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Choline kinase alpha expression during RA-induced neuronal differentiation: Role of C/EBPβ

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ABSTRACT

Neuronal differentiation is a complex process characterized by a halt in proliferation and extension of neurites from the cell body. This process is accompanied by changes in gene expression that mediate the redirection leading to neurite formation and function. Acceleration of membrane phospholipids synthesis is associated with neurite elongation, and phosphatidylcholine (PtdCho) is the major membrane phospholipid in mammalian cells. The transcription of two genes in particular encoding key enzymes in the CDP–choline pathway for PtdCho biosynthesis are stimulated; the *Chka* gene for choline kinase (CK) alpha isoform and the *Pcyt1a* gene for the CTP: phosphocholine cytidylyltransferase (CCT) alpha isoform. We report that the stimulation of CKα expression during retinoic acid (RA) induced differentiation depends on a promoter region that contains two CCAAT/Enhancer-binding Protein-β (C/EBPβ) sites. We demonstrate that during neuronal differentiation of Neuro-2a cells, RA induces *Chka* expression by a mechanism that involves $ERK_{1/2}$ activation which triggers C/EBPβ expression. Elevated levels of C/EBPβ bind to the *Chka* proximal promoter (Box1) inducing CKα expression. In addition we identified a downstream sequence named Box2 which together with Box1 is required for the promoter to reach the full induction. This is the first elucidation of the mechanism by which the expression of *Chka* is coordinately regulated during neuronal differentiation.

1. Introduction

Phosphatidylcholine (PtdCho) biosynthesis is an important event during neuronal differentiation due to the increased PtdCho mass for neurite membrane genesis [1–3]. PtdCho synthesis takes place in all nucleated mammalian cells *via* the Kennedy pathway [4]. Although the rate of PtdCho biosynthesis by this pathway is governed by the rate of conversion of phosphocholine to CDP–choline in a reaction catalyzed by the CTP:phosphocholine cytidylyltransferase (CCT), ample evidence suggests a regulatory role for choline kinase (CK) as well [5]. CK catalyzes the phosphorylation of choline by ATP yielding phosphocholine, and has some reactivity with ethanolamine [6]. At least three protein isoforms of CK, termed CKα1, CKα2 and CKβ, have been identified. The first two isoforms are derived from the same gene, *Chka*, by alternative splicing, whereas the third isoform is the product of a distinct gene named *Chkb* [7].

The supply of PtdCho can be regulated by the biochemical activity of key enzymes [8,9], gene expression of biosynthetic or degradative enzymes [9–13] or by intracellular trafficking [14,15]. It was demonstrated that during RA-induced differentiation of Neuro-2a cells, the increase of PtdCho biosynthesis is promoted by an ordered and coordinated activation of CKα and CCTα activity and expression. *Chka* mRNA accumulates as soon after 3 h of RA treatment, however, its transcription initiates 24 h after RA. It was demonstrated that $ERK_{1/2}$ activation is essential for RA-stimulated neuronal differentiation and for RA-stimulated CCTα and CKα transcription [1]. However, the mechanism that promotes the transcription of both genes coordinately with the program of neuronal differentiation is still unknown.

The ERK signal is disseminated by multiple cascade's substrates including transcription factors, protein kinases or phosphatases, cytoskeletal elements and a variety of other signaling-related molecules. Most of these substrates are localized in the nucleus and participate in the regulation of transcription upon stimulation [16,17].

The CCAAT/Enhancer-binding Proteins (C/EBPs) are one family of transcription factors known to couple growth factor signal transduction to cellular differentiation in numerous developing non-neural tissues [18]. The C/EBP family is composed of basic leucine zipper DNA binding proteins (C/EBPs α, β, γ, δ, ε and ζ) that recognize a common DNA sequence, and is expressed in virtually all tissues, including the developing and adult brain [19]. C/EBPs regulate differentiation and proliferation; in the first case by direct trans-activation and/or repression of gene expression and in the second by multiple mechanisms [20]. In the case of neuronal differentiation it was shown that C/EBPβ is widely expressed in the adult mouse brain [19] and in the brain of

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neonatal rats [21]. It was also demonstrated that C/EBPβ activity increases after NGF stimulation in PC12 cells [19], and that C/EBPβ plays a role in the regulation of neuronal differentiation and apoptosis in Neuro-2a cells [21].

In this study we show how the *Chka* gene encoding CKα, an enzyme involved in PtdCho biosynthesis, is induced coordinately during neuronal differentiation to finally provide PtdCho for membrane biosynthesis. We demonstrate that C/EBPβ is a link between RA-mediated neuritogenesis and the transcription of *Chka* gene. We prove that during neuronal differentiation RA promotes $ERK_{1/2}$ activation which leads to an increase in C/EBPβ expression. Elevated levels of C/EBPβ bind to the promoter region named Box1 and induce *Chka* expression. Furthermore, the increase in *Chka* expression generated by C/EBPβ overexpression promotes neuronal differentiation reinforcing the role of PtdCho metabolism previously described [1]. However, as mutations in the C/EBPβ binding sites do not completely abolish CKα induction after RA, we propose that the downstream inverted repeat region named Box2 is required for the full induction of CKα during neuritogenesis. The Box2 is a novel sequence common to the CKα promoter of different species, which binds a not yet identified transcriptional factor(s). This is the first report demonstrating how the expression of an enzyme involved in phospholipid biosynthesis is coordinately regulated during neuronal differentiation.

2. Materials and methods

2.1. Tissue culture

The mouse neuroblastoma cell line Neuro-2a (ATCC CCL-131) was cultured in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS) and supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml). Cells were maintained in a 5% CO_2 humidified incubator at 37 °C. To induce neuritogenesis, the medium was changed to Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% FBS and 10 μM of all trans-retinoic acid (RA) [1,22].

2.2. Plasmids

The following CKα promoter-luciferase reporter plasmids were gently provided by Dr. Aoyama [23]: Luc.CK(−1625/+57), Luc.CK(−953/+57), Luc.CK(−901/+57), Luc.CK(−866/+57) and Luc.CK(−495/+57). CKα promoter-luciferase reporter plasmid Luc.CK(delBox1) was performed with the Site Directed Mutagenesis-XL kit (Stratagene) according to the manufacturer's instructions. To generate Luc.CK(delBox2), initially the construct Luc.CK(−901/+57) was digested with KpnI enzyme and cohesive ends were blunted with Klenow Fragment (Promega). This linear vector was ligated with a DNA probe, which contains the (−953/−918) region from the *Chka* promoter. Oligos used are shown in Fig. 2C.

