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A Functional N-terminal Domain in C/EBPβ-LAP* is Required for Interacting with SWI/SNF and to Repress Ric-8B Gene Transcription in Osteoblasts

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Abstract

The chromatin remodeling complex SWI/SNF and the transcription factor C/EBPβ play critical roles in osteoblastic cells as they jointly control transcription of a number of bone-related target genes. The largest C/EBPβ isoform, LAP*, possesses a short additional N-terminal domain that has been proposed to mediate the interaction of this factor with SWI/SNF in myeloid cells. Here we examine the requirement of a functional N-terminus in C/EBPβ-LAP* for binding SWI/SNF and for recruiting this complex to the Ric-8B gene to mediate transcriptional repression. We find that both C/EBPβ-LAP* and SWI/SNF simultaneously bind to the Ric-8B promoter in differentiating osteoblasts that repress Ric-8B expression. This decreased expression of Ric-8B is not accompanied by significant changes in histone acetylation at the Ric-8B gene promoter sequence. A single aminoacid change at the C/EBPβ-LAP* N-terminus (R3L) that inhibits C/EBPβ-LAP*-SWI/SNF interaction, also prevents SWI/SNF recruitment to the Ric-8B promoter as well as C/EBPβ-LAP*-dependent repression of the Ric-8B gene. Inducible expression of the C/EBPβ-LAP*R3L protein in stably transfected osteoblastic cells demonstrates that this mutant protein binds to C/EBPβ-LAP*-target promoters and competes with the endogenous C/EBPβ factor. Together our results indicate that a functional N-terminus in C/EBPβ-LAP* is required for interacting with SWI/SNF and for Ric-8B gene repression in osteoblasts.

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Keywords

C/EBPβ transcription factor; SWI/SNF-mediated control of expression; osteoblast gene transcription

INTRODUCTION

CCAAT/Enhancer Binding Protein beta (C/EBPβ) is a critical regulator of tissue-specific gene expression in several cell lineages (Smink and Leutz, 2012). This transcription factor is coded by a single intron-less gene and can be found in mammalian cells as three protein isoforms that are generated by alternative translation initiation: C/EBPβ-1 or LAP* (Liver Activating Protein*), C/EBPβ-2 or LAP, and C/EBPβ-3 or LIP (Liver Inhibitory Protein) (Nerlov, 2008). All three proteins contain a basic leucine zipper (b-zip) domain at their C-terminus that functions as DNA-binding domain. On the other hand, only the two larger C/EBPβ isoforms retain either a complete (C/EBPβ-LAP*) or partial (C/EBPβ-LAP) N-terminal sequence, which includes the transactivation domain. The C/EBPβ-LIP protein that completely lacks this N-terminal end can function as an inhibitor of both C/EBPβ-LAP*- and C/EBPβ-LAP-mediated gene regulation (Nerlov, 2008). Several reports indicate a critical role for C/EBPβ-mediated transcriptional control during osteogenesis. Thus, C/EBPβ knock-out mice exhibit delayed skeleton formation and decreased expression of osteoblast-lineage gene markers (Smink et al., 2009; Tominaga et al., 2008). Also, osteoblast-targeted over-expression of C/EBPβ-LIP in mice results in reduced bone formation (Harrison et al., 2005). Our group has shown that C/EBPβ binds to target genes in osteoblastic cells where it can mediate up- or down-regulation of transcription (Grandy et al., 2011; Gutierrez et al., 2002; Henriquez et al., 2011; Villagra et al., 2006). The regulatory properties exhibited by C/EBPβ factors are based on its ability to bind and recruit chromatin remodeling and co-activator complexes to target genes. Thus, it has been demonstrated that C/EBPβ can interact with SWI/SNF (Kowenz-Leutz and Leutz, 1999) and ISWI (Steinberg et al., 2012) ATP-dependent chromatin remodeling complexes and that it can recruit SWI/SNF to myeloid-, muscle-, and bone-target genes (Grandy et al., 2011; Kowenz-Leutz and Leutz, 1999; Ohkawa et al., 2007; Villagra et al., 2006). C/EBPβ can also interact with components of the Mediator complex (Li et al., 2008; Mo et al., 2004), as well as with transcriptional co-regulators containing histone-modifying enzymatic activities like CBP/p300 (Mink et al., 1997), G9a (Pless et al., 2008), and PRMT4/CARM1 (Kowenz-Leutz et al., 2010).

Recent reports indicate that the ability of C/EBPβ to interact with SWI/SNF is determined by the N-terminal domain present only in C/EBPβ-LAP* (Kowenz-Leutz et al., 2010). Moreover, using chicken C/EBPβ-LAP* it was shown that C/EBPβ-LAP*-mediated transcriptional activation of target genes in myeloid cells can be inhibited by dimethylation of the R3 residue catalyzed by PRMT4/CARM1 (Kowenz-Leutz et al., 2010). In this same study it was found that this post-translational modification can prevent the interaction between C/EBPβ-LAP* and SWI/SNF. Because recent reports indicate that C/EBPβ-LAP* transcription factor can also function as a repressor by either recruiting HDAC4 (Wang et al., 2008) or SWI/SNF (Grandy et al., 2011) activities to target gene promoters, there is a

necessity for establishing whether the N-terminus domain of C/EBPβ-LAP* contributes to C/EBPβ-LAP*-SWI/SNF-mediated gene repression in osteoblasts.

The Ric-8B gene codes for a guanine exchange factor (GEF) that modulates G protein-mediated signaling, exhibiting a relevant role during regulation of cell division (Hinrichs et al., 2012), particularly at vertebrate craniofacial neural crest precursor cells (Fuentealba et al., 2013). We have recently shown that Ric-8B gene expression is down-regulated during osteoblast differentiation in a C/EBPβ-LAP*- and SWI/SNF-dependent manner (Grandy et al., 2011). This transcriptional inhibition involves nucleosome enrichment and reduced accessibility at the Ric-8B promoter regulatory sequences. Here, we examine the requirement of a functional N-terminal domain in C/EBPβ-LAP* for both interacting with SWI/SNF and recruiting this complex to the Ric-8B gene promoter to mediate transcriptional repression.

