Transcriptional Co-activators CREB-binding Protein and p300 Regulate Chondrocyte-specific Gene Expression via Association with Sox9*

Masanao Tsuda**, Shigeru Takahashi**, Yuji Takahashi†, and Hiroshi Asahara§**

From the 3Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037, the 4Laboratory of Environmental Molecular Physiology, School of Life Science, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-0392 Japan, and 5PRESTO, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan

Chondrocytes are critical components for the precise patterning of a developing skeletal framework and articular joint formation. Sox9 is a key transcription factor that is essential for chondrocyte differentiation and chondrocyte-specific gene expressions; however, the precise transcriptional activation mechanism of Sox9 is not fully understood. Here we demonstrate that Sox9 utilizes a cAMP-response element-binding protein (CREB)-binding protein (CBP)/p300 to exert its effects. Sox9 associates with CBP/p300 in the chondrosarcoma cell line SW1353 via its carboxyl termini activation domain in a cell type-specific manner. In promoter assays, CBP/p300 enhances Col2a1, which encodes cartilage-specific type II collagen gene promoter activity via Sox9. Chromatin immunoprecipitation shows that p300 is bound to the Col2a1 promoter region. Furthermore, the CBP/Sox9 complex disrupter peptide suppresses Col2a1 gene expression and chondrogenesis from mesenchymal stem cells. These data demonstrate that CBP and p300 function as co-activators of Sox9 for cartilage tissue-specific gene expression and chondrocyte differentiation.

The Sox9, a high-mobility group (HMG) domain transcription factor, has been identified as a key molecule in chondrocyte differentiation, a multi-step pathway during which multipotent mesenchymal stem cells differentiate into chondrocytes (1–3). Expression of Sox9 shadows that of Col2a1, which encodes cartilage-specific type II collagen gene promoter activity Sox9. Chromatin immunoprecipitation shows that p300 is bound to the Col2a1 promoter region. Furthermore, the CBP/Sox9 complex disrupter peptide suppresses Col2a1 gene expression and chondrogenesis from mesenchymal stem cells. These data demonstrate that CBP and p300 function as co-activators of Sox9 for cartilage tissue-specific gene expression and chondrocyte differentiation.

* This work was supported by grants from PRESTO, Japan Science and Technology Corporation, and the Arthritis Foundation Investigator Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Molecular and Experimental Medicine, The Scripps Research Inst., 10550 N. Torrey Pines Rd., MEM161, La Jolla, CA 92037, Tel.: 858-784-9026; Fax: 858-784-2744; E-mail: asahara@scripps.edu.

† The abbreviations used are: CBP, cAMP-response element-binding protein; CREB, cAMP-response element-binding protein; MSC, mesenchymal stem cells; HA, hemagglutinin; GST, glutathione S-transferase; m.o.i., multiplicity of infection; TAZ, transcriptional adapter zinc-binding; GFP, green fluorescent protein; TK, thymidine kinase; CH, cysteine/histidine-rich domain.

Received for publication, April 3, 2003, and in revised form, April 21, 2003 Published, JBC Papers in Press, May 5, 2003, DOI 10.1074/jbc.M303471200

EXPERIMENTAL PROCEDURES

Tissue Culture Methods and Transfections—Human chondrosarcoma cell line (1353) cells were grown in Dulbecco’s modified Eagle’s medium (Cellgro, Mediatech, Washington, DC) supplemented with 10% fetal calf serum and penicillin/streptomycin (Sigma). Cells were transfected by using FuGENE 6 (Roche Applied Science) as described by the manufacturer. The amounts of transfected plasmids for cotransfection assays were as follows. For Sox9-dependent activation, 50 ng of Gal-TK luciferase reporter plasmid was cotransfected with 50 ng of a CMV-luciferase plasmid and 50 ng of a plasmid expressing GalT-K. For Sox9-dependent suppression, 50 ng of Gal-TK luciferase reporter plasmid was cotransfected with 50 ng of a CMV-luciferase plasmid and 50 ng of a plasmid expressing GFP.
Fig. 1. Sox9 associates with CBP/p300 via its C-terminal activation domain. A, lysates of SW1353 cells transformed with HA-tagged p300 or CBP and FLAG-tagged Sox9 were immunoprecipitated with anti-HA antibody. Precipitates (IP) and 10% on-putts (OP) were subjected to Western blotting with anti-FLAG antibody. B, schematic illustration of Sox9 deletion mutants. Various regions of Sox9 were cloned into pDNA3. C, the carboxyl terminus of Sox9 associates with p300. FLAG-tagged Sox9 mutants, shown in panel A, were transformed into SW1353 cells together with HA-p300. The cell lysates were immunoprecipitated with anti-HA antibody. Precipitates (IP) and 10% on-putts (OP) were run by 10% SDS-PAGE and analyzed for the presence of Sox9 by Western blotting with the anti-FLAG antibody.