CKα-promoter vector constructs were generated by the digestion of the pGL3 promoter vector (Promega) with SmaI and ligated with different inserts. For pV (−953/−858) and pV (−901/−858), the indicated fragments were amplified by PCR using RV primer 3 (5′-CTAGCAAAAT AGGCTGTCCC-3′) and ChIPCK reverse primer (5′-ACATTAGTCATGGTCA CGCG-3′) and the Luc.CK(−953/+57) or Luc.CK(−901/+57) plasmids as template. To construct pV(Box1) and pV(Box2) we used synthetic DNA probes from Box1 (5′-GAGACCAAAGAATGAGGCAATGTGTGGGCA TACAGC-3′ and 5′-GCTGTATGCCCACACATTGCCTCATTCTTTGGTCTC-3′) or Box2 (5′-AGCATAGGGGCCTTGGCCCCCTTCC-3′ and 5′-GGAAGGGGGC CAAGGCCCCTATGCT-3′).

2.3. Luciferase assay

Neuro-2a cells were transfected with 5′ deletion of CKα promoter-luciferase reporter constructs (0.5 μg) using a cationic liposome method (Lipofectamine 2000, Invitrogen) [24]. All dishes received 0.2 μg of pCMV-β-galactosidase (Promega) as a control for transfection efficiency. Luciferase and β-galactosidase assays were performed using the Promega assay systems, as recommended by the manufacturer and luminometric measurements were made using Synergy 2 Multi-Mode Microplate Reader (BioTek). Luciferase activity was normalized to β-galactosidase activity and expressed as a ratio of luciferase/β-galactosidase.

Vectors designed to overexpressed C/EBP isoforms were kindly provided by Dr. Wei [25]. In the overexpression experiments, 0.5 μg of the plasmid designed to overexpress C/EBP was cotransfected with 0.5 μg of Luc.CK plasmids and 0.2 μg of pCMV-β-galactosidase.

2.4. Chromatin immunoprecipitation (ChIP) assay

Neuro-2a cells were grown in MEM containing 10% FBS or in DMEM 2% FBS plus 10 μM RA for 24 h and incubated with 1% formaldehyde for 10 min at 37 °C. Cells were collected, lysed, and sonicated three times for 10 s each at 30% with an ultrasonic processor GEX-600 (Sonics & Materials) and treated for ChIP as recommended by the manufacturer (Upstate). Anti-C/EBPβ antibody was purchased from Santa Cruz Biotechnology. PCR was performed using 5 μl of template DNA, 1.5 mM $MgCl_2$, ChIPCK forward primer (5′-AGTTTTTGGCTTCCAGCA GA-3′) and ChIPCK reverse primer (5′-ACATTAGTCATGGTCACGCG-3′) (20 pmol) for 40 cycles (94 °C for 1 min, 60 °C for 1 min and 1 min at 72 °C), and 10 min at 72 °C.

2.5. Morphometric analysis

To determine the percentage of differentiated cells, 0.5 μg of pcDNA-C/EBPβ or the empty vector were cotransfected with 0.2 μg of phr-eGFP (Promega). In the indicated cases, cells were treated with 75 μM Hemicholinium-3 (Sigma) for 24 h. Microscopy was carried out at room temperature using a confocal microscope (Nikon model Eclipse TE-2000-E2 C1 plus) equipped with Plan Apochromat 20.0X/0.75/1.00 dry objective; NikonC1 standard detector, and Nikon EZ-C1 3.60 software. Images were adjusted for contrast and gamma using Nikon EZ-C1 3.70 Free Viewer software. Green cells bearing at least one neurite equal or longer than soma diameter were considered to be differentiated.

2.6. Western blot analysis

For Western blot analysis, cell lines were plated at a density of 3×10^5/100-mm dish for 48 h, after which the medium was changed to DMEM supplemented with 2% FBS and RA (10 μM). The $MEK_{1/2}$ U0126 inhibitor was preincubated during 30 min at a final concentration of 10 μM. Control cells were maintained in MEM supplemented with 10% FBS. After 24 h of treatment with RA, cells were collected. For nuclear extract preparation, we used the protocol that was previously described [14]. For total extract preparation, cell pellet was resuspended in lysis buffer (20 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, cocktail protease inhibitor (Sigma), 50 μM TPCK, 20 mM NaF and 1 mM PMSF) and sonicated five times for 5 s at 5% (ultrasonic processor GEX-600, Sonics & Materials). Protein concentrations were determined using bovine serum albumin as standard protein and “Sedmak and Grossberg” reagent [26]. 20 μg of cell lysate were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). After blocking with 5% albumin in 0.1% Tween 20 TBS (10 mM Tris–HCl, pH 7.5, 137.8 mM NaCl), blots were incubated overnight with anti-CKα primary antibody (1:500, provided by Dr. Jackowski), anti-C/EBPβ primary antibody (1:500, Santa Cruz Biotechnology) or anti-phospho-C/EBPβ primary antibody (1:1000, Cell Signaling). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody and developed using a chemiluminescence detection kit (Thermo Scientific). For phospho-C/EBPβ immunoblotting, prior cell collection, samples were washed and incubated with

phosphate buffer saline supplemented with 20 mM NaF. Loading protein control was demonstrated by measuring the levels of β-actin using anti-β-actin (1:600, Santa Cruz Biotechnology).

