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Functional Analysis of CBP/p300 in Embryonic Orofacial Mesenchymal Cells

D.R. Warner,* M.M. Pisano, and R.M. Greene

Department of Molecular, Cellular, and Craniofacial Biology, University of Louisville Birth Defects Center, University of Louisville School of Dentistry, Louisville, Kentucky 40292

Abstract CREB binding protein (CBP) and the close structural homolog, p300, are nuclear coactivators of multiple signaling pathways that play important roles in embryonic development and cellular homeostasis. TGFβ regulates the proliferation rate of many cell types and has been demonstrated to inhibit the growth rate of mouse embryonic maxillary mesenchymal (MEMM) cells. The role of CBP and p300 in TGFβ-mediated control of proliferation of MEMM cells was thus investigated using an in vitro gene knockdown approach. TGFβ reporter assays demonstrated that p300 mRNA knockdown via targeted siRNAs led to a reduction in the response to TGFβ, whereas knockdown of CBP by the same approach had an insignificant effect. In MEMM cell proliferation assays, siRNA-mediated knockdown of CBP and/or p300 had little impact upon TGFβ-mediated growth inhibition; however, the basal rate of proliferation was increased. Inhibition of p300 activity via overexpression of a dominant-negative mutant (p300ΔC/H3) led to significant inhibition of TGFβ-mediated activation of p3TP-lux. As with the siRNA knockdown approach, p300ΔC/H3 also increased the basal rate of cell proliferation of MEMM cells. CBP/p300 siRNA knockdown had a significant but incomplete inhibition of TGFβ-induction of matrix metalloproteinase-9 (gelatinase B) expression. These data demonstrate that p300 is involved in Smad-mediated transcription of p3TP-lux, however, its role (and that of CBP) in biological processes such as the control of cell proliferation and extracellular matrix metabolism is more complex and may be mediated via mechanisms beyond coactivator recruitment. J. Cell. Biochem. 99: 1374–1379, 2006.

Key words: CREB; CBP; p300; TGFβ; embryo; orofacial

Embryonic development requires numerous cellular signaling pathways. The list includes those signaling pathways activated by TGFβ, Wnt, and Notch (reviewed in Gerhart [1999]). Many of these pathways transduce extracellular signals to the nucleus via nucleocytoplasmic proteins such as Smads in the case of TGFβ, and β-catenin in canonical Wnt signaling. Although Smads and β-catenin are transcription factors, the broad range of responses to these signaling proteins requires additional levels of specificity and input from multiple signaling pathways to achieve the desired, tissue-specific response. Transcriptional coactivators such as the CREB binding protein (CBP) and its close homolog, p300, are multifunctional proteins that link numerous transcription factors to elements of the transcription machinery, such as RNA polymerase II, and integrate signaling inputs from multiple pathways.

Development of the secondary palate in mice is an elegantly simple developmental process whereby the palatal processes grow bilaterally from the maxillary processes, initially assuming positions alongside the tongue, then elevate above the tongue where the two palatal processes meet and fuse to form the continuous palate, thereby separating the oral and nasal cavities [Greene and Pisano, 2004]. Early palate development is characterized by a period of mesenchymal cell proliferation and synthesis of extracellular matrix proteins. Several molecules have been demonstrated to be necessary for proper development of the secondary palate,

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*Correspondence to: D.R. Warner, PhD, Department of Molecular, Cellular and Craniofacial Biology, University of Louisville School of Dentistry, 501 South Preston Street, Suite 301, Louisville, KY 40292.

E-mail: dennis.warner@louisville.edu

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including the TGFβs, EGF, and cAMP/PKA [Brunet et al., 1993; Greene et al., 1995; Miettinen et al., 1999]. The importance of CBP in this process is underscored by the observation that in humans, specific mutations in CBP result in Rubinstein–Taybi syndrome, characterized, in part, by craniofacial anomalies, including cleft palate [Petrij et al., 1995]. The phenotype is recapitulated to some extent in the mouse CBP knockout model [Tanaka et al., 1997].