MATERIALS AND METHODS

Expression constructs

Constructs pcDNA3.0-C/EBPβ-LAP* and pcDNA3.0-C/EBPβ-LAP were kindly donated by Dr. Jose L. Gutierrez (University of Concepcion, Concepcion, Chile). The construct 3xFlag-C/EBPβ-LAP* was obtained after digesting with ApaI the pcDNA3.0-C/EBPβ-LAP* vector, releasing the coding sequence for rat C/EBPβ-LAP* protein. The isolated fragment was then ligated into a pCMV-3xFlag-CMV10 plasmid (Sigma Aldrich, MI, USA) previously mutated to incorporate an ApaI site, in frame, downstream the sequence coding for the 3xflag epitope. To obtain the C/EBPβ-LAP*R3L variant, the pcDNA3.0-C/EBPβ-LAP* vector was mutated using the Epoch Site Directed Mutagenesis Service (Epoch Life Sciences, TX, USA) and then subcloned into the pCMV-3xFlag-CMV10 plasmid as described above. Plasmids encoding for Flag-tagged histone deacetylases HDAC3, HDAC4 and HDAC11 were kindly donated by Dr. Alejandro Villagra (H. Lee Moffitt Cancer Center and University of South Florida, Tampa, Florida, USA). Constructs containing mouse Ric-8B gene promoter segments of 2,100 bp or 558 bp fused to firefly luciferase gene (p2.1Ric8B-luc or p0.56Ric8B-Luc) were obtained previously (Grandy et al., 2011). Construct containing a 1,020 bp segment of the Osteocalcin gene promoter fused to firefly luciferase gene (pOC-Luc) was previously reported (Sierra et al., 2003). pGL3-basic vector was purchased from Promega (Promega, WI, USA). Expression of the *Renilla* luciferase gene-containing plasmid was under the control of the simian virus 40 (SV40) constitutive promoter (pRL-SV40). To generate the ROS-LAP* and ROS-LAP*R3L stable cell lines, 3xFlag-C/EBPβ-LAP* vectors were digested with MseI and the sequence coding for the fusion proteins was subcloned into a pTRE-HA vector (Clontech, CA, USA), previously digested with BamHI and HindIII to eliminate the HA epitope. Plasmids were treated before ligation with DNA polymerase I, Large (Klenow) fragment to generate blunt ends (New England Biolabs, MA, USA). Sequences for each plasmid were confirmed by automatic sequencing (Macrogen, Korea).

Cell culture and generation of stable cell lines

Rat osteosarcoma-derived ROS 17/2.8 cells (Majeska et al., 1980) were maintained in F-12 medium supplemented with 5% fetal bovine serum (FBS), 1.176 g/l NaHCO3, 0.118 g/l $CaCl_2x2H_2O$, and 6.670 g/l HEPES. Mouse preosteoblastic MC3T3 cells (donated by Dr. Rafael Burgos, Universidad Austral de Chile, Valdivia, Chile) were maintained in α-MEM without ascorbic acid (AA) and supplemented with 10% FBS and 2.29 g/l NaHCO3. When required, MC3T3 cells were grown to confluence and then induced to differentiate into osteoblasts by supplementing the medium with AA (50 μg/ml) from day 3 of culture. The ROS-LAP* and ROS-LAP*R3L cell lines were generated from ROS17/2.8 cells grown in 6-well plates and transfected with 5 ug of the plasmid pTET-tTAk coding for the tetracycline transactivator (tTA) and 0.25 μg of the pCEP4 plasmid carrying a hygromycin resistance gene using Lipofectamine Plus (Life Technologies, CA, USA). The transfected cells were then selected for their ability to grow in the presence of 200 μg/ml hygromycin. These cells were then stably transfected with 2 μg of the 3xFlag-C/EBPβ-LAP* or 3xFlag-C/EBPβ-LAP*R3L plasmid and 0.25 μg of the plasmid pRSV-Neo carrying a geneticin resistance gene. Several cell lines were selected for growing in 400 μg/ml geneticin (Life Technologies), 50 μg/mL hygromycin, and 10 μg/mL tetracycline. Once generated, the ROS-LAP* and ROS-LAP*R3L cell lines were maintained in 100 μg/ml geneticin, 50 μg/ml hygromycin, and 10 μg/ml tetracycline. The different cell lines (monoclonal and polyclonal lines) were evaluated for their ability to express 3xFlag-C/EBPβ-LAP* proteins by Western blot using anti-Flag M2 antibodies (F3165, Sigma Aldrich).