2.7. Electromobility-shift assay (EMSA)

Synthetic oligonucleotides were purchased from Invitrogen and are illustrated in Fig. 2B. Briefly, complementary oligonucleotides (10 μM of each) were heated at 90 ºC for 5 min and then slowly cooled at room temperature. 2.5 pmol of double stranded DNA probes were 5′-labeled using [γ^{32}-P]-ATP and T4 polynucleotide kinase (Fermentas). For each binding reaction (20 μl): 4 μl of 5× binding buffer (50 mM HEPES pH 7.9, 250 mM KCl, 12.5 mM $MgCl_2$, 5 mM DTT, 5 mg/ml BSA, 50% glycerol), 1 μg of poly-dI-dC, 5 μg of nuclear extract and labeled probe (50,000 cpm) were incubated for 30 min at room temperature. Binding reactions were terminated by the addition of gel loading buffer (30% v/v glycerol, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol). The complexes were separated on a non-denaturing 6% (w/v) polyacrylamide gel and visualized by autoradiography of the dried gel.

2.8. Statistical analysis

The data were analyzed using GraphPad Prism software. Significant effects were determined using Student's *t* test. A statistically significant difference was considered to be present at $p < 0.05$.

3. Results

3.1. Identification of the promoter elements that regulate Chka gene expression during neuronal differentiation

During neuronal differentiation of Neuro-2a cells, *Chka* expression is induced at the transcriptional level after 24 h of RA treatment by a mechanism dependent on $ERK_{1/2}$ activation ([1] and Fig. 1A). In order to understand the mechanism that regulates the increased expression of *Chka* gene, we evaluated the activity of 5′ promoter deletions using a promoter reporter assay (Fig. 1B) [23]. Each construct plus pCMV-β-galactosidase as a transfection control were transiently transfected in Neuro-2a cells. Luciferase and β-galactosidase activities were measured in cells that were grown under proliferating (−RA) and differentiating (+RA) conditions. We observed that Luc.CK(−1625/+57) and Luc.CK(−953/+57) are induced after 24 h of RA treatment, however, further deletions of the promoter decreased the RA-dependent induction (Fig. 1B). This result clearly suggested that the region between (−953/−901) contains elements that regulate *Chka* transcription during RA-induced differentiation.

3.2. C/EBP binds to the Chka promoter

The consensus *cis*-elements in the (−953/−901) promoter region were identified using TFSearch (htt://molsun1.cbrc.aist.go.jp/research/db/TFSearch.html). The analysis of this region (−953/−901) using 90% homology revealed consensus sequences for the transcription factor C/EBPα/β (named C/EBP1), and with 80% homology we identified HNF-3b, AML-1a, SRY, C/EBPα/β (named C/EBP2) and Egr-1 binding sites (Fig. 2A). To investigate if these transcription factor(s) bind to this region during RA-induced differentiation we performed *in vitro* EMSA assay. For a better analysis, as a probe we selected the region of 22 bp (−953/−931) containing the C/EBP1 binding sites identified using high score (90%) (Fig. 2B). We used the wild type probe and nuclear extracts obtained from cells that were grown in the presence and in the absence of RA during 24 h. We also generated a probe harboring point mutations in order to abolish the identified binding site named ΔC/EBP1 (Fig. 2B). We identified at least three complexes named "*a*, *b* and *c*" when the wild type probe was incubated with nuclear extract obtained from growing cells. The intensity of the complex with less mobility named "*a*" increases when the assay was performed with nuclear extracts obtained from differentiated cells (RA) (Fig. 3A). When the assay was performed in the same condition but using the ΔC/EBP1 probe, we were unable to detect the identified complexes, instead we observed different complexes with faster mobility (Fig. 3A). With these results we speculate that at least the complexes "*a*" and "*b*" bind to the C/EBP1 binding site and, as expected, contain a transcription factor belonging to the C/EBP family. Moreover, as the complex "*a*" differentially increases when we assayed extracts obtained from cells treated with RA, we

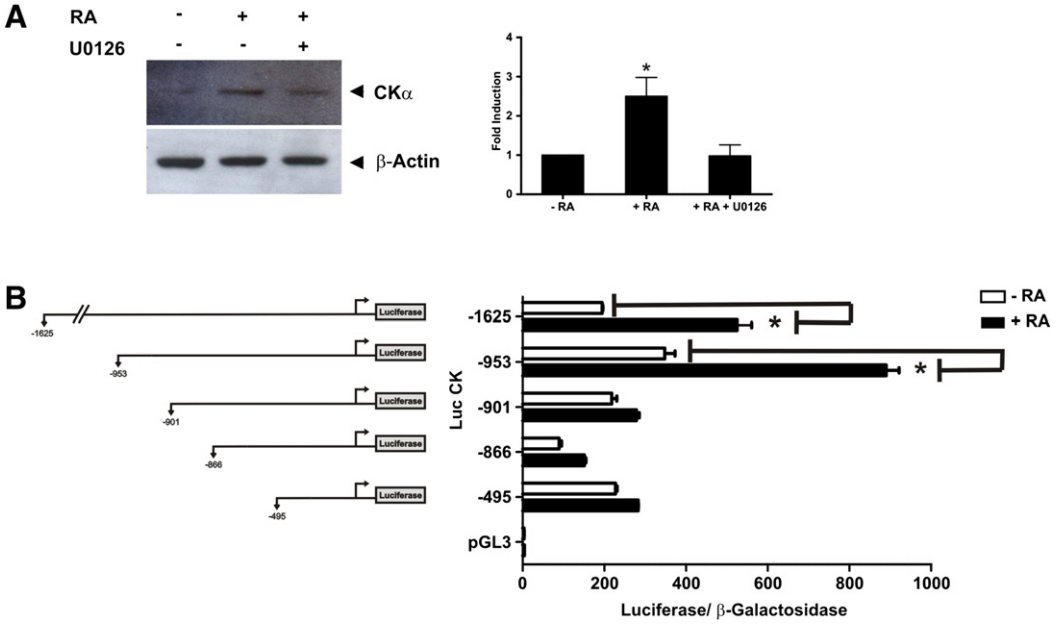

Fig. 1. CKα expression during RA-induced differentiation. A—Western blot analysis of CKα expression using total cellular extracts obtained from cells grown in the presence or in the absence of RA or treated with MEK inhibitor U0126. Densitometric analysis of the CKα-specific band relative to β-actin. These results are representative of two independent experiments, each using freshly isolated extracts (*$p < 0.05$). B—CKα-promoter reporter plasmids (0.5 μg) Luc.CK(−1625/+57), Luc.CK(−953/+57), Luc.CK(−901/+57), Luc.CK(−866/+57), Luc.CK(−495/+57) or pGL3-basic empty vector (Promega), and pCMV-β-galactosidase plasmid (0.2 μg) were transfected in Neuro-2a cells. Luciferase reporter activity is given relative to β-galactosidase activity and was measured 24 h after RA treatment. Graph represents the ratio of luciferase/β-galactosidase ± S.D. obtained from four independent experiments (*$p < 0.0002$).