Previous work from this laboratory has demonstrated the expression and temporal regulation of CBP and p300 in developing mouse palate tissue [Warner et al., 2002] and its role in mediating crosstalk between PKA and TGFβ signaling in cultured palate mesenchymal cells [Warner et al., 2003]. In the present report, evidence is presented that CBP and p300 contribute to at least one important process in palatogenesis, mesenchymal cell proliferation. Moreover, the ability of these coactivators to influence TGFβ-mediated cellular processes appears limited.

MATERIALS AND METHODS

Animals

Mature male and female mice (ICR) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were maintained at a constant temperature of 25°C with a 12 h light cycle and access to food and water ad libitum. To obtain embryos of a specific gestational age, matings were performed overnight and the presence of a vaginal plug the following morning was taken as evidence of copulation and was designated embryonic day 0.5 (E0.5).

Cell Culture and Transfection

For luciferase reporter assays, cell proliferation measurements, and knockdown studies, primary cultures of mouse embryonic maxillary mesenchyme (MEMM) were established. The maxilla and secondary palatal tissue from E13.5 mouse embryos was microdissected, cells liberated by treatment with 0.05% (w/v) trypsin for 10 min, suspended in OptiMEM (Gibco Invitrogen Corp., Gaithersburg, MD) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and plated at a density of approximately 7,000 cells/cm^2 of a 6-well tissue culture plate. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. The following day, cultures were generally 60–70% confluent and ready for transfection. Plasmids or siRNAs were transfected with the lipophilic transfection reagent, Effectene (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations. The use of Effectene for the transfection of MEMM cells routinely yields approximately 5–10% transfection efficiency, based on experiments that utilized a plasmid containing the cDNA for enhanced green fluorescent protein. Many other transfection protocols and reagents have been tested for MEMM cultures, however, Effectene has proven to be the most effective. Where indicated, TGFβ1 (R&D Systems, Minneapolis, MN) was added at a concentration of 2 ng/ml.

Plasmids/siRNAs

p3TP-lux, encoding the plasminogen activator inhibitor promoter upstream of firefly luciferase, was used as a read-out for Smad-mediated TGFβ signal transduction and was obtained from Dr. J. Massague. pRL-CMV (Promega, Madison, WI) encoding *Renilla* luciferase and is expressed constitutively, was included as an internal transfection control. p300ΔC/H3, a dominant-negative mutant of p300, was obtained from Upstate Cell Signaling Solutions (Lake Placid, NY). siRNAs targeting mouse CBP or mouse p300 and a non-targeting control siRNA were obtained from Dharmacon (Lafayette, CO). These siRNAs are composed of a pool of four individual siRNAs designed to different regions of their respective mRNA.

Luciferase Reporter Assays

MEMM cells (in 6-well plates) were transfected with the appropriate siRNA, 1 μg p3TP-lux, and 50 ng pRL-CMV. Twenty-four hours post-transfection, cells were rinsed with fresh culture medium and treated with 2 ng/ml TGFβ1 (R&D Systems Inc.) for an additional 24 h. Each well was rinsed twice with PBS, lysed, and luciferase activity measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity (from p3TP-lux) is reported after normalization to control, *Renilla*, luciferase activity (expressed from pRL-CMV) for each sample. Each condition was assayed in triplicate and the experiment was performed twice with comparable results.

RT-PCR

To determine the relative knockdown efficiency of CBP and p300 mRNA, total RNA was purified from siRNA-transfected cultures of MEMM cells using the RNeasy Protect purification system (Qiagen). cDNAs were synthesized using the SuperScript first strand cDNA synthesis system (Gibco Invitrogen Corp.) and used as the template in real-time PCR assays with probe:primer pairs purchased from Applied Biosystems (Foster City, CA) and analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). All data were normalized to the amplification signal from GAPDH. Fold-change values were determined according to the relationship: fold-change $= 2^{-\Delta\Delta Ct}$, where Ct is the threshold value for real-time PCR amplification detection, ΔCt is the difference in Ct for the same probe:primer pair (e.g., CBP or p300) on CBP or p300 siRNA-transfected samples vs. control samples, and $\Delta\Delta Ct = \Delta Ct$, sample—$\Delta Ct$, GAPDH [Livak and Schmittgen, 2001].