Chromatin Immunoprecipitation (ChIP) and reChIP assays

ChIP studies were performed as described previously (Grandy et al., 2011; Soutoglou and Talianidis, 2002). Precleared cross-linked chromatin samples (200–300 bp) obtained from MC3T3, ROS-LAP* and ROS-LAP*R3L cell cultures were immunoprecipitated overnight using the following antibodies: anti-C/EBPβ C-19 antibody (sc-150, Santa Cruz Biotechnology, CA, USA), anti-RNA polymerase II antibody (8WG16, Covance, WI, USA), anti-histone H3 antibody (ab1791, Abcam, MA, USA), anti-Histone H3 (tri methyl K36) antibody (ab9050, Abcam), anti-acetyl histone H3 antibody (06-599, Merck Millipore, MA, USA), anti-Flag antibody (PRB-132C, Covance). Anti-Brg1 rabbit antiserum was kindly donated by Dr. Anthony Imbalzano (University of Massachusetts Medical School, MA, USA). The PCR primers used to evaluate the distal upstream region of the mouse Ric-8B gene (−4,636/−4,421) by QPCR were: 5′-CATGGACAGGGTTTTGGGAGAC-3′ (forward) and 5′-ACCTGTAGGTTCTGTGCATCTC-3′ (reverse). To evaluate the region −636/−426 of the mouse Ric-8B promoter, the primers were: 5′-TGGTTTCCGGCCTTTAGGGAAC-3′ (forward) and 5′-GACGACAACTGGCGGGCTGTTC-3′ (reverse). To evaluate the mouse and rat Ric-8B promoter region −396/−284 the primers used were: 5′-GGAGAGACAGTTCTGCTCGTGG-3′ (forward) and 5′-GGAGCCACCAGAGACTGAGTCA-3′ (reverse). To evaluate a region inside of the exon 3 of the mouse Ric-8B gene the primers used were: 5′-AGCTCCACGAGTCAGACGAT-3′ (forward) and 5′-CGGTCCTTGCACTTCCTTAG-3′ (reverse). To evaluate the rat Osteocalcin promoter region −198/−28 the primers used were 5′-GGCAGCCTCTGATTGTGTCC-3′ (forward) and 5′-TATATCCACTGCCTGAGCCC-3′ (reverse). The re-ChIP assays were performed as described earlier (Cruzat et al., 2009). The

immunoprecipitated complexes obtained after first ChIP were diluted by incubation for 30 min at 37 °C in 25 μL of 10 mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times with sonication buffer and subjected to the second ChIP procedure.

QPCR analyses

Quantitative PCR (QPCR) was performed using the Brilliant II SYBR Green QPCR master mix in a MX3000P spectrofluorometric thermal cycler (Stratagene-Agilent, CA, USA) according to the manufacturer's recommendations. Efficiencies for each primer pair were adjusted to nearly 100%, modifying the primer concentration in the amplification mix.

Nuclear extracts and protein expression analyses

Nuclear extracts from MC3T3 cells, ROS17/2.8 cells, ROS-LAP* or ROS-LAP*R3L stable cell lines were prepared as previously described (Paredes et al., 2004). Protein levels were quantified using Bio-Rad Protein Assay reagent according to manufacturer's instructions and using bovine serum albumin as standard (Bio-Rad, CA, USA). For Western blot analyses, 5–10 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a nitrocellulose membrane. Immunoblot was performed using the following antibodies: anti-C/EBPβ C-19 antibody (sc-150, Santa Cruz Biotechnology), anti-Flag M2 antibody (F3165, Sigma Aldrich), and anti-TFIIB C-18 antibody (sc-225, Santa Cruz Biotechnology). Immunoblotting was performed with secondary antibodies conjugated to horseradish peroxide (Santa Cruz Biotechnology) and substrates for enhanced chemiluminescence (Thermo Scientific, IL, USA).

Coimmunoprecipitation

MC3T3 cells were transfected with the 3xFlag-C/EBPβ-LAP*-coding vector or its mutant variant 3xFlag-C/EBPβ-LAP*R3L using Superfect reagent (Qiagen, Germany) and nuclear extracts prepared as described previously (Paredes et al., 2004). Coimmunoprecipitations were reported before (Sierra et al., 2003). Flag-tagged C/EBPβ-LAP* or C/EBPβ-LAP*R3L proteins were immunoprecipitated using anti-Flag M2 antibody (F3165, Sigma Aldrich). The immunocomplexes were captured using Protein G-Sepharose beads (Santa Cruz Biotechnology). The immunoprecipitated proteins were detected by Western blot using the following antibodies: anti-Brg1 serum (donated by Dr. Anthony Imbalzano), anti-Ini1 H-300 antibody (sc-13055, Santa Cruz Biotechnology), anti-C/EBPβ C-19 antibody (sc-150, Santa Cruz Biotechnology), and anti-Flag M2 antibody (F3165, Sigma Aldrich).

Transient-transfection and reporter assays

Subconfluent MC3T3 cells were transiently transfected for 24 hrs using Superfect (Qiagen), according to the manufacturer's recommendations. Cells cultured in 6- or 24-well plates were transfected with up to 2.0 or 1.0 μg of DNA, respectively. When required, the total DNA concentration was adjusted using purified plasmid DNA. The analysis of luciferase activity was performed in a GloMax-20/20 luminometer according to the Promega Dual Luciferase Reporter assay protocol (Promega).

RT-PCR

Total cellular RNA was isolated and analyzed by Reverse transcriptase PCR (RT-PCR) amplification as described before (Grandy et al., 2011). Expression of osteocalcin, Ric-8B, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined by QPCR, using the following primers; rat osteocalcin: 5′-CTGAGTCTGACAAAGCCTTC-3′ (forward) and 5′-CGTCCATACTTTCGAGGCAG-3′ (reverse); rat Ric-8B: 5′-GGGGCTATCGAGCGGGTCCTG-3′ (forward) and 5′-GTCTCTGGAGAGAATGCGGAG-3′ (reverse); and GAPDH: 5′-CATGGCCTTCCGTGTTCCTA-3′ (forward) and 5′-CCTGCTTCACCACCTTCTTGAT-3′ (reverse).

RESULTS

C/EBPβ and SWI/SNF simultaneously bind to target genes to mediate repression during osteoblast differentiation

C/EBPβ transcription factor promotes the osteoblast phenotype by directly regulating transcription of bone-related genes (Gutierrez et al., 2002; Henriquez et al., 2011; Villagra et al., 2006). Importantly, our group has recently established that during osteoblast differentiation, the C/EBPβ-LAP*, but not the C/EBPβ-LAP, isoform functions not only as a transcriptional activator but also can mediate down-regulation of proliferation-associated genes like Ric-8B through a SWI/SNF-dependent mechanism (Grandy et al., 2011). Ric-8B codes for a Guanine nucleotide Exchange Factor (GEF) that is critical for proliferation of neural crest-derived progenitor cells, including craneo-facial osteoprogenitors (Maldonado-Agurto et al., 2011). Hence, it is important to define the molecular mechanisms that regulate the function of C/EBPβ-LAP* activating or repressing gene expression in osteoblastic cells.