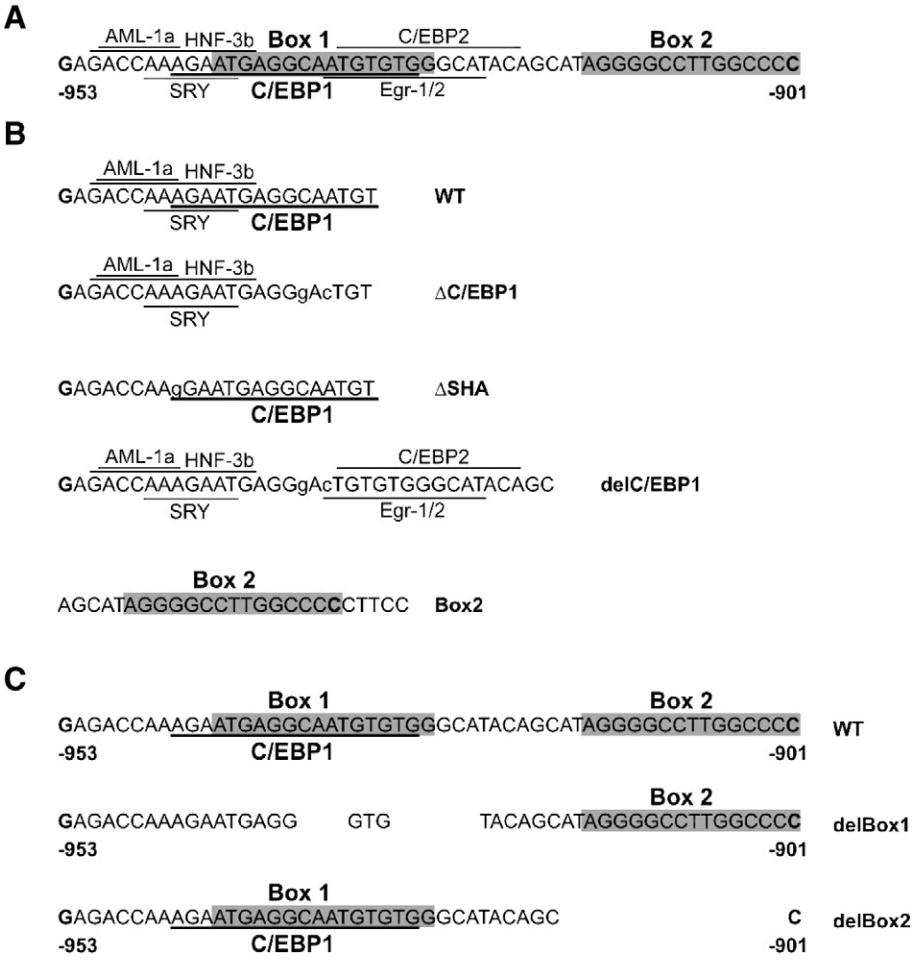

Fig. 2. *Chka* proximal promoter region analysis. A—Consensus elements for C/EBPα/β, Egr-1, AML-1, HNF-3b and SRY nuclear factors identified by TFSearch database. Numbers represent the positions of nucleotides in relationship to the transcription initiation site. B—Probes utilized in EMSA assays. C—Probes utilized to generate promoter plasmids deletions.

propose that a member of the C/EBP family could be involved in regulating *Chka* transcription during RA-dependent neuritogenesis.

Previously we have shown that RA signals differentiation, CCTα and CKα expression and PtdCho biosynthesis in Neuro-2a through the activation of the $ERK_{1/2}$ pathway [1]. To evaluate if the complex that binds to the *Chka* promoter under RA treatment depends on the $ERK_{1/2}$ activation, we obtained nuclear extract from cells treated with RA and the MEK inhibitor U0126 [27] and analyzed by EMSA. As