Cell Proliferation

To measure the rate of MEMM cell proliferation, cells were incubated in Opti-MEM (Gibco Invitrogen Corp.) supplemented with 5% fetal calf serum, and 2 μCi/ml [^{3}H]thymidine (20 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA). [^{3}H] incorporation was determined by cold TCA precipitation followed by solubilization in NaOH. One-half of the sample was assayed for incorporation of [^{3}H] by liquid scintillation spectroscopy and the other half was assayed for protein concentration by the micro BCA method [Smith et al., 1985] using reagents from Pierce Biotechnology (Rockford, IL).

Gelatinase Assay

The activity of gelatinases A and B (MMP-2 and MMP-9, respectively) was determined by gelatin zymography as previously described [Greene et al., 2003].

Statistical Analyses

Statistical significance was determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test, using GraphPad Prism, v. 2.0b (GraphPad Software, Inc., San Diego, CA). P-values <0.05 were considered significant. Each experiment was conducted at least two times with comparable results.

RESULTS

In order to determine the role of CBP and p300 in TGFβ-mediated signaling in primary cultures of MEMM cells, small inhibitory RNAs (siRNAs) targeting either CBP or p300 were transfected into MEMM cells and several endpoints measured. Based upon semi-quantitative RT-PCR assays, transfection with 100 nM of either CBP- or p300-specific siRNAs led to as high as 9- to 16-fold decreases of the level of CBP or p300 mRNA expression, respectively, compared to a non-targeting control siRNA (Table I). These data also demonstrate the specificity of the CBP and p300 siRNAs used in these experiments. To determine the impact of CBP or p300 knockdown on TGFβ-mediated activation of transcription, MEMM cells were transfected with siRNAs targeting CBP or p300, along with the p3TP-lux reporter construct which is highly responsive to Smad-mediated TGFβ signaling (Fig. 1) [Wrana et al., 1992]. TGFβ-mediated transcription activation of p3TP-lux was not significantly affected by CBP-targeting siRNAs. However, knockdown of p300 did attenuate activation of p3TP-lux by TGFβ ($P < 0.01$, one-way ANOVA). These data suggest that reducing the expression of p300 had a greater impact than reduction of CBP on TGFβ-mediated signaling in MEMM cells. The role of p300 was also tested using an alternate approach whereby a dominant-negative mutant of p300, missing the C/H3 domain (also called the Taz2 domain, which contains the E1A binding site), was overexpressed. Overexpression of p300ΔC/H3 had a significant inhibitory effect on TGFβ-mediated activation of the reporter construct, p3TP-lux (Fig. 1), consistent with the above results for siRNA-mediated inhibition of

TABLE I. Inhibition of the Expression of CBP and p300 in MEMM Cells by Targeted siRNAs

siRNA	CBP	p300
	Fold-reduction	
CBP	8.6 ± 0.9	1.5 ± 0.1
p300	1.4 ± 0.2	16.7 ± 1.8

siRNAs (100 nM) targeting either CBP or p300 were transfected into MEMM cells and incubated for 48 h. Real-time PCR was performed on cDNAs generated from each sample and the signal normalized to that from GAPDH. Fold-change (reduction) was calculated using the relationship, fold change $= 2^{-\Delta\Delta Ct}$. Specific details are provided in the Materials and Methods section. Data presented are fold-reduction ± standard deviation from triplicate determinations from a single experiment, representative of two performed.