As an osteoblast differentiation cell system we have used the MC3T3 cells that represent a well-characterized pre-osteoblastic cell line that progressively differentiates to mineralized osteoblasts when grown in the presence of ascorbic acid (Wang et al., 1999), allowing to study gene expression control mechanisms during osteogenesis (Grandy et al., 2011; Yang et al., 2011; Yu et al., 2013). As shown in Figure 1A, sequential chromatin immunoprecipitation (re-ChIP) analyses demonstrate that C/EBPβ (antibody can recognize both C/EBPβ-LAP* and C/EBPβ-LAP proteins) and the SWI/SNF complex bind simultaneously to the same target DNA sequences at the Ric-8B gene promoter, including a consensus and functional C/EBPβ element (Grandy et al., 2011). This interaction occurs only in differentiated osteoblasts (see Figure 1A, 13 DIV) as actively proliferating MC3T3 pre-osteoblastic cells (3 DIV) do not exhibit significant co-enrichment of C/EBPβ and SWI/SNF at the Ric-8B gene promoter. Also, interaction of C/EBPβ and SWI/SNF occurs concomitantly with a reduced association of the RNA polymerase II (RNA PolII) with the Ric-8B promoter and a significant reduction of methylation at lysine 36 of histone H3 (H3K36me3) within the Ric-8B gene coding region (Figure 1B), both indicators of decreased transcriptional activity (Cantone and Fisher, 2013).

Recent reports indicate that C/EBPβ-LAP* transcription factor can function as repressor by recruiting complexes containing HDAC activity to target gene promoters and therefore

inducing a significant reduction in the levels of histone acetylation at key regulatory sequences (Wang et al., 2008). However, we found that repression of the Ric-8B gene (decreased Ric-8B mRNA levels, but not shown) during osteoblast differentiation (Grandy et al., 2011) is not accompanied by a significant change in histone H3 acetylation (H3ac) at the Ric-8B gene promoter (Figure 1C). Histone H3 is one of the most relevant acceptor of acetyl groups in chromatin during acetylation events that accompany regulation of gene transcription in higher eukaryotic cells (Yang and Seto, 2008). An apparent increase in H3ac especially downstream of the regulatory C/EBP site (−504/−497), is mainly due to a previously shown nucleosome enrichment (and therefore histone H3 enrichment) event in this promoter region (see Figure 1C) that results from the nucleosome-remodeling activity of SWI/SNF recruited to this sequence (Grandy et al., 2011). Accordingly, transient transfection studies in proliferating osteoblastic cells showed that forced co-expression of three different HDACs, each representing enzymes of the class I (HDAC3), class II (HDAC4), or class IV (HDAC11), does not affect the activity of the Ric-8B gene promoter (Figure S1). To control for the functional activity of these over-expressed enzymes, we determined that all three HDACs are capable of inhibiting the promoter activity of other bone-related genes (Data not shown).

Together, these results indicate that Ric-8B gene repression during osteoblast differentiation involves a C/EBPβ-LAP*-dependent recruitment of the SWI/SNF chromatin remodeling complex and that this repression process is independent of changes in histone H3 acetylation at the Ric-8B promoter region.

The N-terminus domain of C/EBPβ-LAP* mediates interaction with SWI/SNF and subsequent recruitment of SWI/SNF to target genes in osteoblasts

It has been proposed that the ability of C/EBPβ to interact with SWI/SNF is at least partially determined by the N-terminal domain present only in C/EBPβ-LAP* (Kowenz-Leutz et al., 2010; Smink and Leutz, 2012). Moreover, using chicken C/EBPβ-LAP* it was shown that asymmetric dimethylation of the R3 residue inhibits the interaction with SWI/SNF and prevents transcriptional up-regulation of target genes in myeloid cells (Kowenz-Leutz et al., 2010). A change of this R3 residue to L (C/EBPβ-LAP*R3L) may mimic the asymmetric methylation in this residue and therefore inhibiting the interaction between C/EBPβ-LAP* and SWI/SNF (Kowenz-Leutz et al., 2010). Accordingly, we generated DNA sequences coding for both mutant (C/EBPβ-LAP*R3L) and wild-type (C/EBPβ-LAP*) Flag-tagged proteins and evaluated by co-immunoprecipitation whether the R3L mutant protein lacks the ability of interacting with SWI/SNF in the context of osteoblastic cells. We found that both mutant and wild-type versions of C/EBPβ-LAP* are readily expressed in MC3T3 osteoblastic cells at comparable levels (see Figure S2) following transient transfection. As shown in Figure 2B, in osteoblastic cells C/EBPβ-LAP*R3L protein is unable to interact with two principal components of the SWI/SNF complex like Brg1 (catalytic subunit of the complex) and Ini1 (core component of the complex) (Imbalzano, 1998). In contrast, the wild-type C/EBPβ-LAP* counterpart readily interacts with both components of SWI/SNF (Figure 2, compare A and B).

Next we evaluated whether the R3L mutation also affects the ability of C/EBPβ-LAP* to down-regulate the Ric-8B gene promoter activity. We first performed transient co-expression analyses in MC3T3 osteoblastic cells, to determine the effect of C/EBPβ-LAP* over-expression (wt and R3L) on a Ric-8B promoter-luciferase-reporter construct activity. As shown in Figure 3B, whereas wt C/EBPβ-LAP* effectively inhibits Ric-8B promoter activity in a dose-dependent manner, over-expression of C/EBPβ-LAP*R3L does not produce this inhibitory effect. We confirmed that the presence of the Flag tag (3xFlag) at the N-terminal end of C/EBPβ-LAP*R3L does not affect the expression and functional properties of this transcription factor. Thus over-expression of a tag-less form of C/EBPβ-LAP*R3L remains unable to down-regulate Ric-8B promoter activity (Figure S3).

