Molecular and Cellular Endocrinology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

C/EBPβ LIP and c-Jun synergize to regulate expression of the murine progesterone receptor

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ARTICLE INFO

Keywords:
C/EBPβLIP
c-Jun
Progesterone receptor
Synergistic regulation
Transcription

ABSTRACT

CCAAT/enhancer binding protein β (C/EBPβ) is required for murine mammary ductal morphogenesis and alveologenesis. Progesterone is critical for proliferation and alveologenesis in adult mammary glands, and there is a similar requirement for progesterone receptor isoform B (PRB) in alveologenesis. We examined C/EBPβ regulation of PR expression. All three C/EBPβ isoforms, including typically inhibitory LIP, transactivated the PR promoter. LIP, particularly, strongly synergized with c-Jun to drive PR transcription. Endogenous C/EBPβ and c-Jun stimulated a PR promoter-reporter and these two factors showed promoter occupancy on the endogenous PR gene. Additionally, LIP overexpression elevated endogenous PR protein expression. In pregnancy, both PRB and the relative abundance of LIP among C/EBPβ isoforms increase. Consistent with a role in PRB expression, *in vivo* C/EBPβ and PR isoform A expression showed mutually exclusive localization in mammary epithelium, while C/EBPβ and PRB largely co-localized. We suggest a critical role for C/EBPβ, particularly LIP, in PRB expression.

1. Introduction

CCAAT/enhancer binding protein β (C/EBPβ) is a critical transcription factor in the regulation of murine mammary gland proliferation and development. Experiments with C/EBPβ-deficient mice demonstrate a requirement for C/EBPβ in ductal morphogenesis and alveologenesis (Robinson et al., 1998; Seagroves et al., 1998). C/EBPβ occurs in three isoforms in mammary and other tissues: C/EBPβ p38 (LAP1) and C/EBPβ p35 (LAP2), both potent transcriptional activators, and C/EBPβ p20 (LIP), a truncated form generally reported to inhibit C/EBP-dependent transcription (reviewed in Zahnow, 2002). During the course of pregnancy in the mouse, C/EBPβ protein expression increases, with LIP expression being particularly elevated, in contrast to not being detectable in the virgin mammary gland (Seagroves et al., 1998).

Progesterone signaling through the progesterone receptor (PR) is also a critical factor for proliferation, ductal morphogenesis and alveologenesis in the adult mammary gland (reviewed in Fendrick et al., 1998; Shyamala et al., 1998; Aupperlee et al., 2005; Aupperlee and Haslam, 2007). Furthermore, experiments with mice deficient in PR isoform B (PRB) show a requirement of PRB for alveologenesis (Lydon et al., 1995; Mulac-Jericevic et al., 2003).

The block to alveologenesis in both C/EBPβ- and PRB-deficient mice suggests that these transcription factors might act in the same pathway or may regulate overlapping sets of downstream target genes. An overall decrease in PR observed in sexually mature wildtype mice fails to occur in C/EBPβ-deficient mice, while no alterations in C/EBPβ expression are observed in PR-deficient mice (Seagroves et al., 2000). This is consistent with C/EBPβ acting upstream of PR. PR isoform A (PRA) is the predominant isoform of PR expressed in the mammary glands of virgin adults, with its expression dramatically decreasing at pregnancy and PRB being expressed with alveolar development during pregnancy (Aupperlee et al., 2005). This raises the possibility that C/EBPβ is required for the differential upregulation and localization of PRB expression that is observed during pregnancy.