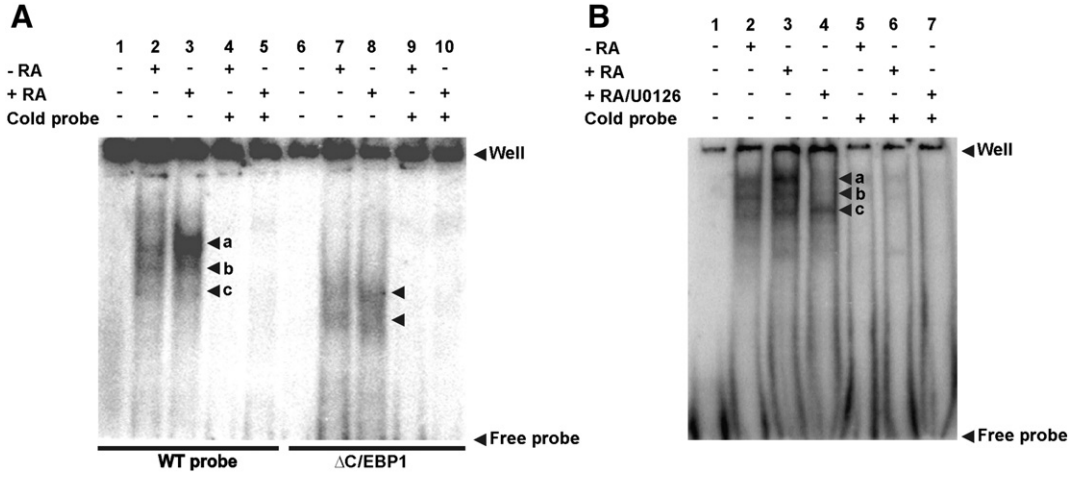

Fig. 3. Association of C/EBP with CKα proximal promoter depends on $ERK_{1/2}$ activation. A—23 bp [γ^{32}P]-ATP labeled probe corresponding to the CKα proximal promoter (WT probe) (lanes 1–5) was incubated with Neuro-2a nuclear extracts obtained from cells that were grown in the presence or in the absence of RA (+RA/−RA) (lanes 2 and 3) or without NE (lane 1). Arrows show the migration of the complexes. Lanes 4 and 5 show competition with excess of unlabelled WT probe (cold probe). Lanes 6–10: the probe harboring mutations in the C/EBP1 consensus binding site (ΔC/EBP1 probe). Incubation with NE promotes the formation of complexes with faster mobility than in lanes 2 and 3 (lanes 7 and 8, arrows). Incubation with unlabelled probe (lanes 9 and 10) disassembles the complexes. B—23 bp [γ^{32}P]-ATP labeled probe corresponding to the CKα proximal promoter (WT) was incubated with Neuro-2a nuclear extracts obtained from cells with or without RA treatment (−RA/+RA) or treated with RA and U0126 (lanes 2–7) or without NE (lane 1). Incubation with unlabelled probe (lanes 5–7) disassembles the complexes. These results are representative of 3 independent experiments, each using freshly isolated nuclear extracts.

Fig. 3B shows, we could not detect the complexes named "*a* and *b*" when we assayed nuclear extract treated with U0126. This result implies a functional role of $ERK_{1/2}$ activation in protein interaction with the *Chka* promoter and in its transcriptional activation.

The role of AML-1a, HNF-3b and SRY binding sites was evaluated using a probe with mutations in these sites, named ΔSHA (Fig. 2B). Despite that the point mutation G/A introduced at position (−945) is located at the beginning of the C/EBP1 binding site (Fig. 2A), the informatic analysis ensured that its binding capacity was not affected as the core of this site is conserved. Interestingly, when the assay was performed with ΔSHA as a probe, we detected the same complexes "*a*, *b* and *c*" observed with the wild type probe (see Supplementary Fig. 1).

As for the EMSA assays, the AML-1a, HNF-3b and SRY binding sites appear not to be involved in RA induction of the *Chka* gene, and to further evaluate the role of the identified C/EBP2 and Egr-1 binding sites, we performed a similar analysis using a probe (−953/−918), with mutations that alter the C/EBP1 binding site (delC/EBP1) (Fig. 2B), which developed the presence of one complex with identical mobility and intensity using nuclear extract obtained in both conditions (+/−RA) (data no shown). This result suggests that C/EBP2 and Egr-1 binding sites could be involved in regulating the basal expression of *Chka* gene.

3.3. C/EBPβ binds Chka promoter in vivo

To address whether C/EBPβ is recruited to the promoter region of the endogenous *Chka* gene we performed chromatin immunoprecipitation (ChIP) assays. Target sequences were detected by PCR using the set of primers described in Materials and methods. Indeed, our analysis showed that a specific anti-C/EBPβ antibody, but not the non-immune IgG control, successfully co-immunoprecipitated C/EBPβ and significant quantities of *Chka* promoter in both conditions (+/−RA) (Fig. 4), indicating that C/EBPβ is recruited by the endogenous *Chka* promoter under proliferation and differentiation conditions.

3.4. C/EBPβ expression increased after RA treatment

$ERK_{1/2}$ regulates gene expression by a direct mechanism which involved the phosphorylation of target transcription factors or indirectly by the activation of other kinases [16,28]. It has been documented that C/EBPβ transactivating activity is stimulated by $ERK_{1/2}$-directed phosphorylation of Thr-235 [29,30]. Considering that the complex named "*a*" which specifically intensified assaying nuclear extracts obtained after RA treatment depends on the integrity of the C/EBPα/β consensus element (Fig. 3A); together with the fact that its binding depends on the $ERK_{1/2}$ activation (Fig. 3B), we analyzed if RA throughout ERK regulates either C/EBPβ expression or its activation by phosphorylation [30,31]. To this aim we analyzed by western blot nuclear extracts obtained from cells growing in proliferating or differentiating conditions and treated with U0126. As Fig. 5 shows, the amount of C/EBPβ increases after RA treatment; and this effect was abolished by U0126 suggesting that the mechanism of induction depends on $ERK_{1/2}$. We could not detect changes in mobility which could suggest the absence of changes

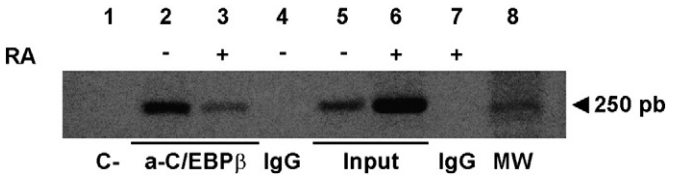

Fig. 4. *In vivo* association between C/EBPβ and the CKα promoter. ChIP assay was performed with anti-C/EBPβ antibody. An unrelated antibody, anti-IgG, was used as a control. The data are representative of two separate experiments. Picture of the electrophoresis gel by which the PCR products obtained after ChiP assay were resolved. Lane 1: PCR negative control (C−), lanes 2 and 3: anti-C/EBPβ antibody (a-C/EBPβ), lanes 4 and 7: anti-IgG (IgG), lanes 5 and 6: input (I). +/−RA indicate cells grown in the presence and absence of RA. MW: molecular weight marker and 250 bp is the size of the PCR product.

in phosphorylation, in fact western blot with specific anti-phospho-C/EBPβ antibody confirmed that the rate of C/EBPβ phosphorylation does not change with RA treatment (Fig. 5B).