CBP and p300 Are Sox9 Coactivators

All mRNA expression data from the quantitative PCR with reverse transcription were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in the corresponding sample.

Adenovirus Infection—Recombinant adenovirus vectors carrying mouse CBP amino acids 1805–1890 with the Gal4 DNA binding domain as a tag or control lacZ gene were constructed in pM17 as described (26) (generous gifts from Dr. Montminy, Salk Institute, La Jolla, CA). Viruses were purified by the CaCl2 method, and titer was checked as described (26). The infection efficiency of this adenovirus in SW1353 cells was examined by immunocytochemistry using an anti-Gal antibody, which showed almost 100% infection efficiency with an m.o.i. of 8 (data not shown).

MSC Cells Culture and Chondrogenesis—Human mesenchymal stem cells were purchased from BioWhittaker (Walkersville, MD). To induce chondrogenesis, pellet cultures were prepared by gently centrifuging 2.5 × 105 cells at 500 × g in 15 ml polypropylene conical tubes. The culture media was Dulbecco’s modified Eagle’s medium, low glucose supplemented with ITS Premix (BD Biosciences) consisting of insulin, transferrin, selenic acid, bovine serum albumin, and linoleic acid, Sodium pyruvate (1 mM), ascorbate 2-phosphate (37.5 μM), and transforming growth factor β3 (TGF-β3) (10 ng/ml). The pellet cultures were incubated at 37 °C, 5% CO2. Cells form an essentially spherical aggregate that does not adhere to the walls of the tube. Medium changes were carried out at 2-3 day intervals, and pellets were harvested for analysis at time points up to 4 weeks. The pellets were embedded, fixed, and stained with a type II collagen antibody.

RESULTS

Transcriptional Co-activators CBP and p300 Associating with Sox9—To test an interaction between CBP/p300 and Sox9 in intact cells, amino-terminally FLAG-tagged, full-length Sox9 was expressed in SW1353 alone or in the presence of HA-tagged p300 or CBP. Cell extracts were prepared, and immunoprecipitations were performed with anti-HA antibodies followed by Western blotting utilizing anti-FLAG antibodies. In the presence of coexpressed HA-CBP, Sox9 was co-immunoprecipitated (Fig. 1A). Similarly, we detected co-immunoprecipitation of Sox9 with HA-tagged p300 (Fig. 1A). These data show a physical association of Sox9 with the transcriptional co-activators p300 and CBP.

Mapping of Interaction Domains of CBP and Sox9 Complex—To determine the Sox9 interaction domain with CBP in SW1353 cells, FLAG-tagged Sox9 truncations were constructed (Fig. 1B). As shown in Fig. 1C, a carboxyl-terminal truncation reporter, 50 ng of Gal4-Sox9, and 100 ng of hemagglutinin (HA)-tagged p300 were used. The Col2a1 promoter (pKN185) (3) was assayed by cotransfection assays using 50 ng of plasmid or empty plasmid control per well. Luciferase activity was assayed, and reporter activities were normalized to activity from a cotransfected Rous sarcoma virus (RSV) promoter, 50 ng of Gal4-Sox9, and 100 ng of hemagglutinin (HA)-tagged p300 via its C-terminal activation domain. The infection efficiency of this adenovirus in SW1353 cells was examined by immunocytochemistry using an anti-Gal antibody, which showed almost 100% infection efficiency with an m.o.i. of 8 (data not shown).
p300 mutants were transfected into SW1353 cells, and protein interactions were examined by co-immunoprecipitation. CH1 also showed promoter activity 4-fold. Interestingly, p300 as a dominant negative inhibitor of transcription by reducing CH3 function also showed strong dominant negative effect on the Col2a1 promoter, suggesting that the CH1 domain might also play a role in this transcriptional activation 