In this report, we examined whether C/EBPβ participates in the transcriptional regulation of PR expression in the murine mammary

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https://doi.org/10.1016/j.mce.2018.06.001
Received 5 December 2017; Received in revised form 31 May 2018; Accepted 1 June 2018

gland. Transient co-transfection of a PR promoter-reporter with expression vectors that individually express C/EBPβ isoforms into a murine mammary tumor cell line revealed that all C/EBPβ isoforms, surprisingly including LIP, can transactivate the PR promoter. Importantly, we found that LIP, in particular, robustly synergizes with c-Jun to drive PR transcription. Consistent with significant roles for C/EBPβ and c-Jun in PR expression, knockdown experiments showed that endogenous levels of C/EBPβ and c-Jun expression are sufficient to stimulate the PR promoter-reporter. Additionally, overexpression of LIP elevates PR protein expression from the intact endogenous gene encoding PR. Furthermore, *in vivo* immunofluorescence studies showed that the localization of C/EBPβ and PRA expression are mutually exclusive in the mammary epithelium, while PRB is expressed in cells that express C/EBPβ. Collectively, our data suggest a critical role for C/EBPβ, particularly LIP, in PRB expression.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from Harlan (Indianapolis, IN, USA). All animal experimentation was conducted in accord with accepted standards of humane animal care under guidelines approved by the All University Committee on Animal Use and Care at Michigan State University.

2.2. Cells and cell culture

MC7-L1, MC4-L2, and MC4-L3 are mammary cell lines of epithelial origin, derived from murine mammary ductal carcinomas (Lanari et al., 2001; a gift from Dr. Claudia Lanari, Universidad de Buenos Aires, Buenos Aires, Argentina). These cell lines express both ER and PR. MC7-L1 and MC4-L2 are hormone responsive *in vitro* and MC4-L3 is hormone responsive *in vivo*. Cells were maintained in DMEM-F12 (1:1) medium supplemented to 5% FCS, 100 units/ml penicillin and 100 mg/ml streptomycin. Experiments were carried out with charcoal-stripped FCS in the absence of antibiotics. Cells were cultured at 37 °C with 5% CO_2.

2.3. Expression vectors and promoter-reporters

For transient transfections, murine C/EBPβ isoforms were individually expressed from pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). pcDNA-LIP has been described (Dearth et al., 2001). pcDNA-LAP2 (plasmid 12557; Addgene, Cambridge, MA, USA) has been described (Basu et al., 2011). pcDNA-LAP1 was derived from a plasmid containing the complete coding sequence of murine C/EBPβ inserted between the EcoRI and HindIII sites of pcDNA3.1(−) (a gift from Dr. Peter Johnson, NCI-Frederick, Frederick, MD, USA). The ATG translational start sites for LAP2 and LIP were mutated to GCG and a consensus Kozak sequence introduced upstream of the LAP1 translational start, mutating GCGTT CATG to GCCACCATG (mutated bases underlined) by site-directed mutagenesis. pCMV-c-jun has been described (McCabe et al., 1996). Murine PRA and PRB were individually expressed from vectors derived from the complete murine PR cDNA (Schott et al., 1991). For PRA, a HincII/EcoRV fragment of the PR cDNA, lacking the PRB ATG translational start site, was ligated into the EcoRV site of pcDNA3.1(−). For PRB, a NheI/NotI fragment of the PR cDNA was ligated into the cognate sites of pcDNA3.1(+). The PRA ATG translational start site was mutated to GCG.