3.5. C/EBPβ overexpression alters CKα transcription

To evaluate the role of the increased amount of C/EBPβ as inductor of *Chka* transcription during RA-induced differentiation, we co-transfected different concentrations of plasmids that drives the overexpression of each C/EBP family members (α, β and δ) (gently provided by Dr. Wei) together with the minimal promoter reporter construct that respond to RA, Luc.CK(−953/+57). The results indicated that only the β isoform of C/EBP is able to induce *Chka* expression (Supplementary Fig. 2). To further evaluate the role of C/EBPβ, we cotransfected Luc.CK(−953/+57) with a plasmid designed to overexpress C/EBPβ or with the empty plasmid as a control, together with pCMV-β-galactosidase as transfection control. Luciferase and β-galactosidase activities were measured in cells treated with or without RA (24 h). As Fig. 6A shows, C/EBPβ overexpression is able to promote CKα transcription not only in cells growing in a differentiation condition (+RA) but also under a proliferating condition (−RA). The increased expression was observed even in cells treated with U0126 suggesting that C/EBPβ overexpression is able to bypass the blockage of $ERK_{1/2}$ signaling. Furthermore, we were able to detect an increase in CKα protein levels by western blot in cells transfected with C/EBPβ overexpressing plasmid (Fig. 6B). As C/EBPβ overexpression induces CKα expression and previous results from our laboratory indicate that CKα overexpression increases PtdCho biosynthesis and promotes neuronal differentiation in Neuro-2a [1], we evaluate the rate of neuronal differentiation. We co-transfected the plasmid designed to overexpress C/EBPβ together with phr-eGFP in order to visualize the transfected cells and measure the number of neurite bearing cells. To demonstrate that C/EBPβ induces CKα which in turn induces neuronal differentiation, we analyzed the effect of C/EBPβ overexpression in cells treated with Hemicholinium-3, as a specific CKα inhibitor [32], or in cells transfected with siRNA specifically designed to knockdown CKα. As Fig. 6C shows, cells that overexpressed C/EBPβ and have more levels of CKα (Fig. 6C), differentiate even in the absence of RA and to a similar extent as those treated with RA, however, the effect was abolished when CKα was inactive due to the pharmacological treatment. The knockdown of CKα *per se* totally abolished neuritogenesis, and the inability to control the amount of knockdown made it difficult to analyze the role of C/EBPβ (data not shown).

3.6. Mutations in C/EBP binding sites do not completely abolish RA dependent induction of the Chka promoter

We demonstrated that under RA treatment, the C/EBP1 binding site specifically recruits a protein complex (Fig. 3). To further investigate the functional role of the C/EBP binding sites in the transcriptional induction of *Chka* promoter, we generated a promoter reporter construct named delBox1 harboring deletions to alter this DNA binding site (Fig. 2C). We performed luciferase reporter assay analysis using Luc.CK(−953/+57) and Luc.CK(delBox1). As Fig. 7A shows, this construct lost 28% of RA induction but is still able to be induced suggesting that an extra actor in this scenario might play an important regulatory role. As the transcription factor search did not reveal any other consensus binding sites (Fig. 2A), we searched for DNA sequence by aligning *Chka* promoters from mouse, rat and human (see Supplementary Fig. 3A). We identified two highly conserved sequences; Box1 includes the two C/EBPβ binding sites previously analyzed and Box2 is an inverted repeat sequence GGGGCCTTGGCCCC (Fig. 2A and Supplementary Fig. 3A). We demonstrated by EMSA assay that protein(s) binds to Box2 in both conditions assayed (−/+RA) (Supplementary Fig. 3B), however, as the transcription factor search did not reveal any consensus binding sites, the nature of the

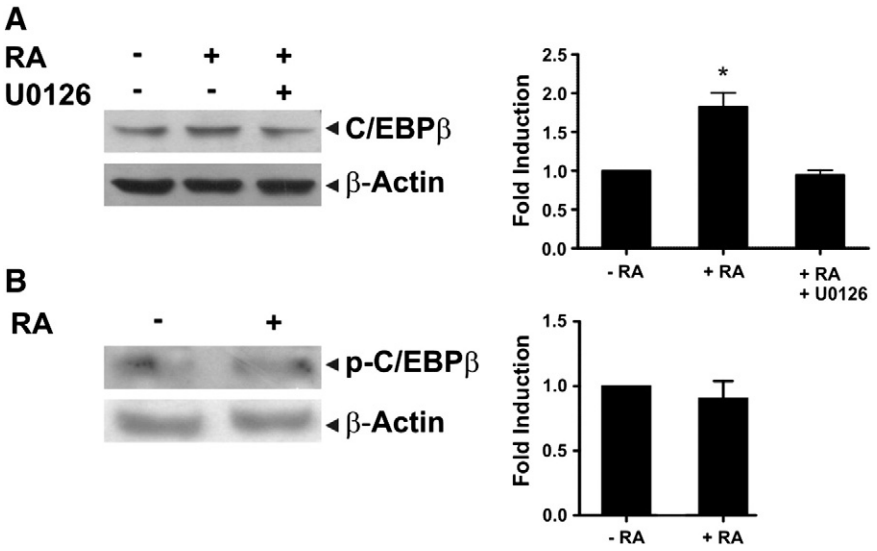

Fig. 5. C/EBPβ expression during neuritogenesis. Western blot analysis of C/EBPβ (A) or p-C/EBPβ (B) expression using nuclear extracts obtained from cells grown in the indicated conditions. Densitometric analysis of the C/EBPs-specific band relative to β-actin. These results are representative of two independent experiments, each using freshly isolated extracts (*$p < 0.05$).

protein is still unknown. The role of Box2 in the *Chka* transcriptional regulation was evaluated by generating a promoter reporter construct with the Box2 deleted named delBox2 (Fig. 2C). The analysis revealed that deletion in Box2 decreases RA-dependent induction by 72% (Fig. 7A). This result might suggest that the identified Box1 and Box2 are together required for the full induction of the *Chka* transcription after RA. In fact, the promoter construct Luc.CK(−901/+57) that lacks both boxes do not show RA induction (Fig. 1 and 7A).