To further verify the CBP/p300 dependence on Sox9-mediated transactivation, we applied the Gal4 fusion system. Sox9 was fused to the Gal4 DNA binding domain (Fig. 2A), and its activity was analyzed with a reporter containing five Gal4 binding sites and the TK promoter (Gal4 TK). Transfection with Gal4-Sox9(182–507) in SW1353 cells led to a 21-fold increase in luciferase activity compared with the Gal4 DNA binding domain (Fig. 2B). However, the Gal4-Sox9 construct lacking a C-terminal activation domain, which was identified as a CBP interaction domain, did not show a strong transcriptional activation (Gal4-Sox9 amino acids 1–423, 1–327). The Gal4-Sox9(182–507) transcriptional activity was further enhanced by cotransfection of p300 or CBP. We observed that the transfection of p300 in conjunction with Gal4-Sox9(182–507) in SW1353 cells showed a 13-fold increase in luciferase activity compared with Gal4-Sox9(182–507) alone. CBP had a synergistic effect similar to that of p300 (Fig. 3C). Collectively, these data support the notion that p300/CBP enhances Sox9 transcription activity.

**Critical Role of CBP/Sox9 Association for Col2a1 Gene Expression**—To determine whether CBP/p300 is bound to the Col2a1 promoter, we performed chromatin immunoprecipitation using CBP- and p300-specific antibodies (Fig. 4A). PCR amplification products from reactions with human Col2a1 first intron 2151–2305, which contains the Sox9 DNA binding site, were analyzed by 2% agarose gel. The Col2a1 intron DNA was efficiently recovered from immunoprecipitates of CBP but not control immunoglobulin (IgG) (Fig. 4B). Confirming the specificity of these antibodies, no PCR product was obtained from CBP immunoprecipitates using 293T cells in which Sox9 is not expressed, and, thus, CBP should not be recruited to the Col2a1 promoter (Fig. 4B).
Fig. 3. p300 promotes Sox9-dependent transcriptional activity. A, wild type but not mutant p300 activates transcription regulated by Sox9 binding sites. The effects of p300 or its mutant were monitored in a transient transfection luciferase assay. SW1353 cells were transfected with reporter plasmids carrying Sox9 binding site upstream of the TK promoter together with expression plasmids for wild type or the mutant of p300. Fold induction represents the ratio of luciferase activity measured in the absence of p300 expression vectors and the activity in the presence of p300 proteins after normalization. B, carboxyl terminal of Sox9 has transcriptional activity. Various regions of Sox9 indicated in Fig. 2A were fused with the Gal DNA binding domain and expressed in SW1353 cells. Reporter gene expression regulated by Gal binding site was measured. C, p300/CBP enhances transcriptional activity of Sox9. Expression vectors of p300 or CBP were transfected into SW1353 cells together with Gal-fused, amino-terminal truncated Sox9 (182-507). Reporter gene expression regulated by the Gal binding site was measured.

Fig. 4. CBP binding to Col2a1 promoter chromatin. A and B, Col2a1 promoter DNA is recovered and amplified from immunoprecipitates with CBP antibodies from SW1353 cells (A) but not from 293T cells (B). PCR amplification products from reactions with human Col2a1 first intron 2151–2305, which contains the Sox9 DNA binding site, were analyzed by 2% agarose gel.

To further examine the role of CBP/p300 and Sox9 interaction for Col2a1 gene expression, we used adenovirus expressing CH3 peptide (mouse CBP amino acids 586–672), which is also characterized as a protein interaction domain for several transcription factors, including CREB, c-Jun, and c-Myb (10, 25). Following the infection of the adenovirus, MSC cells were transferred to micromass cultures and incubated in the presence of transforming growth factor β to induce chondrogenesis. More than 95% of MSCs were co-expressing the green fluorescent protein (GFP) marker (not shown). Col2a1 expression, a marker for chondrogenesis, was not affected by control GFP or KIX peptide (Fig. 6A and B). CH3 peptide infection, however, blocked Col2a1 expression (Fig. 6C). Staining with control rabbit serum was negative for each of the samples (data not shown). These results indicate that CBP/p300 also plays a critical role in MSC differentiation into chondrocyte as well as chondrocyte-specific gene expression.