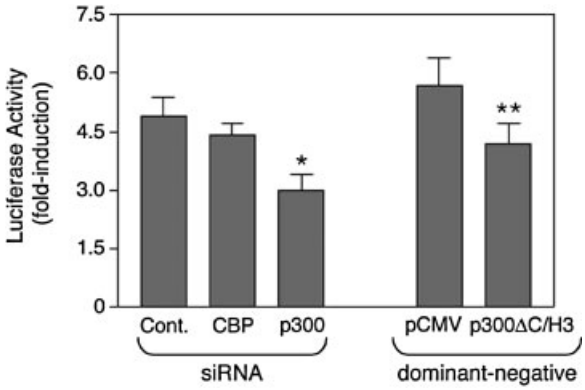

Fig. 1. Effect of CBP or p300 knockdown via specific siRNAs or a p300 dominant-negative mutant on TGFβ-induced transactivation of p3TP-lux. MEMM cells were transfected with siRNAs specific to either CBP or p300 or with a dominant-negative mutant of p300 (p300ΔC/H3; missing the Smad-binding, C/H3 domain), along with the reporter construct p3TP-lux and pRL-CMV, which constitutively expresses *Renilla* luciferase and serves as an internal control. Twenty-four hours after transfection, cells were treated with 2 ng/ml TGFβ1 for an additional 24 h, at which time the cells were harvested and luciferase activity measured as detailed in the Materials and Methods section. Expression of the siRNA targeting CBP had little effect on the ability of TGFβ to activate p3TP-lux. However, both the p300-specific siRNA and p300ΔC/H3 dominant-negative mutant resulted in a small, but statistically significant decrease in TGFβ induction of p3TP-lux reporter activity (*$P < 0.01$ and **$P < 0.05$, respectively; one-way ANOVA). These results demonstrate that p300 (if not CBP) is required for Smad-dependent TGFβ signaling in MEMM cells.

p300, and suggesting critical involvement of p300 in this process. It is not known, however, if p300ΔC/H3 also inhibited CBP activity.

Overexpression of p300ΔC/H3 in MEMM cells did not block the growth-inhibitory effect of TGFβ on the rate of proliferation of MEMM cells, as measured by [^{3}H]thymidine incorporation into DNA, but did result in an enhanced basal rate of proliferation of these cells (Fig. 2). The effect of reducing the expression of CBP and p300, either in combination, or individually, on MEMM cell proliferation was also determined via transfection of targeted siRNAs (Table II). Transfection of CBP and p300-specific siRNAs in combination increased the rate of proliferation of MEMM cells by ~1.2-fold compared to a non-targeting siRNA ($P < 0.05$), however, the growth-inhibitory effect of TGFβ was unchanged. Similarly, the transfection of either CBP- or p300-specific sRNAs individually increased the basal rate of proliferation by ~1.3-fold ($P < 0.001$, compared to a control siRNA), but again had little effect on TGFβ-mediated MEMM growth inhibition. These data demonstrate that both CBP and p300 are important for the regulation of MEMM cell proliferation, but that the growth-inhibitory effect of TGFβ is mediated through additional mechanisms beyond recruitment of CBP or p300. Overexpression of p300ΔC/H3 failed to alter TGFβ-induced MMP-9 activity as determined by zymography (Fig. 3). However,

