rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Miner Res* 1996, 11:88-95.
14. Fox DA: **The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives.** *Arthritis Rheum* 1997, 40:598-609.
15. Udagawa N, Horwood NJ, Elliott J, Mackay A, Owens J, Okamura H, Kurimoto M, Chambers TJ, Martin TJ, Gillespie MT: **Interleukin-18 (interferon-γ-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast formation.** *J Exp Med* 1997, 185:1005-1012.
16. Horwood NJ, Udagawa N, Elliott J, Grail D, Okamura H, Kurimoto M, Dunn AR, Martin T, Gillespie MT: **Interleukin 18 inhibits osteoclast formation via T cell production of granulocyte macrophage colony-stimulating factor.** *J Clin Invest* 1998, 101: 595-603.
17. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, Martin TJ, Suda T: **IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis.** *J Clin Invest* 1999, 103:1345-1352.
18. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM: **Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand.** *Nature* 1999, 402:304-309.
19. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT: **Activated T lymphocytes support osteoclast formation in vitro.** *Biochem Biophys Res Commun* 1999, 265:144-150
20. Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, Takahashi K, Furuya T, Ishiyama S, Kim KJ, Saito S, Nishikawa T, Takahashi N, Togari A, Tomatsu T, Suda T, Kamatani N: **Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients.** *Arthritis Rheum* 2001, 44:1003-1012.
21. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T: **T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ.** *Nature* 2000, 408:600-605.
22. Tamura T, Udagawa N, Takahashi N, Miyaura C, Tanaka S, Yamada Y, Koishihara Y, Ohsugi Y, Kumaki K, Taga T, Kishimoto T, Suda T: **Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6.** *Proc Natl Acad Sci USA* 1993, 90: 11924-11928.
23. Udagawa N, Takahashi N, Katagiri T, Tamura T, Wada S, Findlay DM, Martin TJ, Hirota H, Taga T, Kishimoto T, Suda T: **Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors.** *J Exp Med* 1995, 182:1461-1468.
24. Broxmeyer HE: **Is interleukin 17, an inducible cytokine that stimulates production of other cytokines, merely a redundant player in a sea of other biomolecules?** *J Exp Med* 1996, 183: 2411-2415.
25. Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P: **CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene.** *J Immunol* 1993, 150:5445-5456.
26. Kennedy J, Rossi DL, Zurawski SM, Vega F Jr, Kastelein RA, Wagner JL, Hannum CH, Zlotnik A: **Mouse IL-17: a cytokine preferentially expressed by αβTCR + CD4-CD8-T cells.** *J Interferon Cytokine Res* 1996, 16:611-617.
27. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S: **T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines.** *J Exp Med* 1996, 183:2593-2603.
28. Chabaud M, Fossiez F, Taupin JL, Miossec P: **Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines.** *J Immunol* 1998, 161:409-414.
29. Chabaud M, Lubberts E, Joosten L, van Den Berg W, Miossec P: **IL-17 derived from juxta-articular bone and synovium contributes to joint degradation in rheumatoid arthritis.** *Arthritis Res* 2001, 3:168-177.
30. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, Miossec P: **Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium.** *Arthritis Rheum* 1999, 42:963-970.
31. Lubberts E, Joosten LA, Chabaud M, van Den Bersselaar L, Oppers B, Coenen-De Roo CJ, Richards CD, Miossec P, van Den Berg WB: **IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion.** *J Clin Invest* 2000, 105:1697-1710.
32. Jovanovic DV, Martel-Pelletier J, Di Battista JA, Mineau F, Jolicoeur FC, Benderdour M, Pelletier JP: **Stimulation of 92-kd gelatinase (matrix metalloproteinase 9) production by interleukin-17 in human monocyte/macrophages: a possible role in rheumatoid arthritis.** *Arthritis Rheum* 2000, 43:1134-1144.
33. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP: **IL-17 stimulates the production and expression of proinflammatory cytokines, IL-β and TNF-α, by human macrophages.** *J Immunol* 1998, 160:3513-3521.