DISCUSSION

Sox9 has been identified as a critical molecule in chondrocyte differentiation (2, 4). Expression of Col2a1 shadows Sox9 expression. In the genital ridge, however, Col2a1 is not expressed despite a high level of Sox9 expression (4). This suggests the existence of molecular partners of Sox9 that are required for cartilage-specific Col2a1 gene expression.

Here we identified the interaction of Sox9 with CBP/p300. The paralogous proteins CBP and p300 were originally identified as interaction partners for CREB (38) and the adenoviral
A cartilage matrix of MSC pellets with A drogenesis ( ), whereas there was no effect on control adenovirus- antibody to collagen type II. CH3 peptide specifically inhibited chon- GFP ( ), KIX ( ) adenovirus infection was examined with transcriptional activation. The CH1 domain may play a role in recruiting RNA polymerase II or other basic transcriptional machinery in Sox9-dependent the CH3 domain is critical for its recruitment of RNA polymerase II (41, 42). Because the CH3 domain is occupied by Sox9 interaction, we observed that the CH1 domain, which is identified as an interaction domain for Sox9 binding, we observed that the CH1 domain of p300 is also critical for co-activator function. The CH1 domain of CBP/p300 has been shown to interact with transcriptional factor Hif-1 (34, 40). One of the CBP/p300 functions is to recruit RNA polymerase machinery. For example, in the case of CREB transcription, Montminy and co-workers showed that CH3 is critical for its recruitment of RNA polymerase II (41, 42). Because the CH3 domain is occupied by Sox9 interaction, the CH1 domain may play a role in recruiting RNA polymerase II or other basic transcriptional machinery in Sox9-dependent transcriptional activation.

Recently, mutations in the gene encoding CBP were found to cause Rubinstein-Taybi syndrome (20). The CBP+/− mice exhibited the clinical features of Rubinstein-Taybi syndrome, including skeletal abnormalities (21, 22). This phenotype could be, at least in part, explained by our findings that demonstrate the critical role of CBP/p300 in transcriptional activity of Sox9. Sox9 has been shown to plays an important role for developing skeletal framework (5, 6). The absence of CBP may reduce Sox9 activity and, hence, may indirectly affect skeletal development (22). Research on molecular function of co-activator CBP/p300 in accordance with Sox9 will advance our understanding of skeletal development and has the potential to identify new approaches to the treatment of skeletal and articular joint diseases (43).

Acknowledgments—We thank M. Montminy for the gift of adenovirus constructs and B. de Crombrugghe and Y. Yamada for the gift of Sox9 plasmids and Col2a1 promoter plasmids. We also thank M. Conkright and D. Brinson for helpful discussion, L. Creighton-Achermann for immunohistochemistry, and M. Lotz for encouragement and providing access to critical equipment.