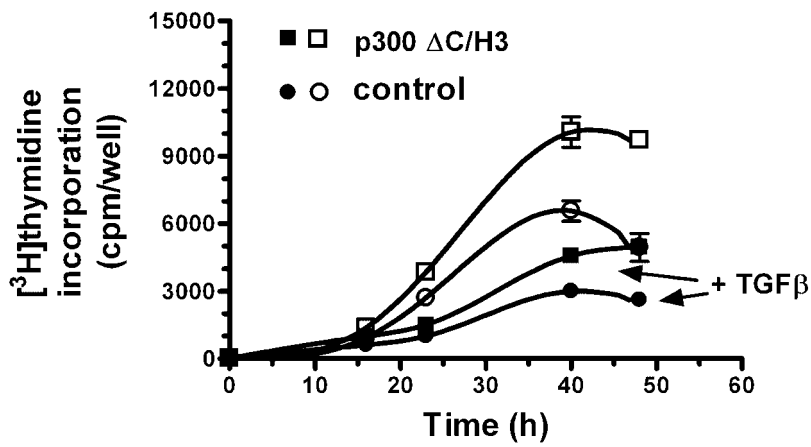

Fig. 2. Effect of p300 inhibition by a dominant-negative mutant on TGFβ-mediated reduction in MEMM cell proliferation. Primary cultures of MEMM cells were transfected with a dominant-negative mutant of p300 (p300ΔC/H3; □, ■) or empty vector (Control; ○, ●), grown in reduced serum for 48 h to synchronize cells in the G_1-S interface of the cell cycle [Pisano et al., 1986], and released with 5% fetal bovine serum in the absence (□, ○) or presence (■, ●) of 2 ng/ml TGFβ1. Each well also contained 4 μCi/ml [^{3}H]thymidine. At the indicated time points, new DNA synthesis was measured by determination of incorporation of [^{3}H]thymidine into TCA-precipitable material. Overexpression of p300ΔC/H3 increased the basal rate of proliferation by MEMM cells ($P < 0.001$, one-way ANOVA), but had no effect on the ability of TGFβ to inhibit cell proliferation.

TABLE II. Effect of CBP/p300 siRNA on MEMM Cell Proliferation

	Vehicle	TGFβ
	cpm [^{3}H]/μg protein	
Control siRNA	1156 ± 47	564 ± 19
CBP/p300 siRNA	1375 ± 40*	669 ± 22
Control siRNA	1506 ± 129	1035 ± 109
CBP siRNA	1952 ± 60**	1193 ± 60
p300 siRNA	2014 ± 53**	1248 ± 39*

MEMM cells were transfected with 100 nM siRNAs targeting CBP and/or p300. Forty-eight hours later, cultures were stimulated with 2 ng/ml TGFβ and 4 μCi/ml [^{3}H]thymidine. Thirty-six hours later, the incorporation of [^{3}H]thymidine was determined as detailed in the Materials and Methods section.
*$P < 0.05$ versus vehicle control.
**$P < 0.001$ versus vehicle control.
The data reported are the mean ± standard deviation of triplicate determinations from one of two experiments.

TABLE III. Effect of CBP/p300 siRNA on MMP Transcription

siRNA	TGFβ	MMP-2	MMP-9
	Fold-change		
Control	−	−1.2	+1.8
	+	−1.4	+31.1
CBP + p300	−	+1.2	+3.5
	+	+1.7	+36.8

MEMM cells were transfected with 100 nM siRNAs targeting CBP and stimulated with 2 ng/ml TGFβ for 32 h. The relative level of expression of MMP-2 and MMP-9 were determined by real-time PCR as described in the Materials and Methods section. Results are representative of two experiments and are presented as the fold-change at 32 h post-TGFβ treatment versus 0 h time point. TGFβ increased the expression of MMP-9 by 17.3- and 10.5-fold in the control versus CBP + p300 siRNA treated cells, respectively ($P < 0.01$, one-way ANOVA; TGFβ induction = fold-change + TGFβ/fold-change in absence of TGFβ).

siRNA-mediated inhibition of CBP and p300 had a significant effect on the ability of TGFβ to induce MMP-9 expression, reducing the effect of TGFβ from 17-fold in the control sample versus 10.5-fold in the CBP/p300 siRNA-transfected cells (Table III). Consistent with earlier observations, TGFβ did not significantly induce MMP-2 expression [Greene et al., 2003].

DISCUSSION

CBP and p300 are transcriptional coactivators involved in facilitating an increasingly diverse array of signaling pathways. The role of CBP and p300 in TGFβ-mediated signaling is well documented and mutations in either can lead to developmental defects [Yao et al., 1998; Tanaka et al., 2000]. CBP and p300 have distinct functions. For example, Kawasaki et al. [1998] demonstrated that induction of $p21^{Cip1}$ expression is dependent on p300 while CBP controls expression of $p27^{Kip1}$ in F9 cells. Moreover, $p300^{-/-}$ mice present with cardiac defects [Yao et al., 1998], while $CBP^{-/-}$ mice have craniofacial anomalies not seen in $p300^{-/-}$ mice [Tanaka et al., 2000].