To confirm the role of each Box in RA-dependent induction of the *Ckha* promoter, we generated promoter vector constructs named pV(−953/−858), pV(Box1), pV(Box2) and pV(−901/−858) in which the indicated DNA promoter sequences were cloned upstream of the SV40 promoter (see Material and methods). Each plasmid together with pCMV-β-galactosidase was transfected in Neuro-2a cells and after 24 h of RA treatment both activities were measured. As Fig. 7B shows, only pV(−953/−858) which contains Box1 and

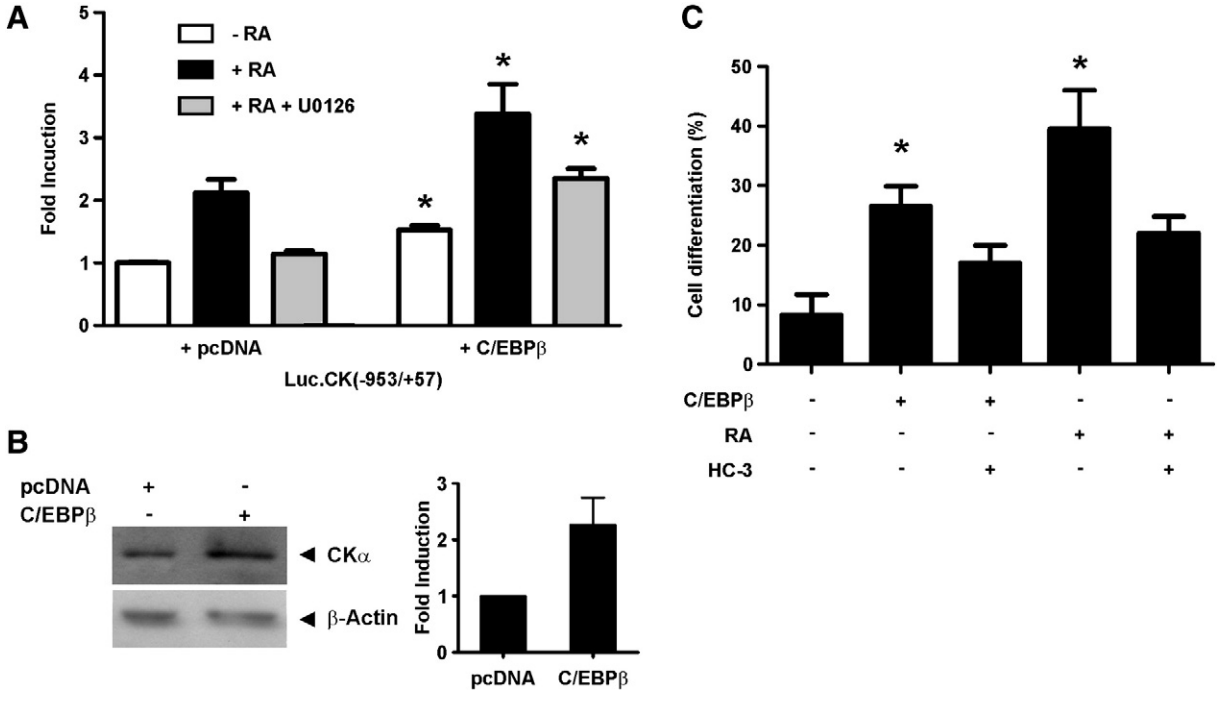

Fig. 6. C/EBPβ overexpression promotes CKα expression and neuronal differentiation. A—Neuro-2a cells were transfected with the indicated Luc.CK(−953/+57) proximal promoter reporter construct together with a plasmid designed to overexpress C/EBPβ (C/EBPβ) or the empty plasmid (pcDNA) as a control, together with pCMV-β-galactosidase as a transfection control. 24 h after transfection, cells were grown in proliferating media (MEM 10% FBS) or in differentiating media (DMEM 2% FBS) supplemented with 10 μM RA with or without U0126 (10 μM). Luciferase and β-galactosidase activities were measured 24 h after differentiation was initiated. Graph represents the ratio between luciferase/β-galactosidase obtained in each condition *versus* pcDNA transfected cells grown in non-differentiating conditions from three independent experiments. The values are means ± S.E. (*$p < 0.05$). B—Western blot analysis of CKα expression using total cellular extracts obtained from cells grown in the indicated conditions. Densitometric analysis of the CKα-specific bands relative to β-actin. These results are representative of two independent experiments, each using freshly isolated extracts. C—Cells were transfected with C/EBPβ overexpressing plasmid (+) or the empty plasmid (−). Graph represents the percentage (%) of neurite-bearing cells grown in the presence and in the absence of RA (RA) (*$p < 0.01$), and in cells treated with CKα inhibitor (HC-3).