Interestingly, over-expression of C/EBPβ-LAP*R3L in this same osteoblastic cells inhibited the activity of a co-transfected rat osteocalcin (OC) gene promoter-luciferase-reporter construct (Figure S4). Transcription of the bone-specific OC gene in rat-derived osteoblasts has been shown to be cooperatively up-regulated by a heterodimer formed between C/EBPβ-LAP and the transcription factor Runx2 (Gutierrez et al., 2002) in part due to their ability to recruit a large complex to the OC promoter that includes histone acetylases like p300/CBP as well as SWI/SNF (Gutierrez et al., 2002; Villagra et al., 2006). Therefore, likely the expressed C/EBPβ-LAP*R3L binds to the promoter and competes with wild type C/EBPβ-LAP. This interpretation is further supported by the enhanced OC promoter activity observed in parallel experiments where the effect of over-expression of wt C/EBPβ-LAP* was evaluated (Figure S4). This stimulatory effect over the OC promoter was equivalent to that obtained after C/EBPβ-LAP over-expression (Figure S4).

Together, these results indicate that a functional interaction between C/EBPβ-LAP* and SWI/SNF requires an intact and unmethylated R3 residue in the N-terminus of the C/EBPβ-LAP* protein. In addition, our results confirm that the change R3L represents a valuable molecular tool to explore the regulatory role of C/EBPβ-LAP*-SWI/SNF function in osteoblasts.

C/EBPβ-LAP*R3L can target C/EBPβ-LAP- and C/EBPβ-LAP*-regulated genes in proliferating osteoblasts

We next determined whether transcription of endogenous bone-related target genes in proliferating osteoblastic cells that do not express the larger isoform of C/EBPβ is affected by the presence of C/EBPβ-LAP* and C/EBPβ-LAP*R3L. We generated osteoblastic cell lines that inducibly express Flag-tagged C/EBPβ-LAP* and Flag-tagged C/EBPβ-LAP*R3L. We used the tetracycline-inducible expression system following protocols previously established in our group (Cruzat et al., 2009; Villagra et al., 2006). Rat-derived osteosarcoma ROS 17/2.8 cells (Majeska et al., 1980), that poorly express the C/EBPβ-LAP* isoform, were stably transfected with tetracycline-VP16 regulator and the gene coding hygromycin resistance. Drug-resistant cell lines were screened for the ability to activate a transiently transfected luciferase reporter construct under the control of tetracycline operator elements (Gossen and Bujard, 1992). The selected cell lines were then stably transfected with a gene coding neomycin resistance and a tetracycline operator-controlled vector encoding either 3xFlag-C/EBPβ-LAP* or 3xFlag- C/EBPβ-LAP*R3L. Several drug-

resistant monoclonal (as well as polyclonal) cell lines were selected and analyzed for expression of Flag-tagged proteins. A time-course of optimal Flag-tagged protein expression upon tetracycline deprival was established for each cell line by western blot using both anti Flag and anti C/EBPβ antibodies (see a representative study in Figure S5).

A shown in Figure 4A, 4 days of culturing in the absence of tetracycline was sufficient for most of the cell lines evaluated to produce significant levels of the Flag-C/EBPβ-LAP* and Flag-C/EBPβ-LAP*R3L proteins. We find that in the presence of tetracycline our transgenic ROS17/2.8 cells principally express the endogenous C/EBPβ-LAP isoform of this transcription factor, together with the OC and Ric-8B genes that are expressed at high levels (Grandy et al., 2011; Gutierrez et al., 2002; Villagra et al., 2006). ChIP analyses using anti Flag antibodies showed that inducibly-expressed (−Tet) wt and mutant C/EBPβ-LAP* proteins bind to target gene promoters, including OC and Ric-8B (Figure 4B). Importantly, interaction of wt C/EBPβ-LAP* with the Ric-8B promoter is accompanied by enhanced enrichment of Brg1 at this promoter sequence (Figure 4B, right graph) and results in reduced expression of the Ric-8B gene as reflected by decreased Ric-8B mRNA levels (Figure 4D, upper graph). This result confirms that C/EBPβ-LAP* can bind to the Ric-8B gene promoter in osteoblastic cells and that this interaction mediates SWI/SNF recruitment to the promoter sequence leading to Ric-8B gene repression (Grandy et al., 2011). Expressed C/EBPβ-LAP* also binds to the OC promoter (Figure 4B) and this interaction is accompanied by a significant increase in OC mRNA levels (Figure 4D, upper graph). This result is in agreement with our transient expression analyses (Figure 4S) and also demonstrates that, as it has been previously shown for C/EBPβ-LAP (Gutierrez et al., 2002; Villagra et al., 2006), the C/EBPβ-LAP* isoform is also capable to stimulate the expression of the OC gene in osteoblasts.

When binding of C/EBPβ-LAP*R3L to target genes was analyzed by ChIP, we found that this mutant protein can interact with both OC and Ric-8B endogenous gene promoters in osteoblastic cells (Figure 4C). Importantly, binding of C/EBPβ-LAP*R3L to the Ric-8B gene further reduces the already low enrichment of Brg1 at this promoter sequence (Figure 4C). This result indicates that C/EBPβ-LAP*R3L is unable to bind, and hence to recruit, SWI/SNF, to the Ric-8B gene promoter in intact osteoblastic cells. As expected, this reduced association of C/EBPβ-LAP*R3L and SWI/SNF leads to increased Ric-8B gene transcription (Figure 4D, lower graph), further confirming their role as negative regulators of Ric-8B gene expression in osteoblasts. In accordance with our transient expression studies (Figure 4S), binding of C/EBPβ-LAP*R3L to the OC gene promoter results in a significant inhibition of OC mRNA synthesis (Figure 4D, lower graph), suggesting that this protein may function as a dominant-negative mutant for OC gene expression.