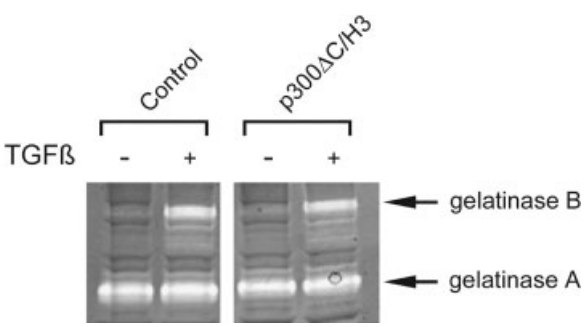

Fig. 3. Effect of p300 inhibition by a dominant-negative mutant on gelatinase B activity in MEMM cells. p300ΔC/H3 was overexpressed in primary cultures of MEMM cells by transfection and subsequently stimulated with 2 ng/ml TGFβ1 for 24 h as described for Fig. 2. The activity of gelatinases A and B were determined in whole-cell lysates by zymography using polyacrylamide gels infused with 0.1% gelatin. Cleared zones represent areas of gelatinase activity and were quantified with NIH image, version 1.63f. No effect of p300 inhibition was observed on the ability of TGFβ to induce either gelatinase A or B activity.

The goal of the present report was to determine the effect of CBP and p300 inhibition on cellular end-points regulated by TGFβ in MEMM cell cultures: mesenchymal cell proliferation and synthesis of gelatinases (matrix metalloproteinases). It has been previously demonstrated that Smads utilize CBP/p300 to mediate the effects of TGFβ [Pouponnot et al., 1998]. Blocking CBP synthesis via specific siRNAs had no effect on TGFβ-activation of p3TP-lux, a reporter plasmid that responds to Smad-mediated transcriptional control, suggesting that CBP alone is insufficient to mediate Smad dependent TGFβ signaling, or that possibly p300 can compensate for the loss of CBP. Conversely, blocking p300 activity by overexpression of a dominant-negative mutant, or blocking expression via siRNAs, however, led to significant effects on both TGFβ-mediated induction of p3TP-lux and the basal rate of MEMM cell proliferation. Although synthesis/activation of gelatinase was unaffected. These data suggest that p300 plays a significant role in some TGFβ-mediated processes, but not in such physiological processes such as cell proliferation or gelatinase activation.

While reducing intracellular CBP and p300 increased the basal proliferative rate, TGFβ inhibition of MEMM cell proliferation was

unaffected. These data are similar to that for p300ΔC/H3, suggesting that the contribution of p300 to regulation of MEMM cell proliferation is greater than that for CBP. Reduction of intracellular levels of either CBP or p300 separately also increased the basal rate of proliferation (Table II), suggesting *both* CBP and p300 are required to alter the rate of proliferation.

The fact that inhibition of CBP and p300 had little effect on TGFβ-mediated inhibition of cell proliferation, and a greater, but incomplete, block of gelatinase expression, suggests that other, CBP/p300-independent pathways are also utilized in these processes, such as, perhaps, the MAP kinase pathway [Santibanez et al., 2002]. It is clear, however, from these studies that precise control of p300 and CBP protein levels is crucial for normal cellular homeostasis and that relatively small changes (in some cases as low as twofold reduction) in the level of mRNA can lead to profound downstream signaling effects. Because CBP and p300 are available in limiting amounts, multiple signaling pathways utilizing these cofactors can influence the strength of response from competing pathways. For example, the intracellular domain of Notch competes with Smad 3 for binding sites on p300 and signaling through Notch inhibits the growth-inhibitory effects of TGFβ in Mv1Lu cells [Masuda et al., 2005]. Therefore, tight control over both availability and protein levels of CBP and p300 is necessary for mediating inputs from the array of signaling proteins controlling embryogenesis.

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