was measured as % of MTS reduction compared to the Control condition. The graph shows the average of four independent assays. The APPSw clone (black bars) shows a higher susceptibility to H₂O₂ treatment, compared to the Control clone (white bars). The difference is statistically significant at 1 mM concentration ($P < 0.05$; unpaired t-test). **B:** TUNEL assay quantification. Percentage of TUNEL positive cells in a H₂O₂ dose-curve, for Control (white bars) and APPSw clones (black bars). The graph shows the average of three independent assays. The APPSw cells are more labile to H₂O₂ injury than Control cells and this difference is statistically significant ($P < 0.05$ for 50, 100, and 200 μ M, unpaired t-test). **C:** Transient transfections of Fe65, WT-c-Abl, KD-c-Abl, each one alone or in pairs. The graph shows the apoptotic response as percentage of HOECHST positive nuclei for each transfection. c-Abl KD transfection prevents the apoptotic response induced by Fe65 in the Control clone ($P < 0.05$; no-paired t-test). In addition, the apoptotic response is diminished compared with c-Abl WT transfection ($P < 0.05$; unpaired t-test), and in the APPSw clone the percentage of apoptosis is higher than in the Control clone despite the presence of c-Abl KD ($P < 0.05$; unpaired t-test).

sensitivity to these stimuli is significantly higher than in Control cells. This was correlated with an increase in the apoptotic response after hydrogen peroxide treatment, as we established using the TUNEL assay. We observed a basal increase of

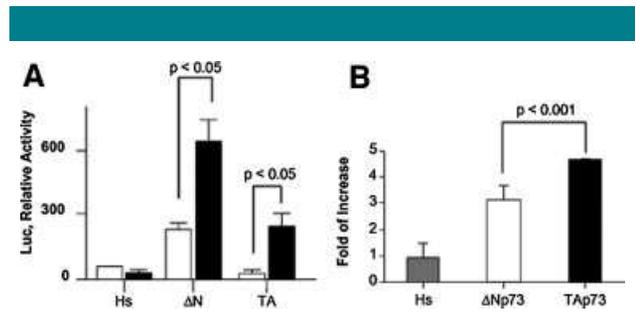


Fig. 5. Expression levels of TAp73 and Δ N p73 isoforms. **A:** Luciferase reporter assay for TA and Δ N promoters in the Control and APPSw clones. Both isoforms are increased in the APPSw clone compared to Control ($P < 0.05$ for Δ Np73 and TAp73, unpaired t-test). **B:** Ratio of increase between clones for both isoforms. TA increment was higher than Δ N ($P < 0.001$, unpaired t-test).

apoptotic nuclei in APPSw cells. This increase was even more pronounced after hydrogen peroxide treatment, and was also dose-dependent. Altogether, these results suggest that in conditions of high AICD levels the threshold for apoptotic response is decreased. Such effect could be explained by gene transcription modulation, increment of pro-apoptotic gene expression, or decrease expression of anti-apoptotic genes. Since we transfected full-length APP, the apoptotic response could also be mediated, at least in part, by A β production. However, there is evidence in the literature supporting an active role in apoptosis induction for the AICD fragment alone, which is even stronger than triggered by the full-length APP (Nakayama et al., 2008). To further confirm our hypothesis, the effect of AICD must be determined by over-expressing this fragment alone.

The pro-apoptotic role of c-Abl has already been described (Wang et al., 1984; Ito et al., 2001). In addition, a pro-apoptotic function for Fe65 was recently suggested. Therefore, we evaluated the apoptotic response after transient co-transfection with Fe65 and c-Abl, the later one in its wild-type (WT) or kinase death (KD) variants, in Control and APPSw cells. Interestingly, we found that transfection of WT-c-Abl or Fe65 induces a stronger apoptotic response than transfection with KD-c-Abl. However, co-transfection of Fe65 and KD-c-Abl prevents apoptotic death in Control cells but not in APPSw cells. These results lead us to propose that in conditions of elevated AICD levels, such as in AD pathology, c-Abl could be involved in a pro-apoptotic pathway through its interaction with the AICD fragment, modulating the expression of pro-apoptotic genes.

We have shown that the kinase c-Abl plays a central role in neurodegeneration induced by A β fibrils (A β _f) in vitro (Alvarez et al., 2004; Cancino et al., 2008). The A β _f induce an increase of c-Abl levels and activity in rat hippocampal neurons. There is also an increment of the p73/c-Abl complex, as well as of total and nuclear p73 protein levels (Alvarez et al., 2004; Cancino et al., 2008).

c-Abl is present in different sub-cellular locations; in the nucleus c-Abl regulates cell cycle and apoptosis signals and in the cytoplasm is involved in cell adhesion and cytoskeleton dynamics. p73 phosphorylation is the main c-Abl nuclear function that underlies its ability to regulate apoptosis or cell cycle arrests. Furthermore, it has been recently proposed that p53, a pro-apoptotic transcription factor, could be an AICD target gene (Costa et al., 2006; Checler et al., 2007). Another member of p53 family is p73, which can be mainly found in two isoforms. The TA or full-length that includes the trans-activation domain and is pro-apoptotic; or the Δ N isoform, where the trans-activation domain has been deleted and is therefore anti-apoptotic. Considering that p73 is usually activated by the same stimuli as p53, we evaluated p73 expression in APPSw cells. We found an increase of the pro-apoptotic isoform, TAp73, and a decrease of Δ Np73, the anti-apoptotic isoform (data not shown). This result was confirmed by luciferase reporter assay for each of the isoform's promoters, showing a significantly higher induction of the TAp73 promoter.

In view of these later results we propose that p73 could be a new target gene for AICD and that c-Abl could modulate p73 through a pathway parallel to the previously described p73 stabilization by phosphorylation (Tsai and Yuan, 2003). This c-Abl alternative pathway, could be particularly relevant in the apoptotic response during the neurodegenerative process in AD pathology.

There is still controversy about the role of AICD as a gene transcription inducer (Hébert et al., 2006; Waldron et al., 2008), since AICD potency as transcriptional activator is questioned when compared to the effect of Notch on its target genes. Because of that, it is necessary to determine in which

cellular context AICD activity is important. We propose c-Abl as an AICD transcriptional activity modulator, linking it with the apoptotic response induction.

Finally, from our results we can conclude that there is a correlation between c-Abl kinase activity and Nephrilysin expression in conditions of increased AICD levels, but it is still necessary to demonstrate the binding of AICD to the promoter region of Nephrilysin or another putative target gene. In this context, it is remarkably important to look for consensus binding sites for c-Abl in the promoter regions of the proposed target genes, such as Nephrilysin, p53 or p73 and its isoforms. The identification of these consensus binding sequences will allow identifying new AICD target genes, probably related to AD pathology.

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