Together, our results demonstrate that in osteoblastic cells abrogation of the C/EBPβ-LAP*-SWI/SNF interaction prevents the efficient C/EBPβ-LAP*-mediated recruitment of SWI/SNF to target genes, therefore affecting C/EBPβ-mediated up- or down-regulation of transcription.

DISCUSSION

Bone formation during embryonic development and skeletal remodeling involve temporal control of expression of cell growth and phenotypic genes. There is a requirement for controlling the transition from exiting the cell cycle and committing to the osteoblast lineage, involving down-regulation of genes associated with proliferation and up-regulation of genes associated with the osteoblast phenotype (Stein et al., 2004). Ric-8 is an evolutionary conserved cytosolic GEF playing critical roles during cell division at different stages of development (Hinrichs et al., 2012). This protein is expressed at craniofacial arches (Maldonado-Agurto et al., 2011) particularly regulating migration of vertebrate craniofacial-neural crest cells (Fuentealba et al., 2013). Thus marked craniofacial defects have been observed in frog embryos where Ric-8 expression is down-regulated, mainly due to reduced proliferation and migration of osteogenic precursor cells at this embryonic tissue. Our group has previously shown that during osteoblast differentiation, Ric-8B gene expression is strongly down-regulated concomitant with exiting the cell cycle and committing to the mature osteoblast phenotype and in a C/EBPβ-LAP*- and SWI/SNF-dependent manner (Grandy et al., 2011). Importantly, C/EBPβ-LAP* isoform is significantly expressed in differentiating osteoblasts once these cells are no longer proliferating and the expression of late-osteoblast phenotypic genes is enhanced (Grandy et al., 2011). Here we demonstrate the contribution of the N-terminal domain of C/EBPβ-LAP* factor for interacting with SWI/SNF and for mediating transcriptional repression of the Ric-8B gene in osteoblasts.

Recent results from the group of Leutz and colleagues indicate that the ability of C/EBPβ-LAP* to interact with SWI/SNF may depend, at least in part, on post-translational modifications that occur at residues located within the N-terminus of C/EBPβ-LAP* (Kowenz-Leutz et al., 2010). Hence it was found that in myeloid cells a dimethylation of the R3 residue by arginine methylase PMRT4/CARM1 prevents C/EBPβ-LAP*-SWI/SNF interaction, thereby inhibiting C/EBPβ-LAP*-dependent up-regulation of target genes during myeloid differentiation. Whether this particular regulatory mechanism is also operating in osteoblastic cells remains undetermined. Also, it remains to be established what specific subunit(s) of the SWISNF complex are mediating the interaction with C/EBPβ-LAP* or whether there is an additional protein component that mediates or stabilizes the formation of the C/EBPβ-LAP*-SWI/SNF complex. C/EBPβ factors have been shown to form stable heterodimers with other transcription factors (e. g. Runx2 (Gutierrez et al., 2002; Villagra et al., 2006)) in osteoblasts, which in turn bind to other transcriptional co-activators, therefore forming large complexes at regulatory regions of target gene promoters (Stein et al., 2010). Therefore, it is important to define new experimental approaches that allow researchers in the field to dissect the specific components of these large regulatory complexes and to determine the signaling pathways regulating their formation and function. Because the aminoacid residue change R3L may be mimicking a dimethylated R3 residue at the chicken C/EBPβ-LAP* N-terminus, hence preventing its interaction with SWI/SNF (Kowenz-Leutz et al., 2010), we generated a similar mutation on the rodent versions of this factor and determined the impact on C/EBPβ-LAP*-dependent repression in osteoblasts. Our results using inducible stably-transfected osteoblastic cell lines indicate that C/EBPβ-

LAP*R3L not only can bind to target promoters in osteoblastic cells but also exhibits a dominant-negative function that inhibits C/EBPβ-LAP*- and C/EBPβ-LAP-dependent transcriptional control due, at least in part, to a decreased SWI/SNF activity at these target genes. Future studies will need to address what specific regulatory components (e. g. transcription factors, co-activators, and/or co-repressors) remain associated with or are displaced from target genes under these experimental conditions.

C/EBPβ-LAP* has been shown to repress gene transcription (COX-2 gene) in human cells by means of its ability to recruit HDAC4 to target promoter sequences, therefore decreasing total histone acetylation at chromatin regions that include critical regulatory elements (Wang et al., 2008). Importantly, this C/EBPβ-LAP*-HDAC4 interaction strongly depends on the N-terminal domain present in C/EBPβ-LAP* but not in C/EBPβ-LAP (Wang et al., 2008). Nevertheless, we find that repression of the Ric-8B gene in osteoblasts does not involve a decrease in histone H3 acetylation (neither a decrease in total histone H4 acetylation, but not shown) at the Ric-8B promoter. In agreement with this result, it was determined that overexpression of three different HDACs, each representing class I, class II and class IV members (HDAC3, HDAC4, and HDAC11, respectively) of this family of enzymes that remove acetyl groups from nucleosomal histones, do not affect Ric-8B promoter activity in transient transfection analyses. These results, together with previous findings of our group, allow us to propose that in osteoblasts, C/EBPβ-LAP*-dependent repression of the Ric-8B gene mainly occurs through chromatin remodeling events mediated by the SWI/SNF complex at the Ric-8B promoter (Grandy et al., 2011) and does not involve a C/EBPβ-LAP*-HDAC4 complex.