The tandem PR promoter-reporter (Fig. 1) consists of the region −2494 to +769 base pairs (bp) in relation to the predicted PRB transcriptional start site inserted between the Asp718 and NcoI sites of pGL3-Basic (pGL3B) (Promega, Madison, WI, USA). This region contains the putative transcriptional start sites for both PRB and PRA. The murine PR promoter region was isolated by PCR amplification of C57/Bl6 genomic mouse DNA using the following primers: −2502 to −2471 bp, 5′-ACATGGTACCAGCGTGTCACCTGGCACAGA-3' (containing an underlined Asp718 site); +771 to +753 bp, 5′-CTGTCCATGGACACG TCCGAGTGCTGGCT-3' (containing an underlined NcoI site). TA cloning placed the PCR fragment into pCR2.1 (TA Cloning Kit; Invitrogen, Life Technologies, Grand Island, NY, USA). The promoter fragment was then excised with Asp718 and NcoI, and inserted into pGL3B. The minimal PR promoter-reporter consists of the region −117 to +63 base pairs (bp) in relation to the predicted PRB transcriptional start site (Fig. 1) and was constructed in two steps. First, PCR amplification using 5′-AGCACCTGCAACTTCACCTCTG-3' (−444 to −423 bp) and 5′-TAGCAGAATGTCAGAATCCTC-3' (+43 to +63 bp) produced a promoter fragment that was placed into pCR2.1 by TA cloning, after which the promoter fragment was excised with Sac I and Xho I, and inserted into pGL3B. This construct was then digested with Spe I and religated to produce the −117/+63 PR promoter-reporter. 2xPRE-TK-luc (plasmid 11350; Addgene) (Giangrande et al., 2000) contains two copies of a consensus progesterone response element (PRE) upstream of the human thymidine kinase promoter. pRL-SV40 expresses *Renilla* luciferase from the SV40 early enhancer-promoter (Promega).

2.4. Transient transfections

Transient transfections were conducted in either 12-well cell culture plates or 6 cm culture plates containing 1 ml/well or 4 ml/plate, respectively, of DMEM-F12 supplemented to 5% charcoal-stripped FCS. 12-well cell culture plates were seeded with 5×10^4 cells/well and 6 cm culture plates with 5×10^5 cells 24 h prior to transfection. FuGENE 6 Transfection Reagent (Promega) and plasmid DNAs were mixed at a 2:1 ratio (volume:weight) in 50 or 100 µl of serum-free medium for 12-well and 6 cm culture plates, respectively. This mixture was incubated at room temperature for 30 min before addition to cell cultures. The DNA, totaling 500 ng or 5 µg per individual 12-well and 6 cm culture plate transfection, respectively, comprised 100 ng or 1 µg of the −2494/+769 PR promoter-reporter, 1 ng or 10 ng of pRL-SV40, and varying amounts of control "empty" pcDNA3.1, C/EBPβ expression vector and/or c-Jun expression vectors, as indicated in figure legends. All quantities of expression vector are expressed as ng per 5×10^4 cells. Cells were assayed 24 h after transfection. In experiments involving the −117/+63 PR promoter-reporter, luciferase values derived from pGL3B lacking any inserted sequences were subtracted from those of the −117/+63 PR promoter-reporter to eliminate background levels of luciferase expression driven by C/EBPβ and c-Jun responsive sequences in the parent vector. For experiments utilizing 2xPRE-TK-luc, a slightly modified transfection protocol was used. 400 ng of the 2xPRE-TK-luc promoter-reporter was transiently co-transfected with expression vector DNA totaling 200 ng, comprising 100 ng pcDNA-LIP and/or 100 ng pCMV-c-jun, or 200 ng pcDNA3.1. For experiments examining PRA and PRB activity, 2xPRE-TK-luc was co-transfected with expression vector DNA totaling 500 ng, comprising ratios of PRA and PRB vectors as described in Fig. 4B. These transfections were performed in a 12-well plate format and assayed 36 h after transfection. In some cases, progestin R5020 (PerkinElmer, Waltham, MA, USA) was added to transfected cells 3 h after transfection at a final concentration of 20 nM. Transfected cells were harvested, lysed, and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were normalized to the *Renilla* luciferase values as a control for transfection efficiency between individual samples. All transfections were carried out in duplicate and repeated at least three times.

In transient transfections assayed by the isolation of RNA or nuclear protein extracts, 5×10^5 cells were transfected in 6 cm culture plates under conditions similar to those described above. The quantities of expression plasmids are indicated in figure legends. Cells were harvested for RNA isolation or nuclear extracts as described below.

In transient transfections assayed by immunofluorescence, cells were grown to 30% confluence in 6-well cell culture plates on 22 mm