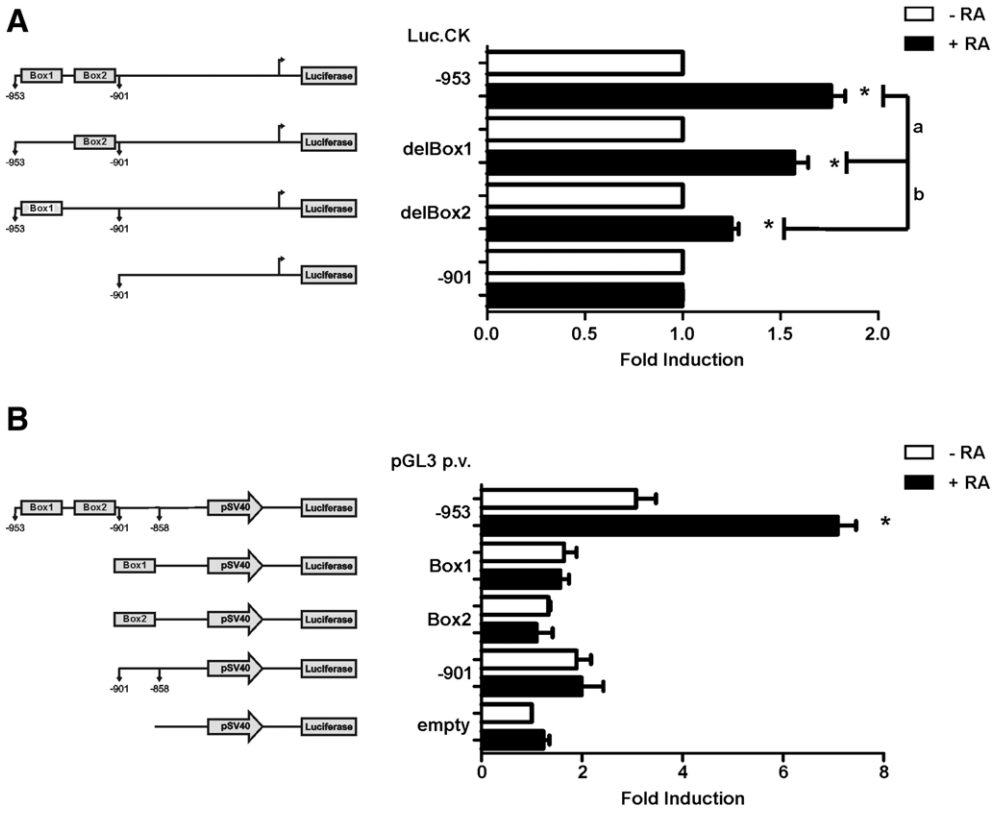

Fig. 7. Box1 and Box2 are required for the full induction of CKα promoter. A—Neuro-2a cells were transfected with the indicated Luc.CK proximal promoter reporter constructs together with pCMV-β-galactosidase as a transfection control. 24 h after transfection, cells were grown in proliferating media (MEM 10% FBS) or in differentiating media (DMEM 2% FBS) supplemented with 10 μM RA. Luciferase and β-galactosidase activities were measured 24 h after differentiation was initiated. Graph represents the ratio between luciferase/β-galactosidase obtained in differentiating *versus* non-differentiating conditions (fold induction) from three independent experiments. The values are means ± S.E. (a, $p < 0.05$; *, $p < 0.005$; b, $p < 0.0001$). B—Neuro-2a cells were transfected with the indicated pGL3 promoter vector (pV) construct together with pCMV-β-galactosidase as a transfection control. 24 h after transfection, cells were grown in proliferating media (MEM 10% FBS) or in differentiating media (DMEM 2% FBS) supplemented with 10 μM RA. Graph represents the ratio between luciferase/β-galactosidase obtained in each condition *versus* the value of the empty pV in the non-differentiating conditions (fold induction) from two independent experiments. The values are means ± S.E. (*$p < 0.05$).

Box2 responds to RA and confers the RA induction to the SV40 promoter. Interestingly, Box1 or Box2 individually does not alter SV40 expression and the effect is likely the same shown by pV(−901/−858) that lacks both boxes, or the empty vector. This result demonstrates that the identified Box1 and Box2 are together required for the full induction of the *Chka* transcription after RA.

4. Discussion

How is the supply of new membrane tailored to the demands of neuronal growth? And what are the molecular mechanisms by which the supply of new membrane correlated with the demands for neuronal growth? These are two important questions that still have poor answers due in part to their complexity; membrane lipids are present in different amounts and fall into different classes which are synthesized in different compartments [4,9]. As PtdCho is the most abundant phospholipid in all mammalian membranes [4] we are focusing on its biosynthesis to give an insight of how its synthesis is coordinately regulated to supply the demands for neuronal differentiation. We previously demonstrated that during RA-induced differentiation of neuroblastoma cells, the demand for membrane biosynthesis that accompanied neuritogenesis is covered by an increase in PtdCho biosynthesis [1]. PtdCho biosynthesis increases by two mechanisms named early and late stimulation. The first one involves the enzymatic activation of CDP–choline:1,2-diacylglycerol cholinephosphotransferase (CPT) and CCT, and the late mechanism, which depends on $ERK_{1/2}$ activation, involves the transcriptional activation of CKα and CCTα expression [1]. In this work we define for the first time the mechanism that regulates CKα expression coordinately during neuronal differentiation. We demonstrated that after RA, CKα expression is regulated by a promoter region located (−953/−901) bp upstream of the start transcriptional point (Fig. 1) [7]. This region contains two highly conserved sequences named Box1 and Box2 (Fig. 2). The following evidences allow us to propose that a member of the C/EBP family is involved in *Chka* transcriptional regulation during neuronal differentiation: we demonstrated that C/EBPβ binds *in vivo* to the *Chka* promoter (Fig. 4), and by *in vitro* assays we detected a complex on the C/EBP1 binding site whose intensity increases when the cells were treated with RA (Fig. 3).

We previously demonstrated that RA induces neuronal differentiation and coordinately activates CCTα and CKα expression through the MAPK pathway [1,33]. As the recruitment of C/EBPβ on the C/EBP1 binding site depends on RA and also on $ERK_{1/2}$ activation (Fig. 3B), we asked whether or not RA and $ERK_{1/2}$ directly modulate C/EBPβ. We demonstrated that $ERK_{1/2}$ regulates *Chka* gene expression by increasing the levels of C/EBPβ and not by a more canonical phosphorylation way (Fig. 5). To evaluate the role of the increased levels of C/EBPβ, we overexpressed the transcription factor in cells and demonstrated that the only overexpression of C/EBPβ increases the transcriptional activity of *Chka* promoter and CKα protein expression similarly to RA (Fig. 6 and Supplementary Fig. 2). Moreover, and reinforcing the role of PtdCho metabolism in regulating cell fate [1], these cells with increased levels of CKα due to C/EBPβ overexpression undergo neuronal differentiation (Fig. 6C). This result supports the occurrence of sequential events in the following order RA–$ERK_{1/2}$–C/EBPβ–CKα-neuronal differentiation, and may explain

a previous report demonstrating that C/EBPβ over-expression in Neuro-2a cells promotes neuronal differentiation [21]. Even though the role of C/EBPβ as an inductor of CKα expression is supported by all the previously discussed experiments, we evaluated a detailed mechanism of transcriptional regulation by analyzing the role of the C/EBPβ binding sites. Surprisingly, the promoter reporter construct harboring the Box1 deleted still responds to RA promoting the transcription (Fig. 7). As the HNF-3b, AML-1a, and SRY binding sites present in the promoter do not bind any factor under the assay conditions (Supplementary Fig. 1) and no other transcription binding sites were identified (Fig. 2A), we search for a conservative sequence among the *Chka* promoter of different species. We identified an inverted repeat sequence (Box2) which, as demonstrated using reporter constructs, is essential for the promoter to reach the full transcription (Fig. 7A), but unable to promote the transcription by itself (Fig. 7B). This result clearly suggests that the identified Box1 and Box2 are together required for the full induction of CKα transcription under RA treatment. As we stated below, the transcription factor search did not reveal any consensus binding sites in Box2 (Fig. 2) which suggests either that Box2 is a novel binding site for a well known transcriptional factor or a consensus binding site for a yet unidentified transcription factor; in any case, this will be the subject of future studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2014.01.007.

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