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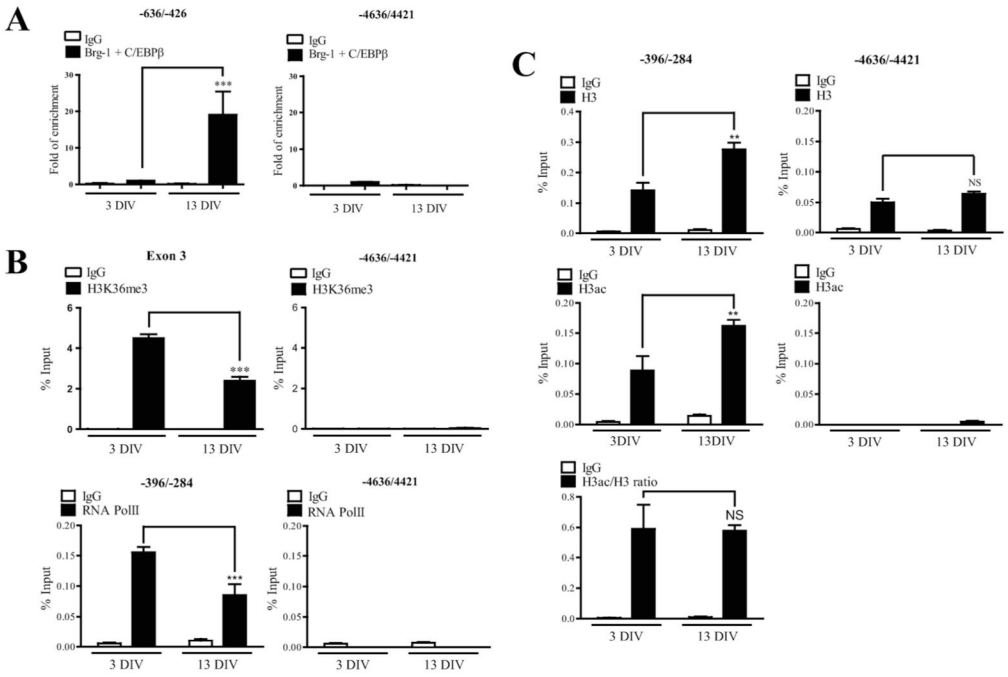

FIGURE 1.

Ric-8B repression during osteoblastic differentiation involves simultaneous recruitment of C/EBPβ and SWI/SNF at the Ric-8B gene promoter, but is independent of histone H3 acetylation. Mouse preosteoblastic MC3T3 cells were cultured between 3 and 13 DIV (days of differentiation *in vitro*) in the presence of 50 μg/mL ascorbic acid. At the indicated days, cells were crosslinked with 1% formaldehyde and sonicated chromatin fragments were immunoprecipitated with specific antibodies. **A.** Sequential chromatin immunoprecipitation (re-ChIP) studies were performed using antibodies against C/EBPβ and the SWI/SNF-catalytic subunit Brg-1. As a negative control a distal upstream sequence of the Ric-8b gene promoter (−4,636/−4,421) was also amplified. The results are expressed as fold change relative to the binding determined at 3 DIV on the same region. **B.** ChIP studies were performed using antibodies against the active elongation-associated epigenetic mark H3K36me3 and RNA polymerase II (RNA PolII). **C.** Additional ChIP studies were performed using antibodies against histone H3 (H3) and acetylated histone H3 (H3ac). The enrichment levels of Ric-8B gene sequences in the precipitated chromatin were determined by QPCR using specific primers. The DNA sequences analyzed at the Ric-8B gene locus are indicated at the top of each graph. The bars represent mean ± standard error of three independent experiments quantified by duplicate. Statistically significant differences were determined by the analysis of variance (ANOVA) test. ***, P<0.001; **, P<0.01; NS, non-significant.

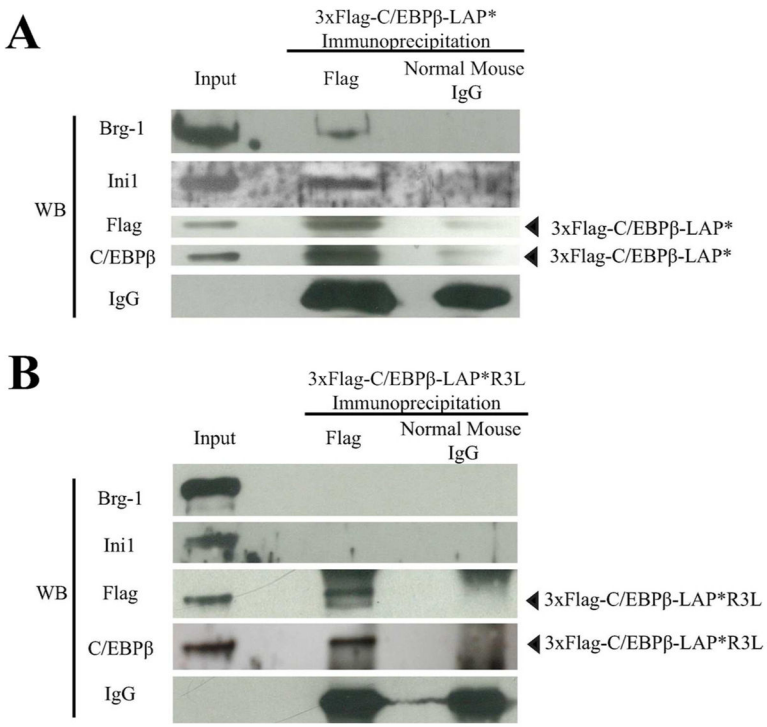

FIGURE 2.

3xFlag-C/EBPβ-LAP*R3L mutant protein is unable to interact with SWI/SNF in osteoblastic cells. Sub-confluent MC3T3 cells were transfected with vectors encoding for 3xFlag-C/EBPβ-LAP* (**A**) or its mutant variant 3xFlag-C/EBPβ-LAP*R3L (**B**). 24 hrs later, nuclear extracts were obtained and immunoprecipitation studies were performed using anti-Flag antibodies. Precipitation with normal mouse IgG was carried out as specificity control. Coimmunoprecipitated proteins were recovered and Western blot analyses performed using antibodies against the Brg-1 and Ini1 subunits of the SWI/SNF complex. Precipitation of each Flag-tagged variant of C/EBPβ-LAP* was controlled by re-blotting against Flag and C/EBPβ. The band corresponding to the IgG high chain (approx. 60 kDa) is also shown.