REFERENCES

1. Cancendez, R., Descalzi Cancedda, F., and Castagnola, P. (1995) Int. Rev. Cytol. 150, 265–358
2. de Crombrugghe, B., Lefèvre, V., Behringer, R. R., Bi, W., Murakami, S., and Huang, W. (2000) Matrix Biol. 19, 389–394
3. Tanaka, K., Matsumoto, Y., Nakatani, F., Iwamoto, Y., and Yamada, Y. (2000) Mol. Cell. Biol. 20, 4428–4435
4. Zhao, Q., Eberpaecher, H., Lefèvre, V., and de Crombrugghe, B. (1997) Dev. Dyn. 209, 377–386
5. Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (1999) Nat. Genet. 22, 85–89
6. Bi, W., Huang, W., Whitworth, D. J., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6698–6703
7. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) Genes Dev. 10, 2657–2663
8. Roeder, R. G. (1996) Trends Biochem. Sci. 21, 327–335
9. Plaschke, M., and Gann, A. (1997) Nature 388, 569–577
10. Janknecht, R., and Hunter, T. (1998) Nature 383, 22–23
11. Brownell, J. E., and Allis, C. D. (1996) Curr. Opin. Genet. Dev. 6, 176–184
12. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) Nature 382, 319–324
13. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 953–959
14. Bannister, A. J., and Kouzarides, T. (1996) Nature 384, 641–643
15. Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., Nakatani, Y., and Allis, C. D. (1996) Cell 87, 1261–1270
16. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
17. Jones, K. A., and Kadonaga, J. T. (2000) Genes Dev. 14, 1992–1996
18. Tyler, J. K., and Kadonaga, J. T. (1999) Cell 99, 443–446
19. Pazin, M. J., and Kadonaga, J. T. (1997) Cell 89, 325–328
20. Petrij, F., Giles, R. H., Dauwerse, H. G., Saris, J. J., Hennekam, R. C., Masuno, M., Tommerup, N., van Ommen, G. J., Goodman, R. H., Peters, D. J., and Bruening, M. H. (1995) Nature 376, 348–351
21. Tanaka, Y., Naruse, I., Maekawa, T., Masuya, H., Shiroishi, T., and Ishii, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10215–10220
22. Yao, T. P., Oh, S. P., Fuchs, M., Zhou, N. D., Chng, L. E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. M., and Eckner, R. (1998) Cell 93, 361–372
23. Asahara, H., Dutta, S., Kao, H. Y., Evans, R. M., and Montminy, M. (1999) Mol. Cell. Biol. 19, 8219–8225
24. Asahara, H., Tartare-Deckert, S., Nakagawa, T., Ikebara, T., Hirose, F., Hunter, T., Ito, T., and Montminy, M. (2002) Mol. Cell. Biol. 22, 2974–2983
25. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1320–1324
26. Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., and Koopman, P. (1997) Dev. Biol. 185, 108–121
27. Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., and Koopman, P. (1997) Dev. Biol. 185, 108–121
28. Arany, Z., Newsome, D., Oldread, E., Livingston, D. M., and Eckner, R. (1995) Nature 374, 81–84
29. Miyagishi, M., Fujii, R., Hatta, M., Yoshida, E., Araya, N., Nagauchi, A., Ishihara, S., Nakajima, T., and Fukamizu, A. (2000) J. Biol. Chem. 275, 35170–35175
30. Takemaru, K. I., and Moon, R. T. (2000) J. Cell Biol. 149, 249–254
31. Hecht, A., Vleminkx, K., Stemmler, M. P., van Roy, F., and Kemler, R. (2000) EMBO J. 19, 1839–1850
32. Waltzer, L., and Rienz, M. (1998) Nature 395, 521–525
33. Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997) Nature 387, 825–827
34. Dames, S. A., Martinez-Yamout, M., De Guzman, R. N., Dyson, H. J., and Wright, P. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5271–5276
35. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Science 284, 144–147
36. Pittenger, M. F., Mosca, J. D., and McIntosh, K. R. (2000) Curr. Top. Microbiol. Immunol. 251, 3–11
37. Sekiya, I., Vuoriisto, J. T., Larson, B. L., and Prockop, D. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4397–4402
38. Chiriva, J. C., Kwock, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859
39. Goodman, R. H., and Smolik, S. (2000) Genes Dev. 14, 1553–1577
40. Freedman, S. J., Sun, Z. Y., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5367–5372
41. Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D., and Montminy, M. (1997) Genes Dev. 11, 738–747
42. Nakajima, T., Uchida, C., Anderson, S. F., Lee, C. G., Hurwitz, J., Parvin, J. D., and Montminy, M. (1997) Cell 90, 1107–1112
43. Aigner, T., and McKenna, L. (2002) Cell Mol. Life Sci. 59, 5–18
Transcriptional Co-activators CREB-binding Protein and p300 Regulate Chondrocyte-specific Gene Expression via Association with Sox9
Masanao Tsuda, Shigeru Takahashi, Yuji Takahashi and Hiroshi Asahara

J. Biol. Chem. 2003, 278:27224-27229.
doi: 10.1074/jbc.M303471200 originally published online May 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303471200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 43 references, 18 of which can be accessed free at http://www.jbc.org/content/278/29/27224.full.html#ref-list-1