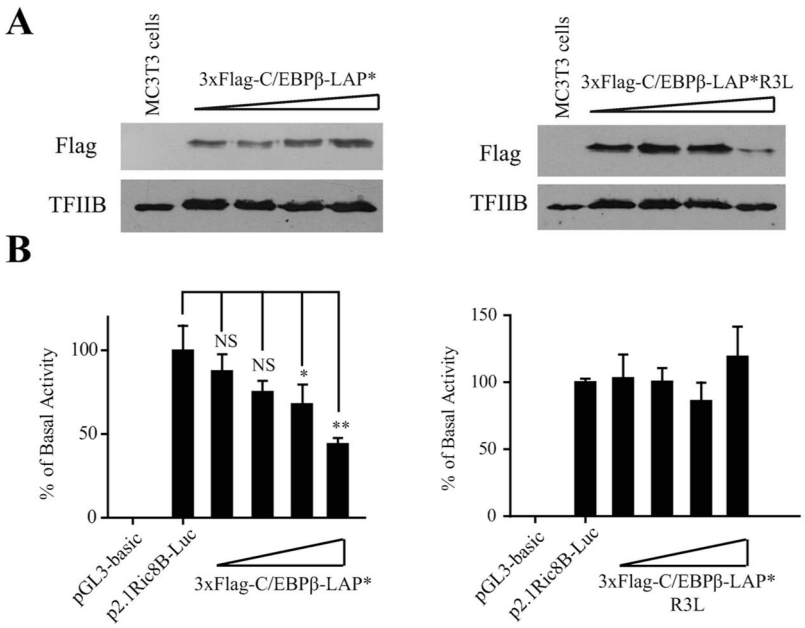

FIGURE 3.

C/EBPβ-LAP*-mediated repression of the Ric-8B promoter in osteoblastic cells is impaired in the R3L mutant protein unable to interact with SWI/SNF. Subconfluent MC3T3 cells were cotransfected with p2.1Ric-8B-Luc together with increasing amounts (100–800 ng) of a vector coding for 3xFlag-C/EBPβ-LAP* or its mutant 3xFlag-C/EBPβ-LAP*R3L protein. p2.1Ric8B-Luc construct contains a 2,124 bp segment of the Ric-8B promoter fused to the Luciferase reporter gene. **A.** 5 ug of nuclear extracts were obtained from cells transfected for 24 hrs and overexpression of Flag-tagged C/EBPβ proteins confirmed by Western blot using anti-Flag antibodies. Reblotting against TFIIB was used to control for equal loading. **B.** Upon 24 hrs of transfection with the plasmids, luciferase activity was determined. The bars represent mean ± standard error of at least three independent experiments quantified by duplicate. Statistically significant differences were determined by the analysis of variance (ANOVA) test. **, $P<0.01$; *, $P<0.05$; NS, non-significant.

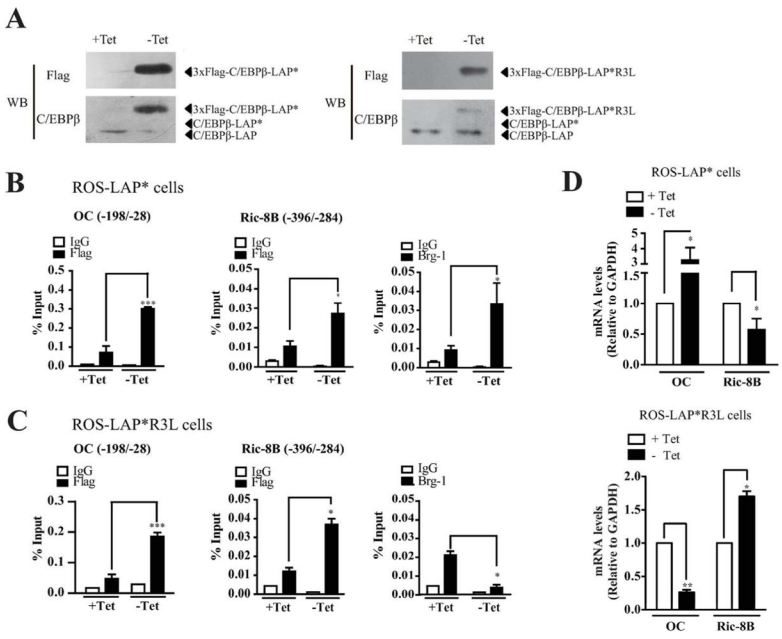

FIGURE 4.

C/EBPβ-LAP* differentially regulates transcription of bone-related genes Ric-8B and Osteocalcin genes in osteoblastic cells. ROS-LAP* and ROS-LAP*R3L cells expressing 3xFlag-C/EBPβ-LAP* or its R3L mutant protein, respectively, under control of the tetracycline-inducible Tet-off system were cultured for 4 days in presence (+Tet) or absence (−Tet) of 10 μg/mL tetracycline. **A.** To confirm the expression of Flag-tagged C/EBPβ-LAP* proteins, cells were collected and nuclear extracts were analyzed by Western blot using specific antibodies against the Flag epitope. Re-blotting using an antiC/EBPβ antibody allowed simultaneous detection of the Flag-tagged and endogenous isoforms of C/EBPβ. **B, C.** To demonstrate the binding of the Flag-tagged proteins to the Osteocalcin (OC) and Ric-8B gene promoter in ROS-LAP* (**B**) or ROS-LAP*R3L (**C**) cells, ChIP experiments were performed and purified DNA analyzed by QPCR using specific primers. Binding of the SWI/SNF catalytic subunit Brg-1 to the Ric-8B promoter was also analyzed. The DNA sequences amplified during each quantitation are indicated at the top of each graph. **C**. OC and Ric-8B mRNA expression levels in each cell line were determined by QPCR using specific primers. The bars represent mean ± standard error of three independent experiments quantified by duplicate. Statistically significant differences were determined by the Student's t-test. ***, P<0.001; **, P<0.01; *, P<0.05.