34. Ziolkowska M, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H, Maslinski W: **High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism.** *J Immunol* 2000, 164:2832-2838.
35. Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, Goldring SR: **Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor.** *Arthritis Rheum* 2000, 43:250-258.
36. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S: **Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis.** *Arthritis Rheum* 2000, 43:259-269.
37. Shigeyama Y, Pap T, Kunzler P, Simmen BR, Gay RE, Gay S: **Expression of osteoclast differentiation factor in rheumatoid arthritis.** *Arthritis Rheum* 2000, 43:2523-2530.
38. Romas E, Bakharevski O, Hards DK, Kartsogiannis V, Quinn JM, Ryan PF, Martin TJ, Gillespie MT: **Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis.** *Arthritis Rheum* 2000, 43:821-826.
39. Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, Benoist C, Gravallese EM: **TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis.** *Am J Pathol* 2001, 159:1689-1699.
40. Teng YT, Nguyen H, Gao X, Kong YY, Gorczynski RM, Singh B, Ellen RP, Penninger JM: **Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection.** *J Clin Invest* 2000, 106:R59-R67.
41. Juji T, Aoki K, Horie D, Ohya K, Herz M, Gautam A, Mouritsen S, Oda H, Nakamura K, Tanaka S: **A novel therapeutic vaccine that prevents pathological bone destruction in models of osteoporosis and RA [abstract].** *J Bone Miner Res* 2001, 16(suppl 1):S150.
42. Chambers TJ, Owens JM, Hattersley G, Jat PS, Noble MD: **Generation of osteoclast-inductive and osteoclastogenic cell lines from the H-2KbtsA58 transgenic mouse.** *Proc Natl Acad Sci USA* 1993, 90:5578-5582.
43. Owens JM, Gallagher AC, Chambers TJ: **Bone cells required for osteoclastic resorption but not for osteoclastic differentiation.** *Biochem Biophys Res Commun* 1996, 222:225-229.
44. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H: **Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu.** *Cytokine Growth Factor Rev* 2001, 12:53-72.
45. Horwood NJ, Elliott J, Martin TJ, Gillespie MT: **IL-12 alone and in synergy with IL-18 inhibits osteoclast formation in vitro.** *J Immunol* 2001, 166:4915-4921.
46. Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, Kennedy K, Carter R, Wei XQ, Xu D, Field M, Foulis A, Liew FY, McInnes IB: **A proinflammatory role for IL-18 in rheumatoid arthritis.** *J Clin Invest* 1999, 104:1393-1401.
47. Makiishi-Shimobayashi C, Tsujimura T, Iwasaki T, Yamada N, Sugihara A, Okamura H, Hayashi S, Terada N: **Interleukin-18 upregulates osteoprotegerin expression in stromal/osteoblastic cells.** *Biochem Biophys Res Commun* 2001, 281:361-366.
48. Nakata A, Tsujimura T, Sugihara A, Okamura H, Iwasaki T, Shinkai K, Iwata N, Kakishita E, Akedo H, Terada N: **Inhibition by interleukin 18 of osteolytic bone metastasis by human breast cancer cells.** *Anticancer Res* 1999, 19:4131-4138.

49. Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T, Inoue J: **Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice.** *Genes Cells* 1999, 4:353-362.
50. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM, Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW: **TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling.** *Genes Dev* 1999, 13:1015-1024.
51. Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P: **Accelerated collagen-induced arthritis in IFN-γ receptor-deficient mice.** *J Immunol* 1997, 158:5507-5513.
52. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T: **Tumor necrosis factor-α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction.** *J Exp Med* 2000, 191:275-286.
53. Matsumoto M, Sudo T, Maruyama M, Osada H, Tsujimoto M: **Activation of p38 mitogen-activated protein kinase is crucial in osteoclastogenesis induced by tumor necrosis factor.** *FEBS Lett* 200, 486:23-28.
54. Quinn JM, Itoh K, Udagawa N, Hausler K, Yasuda H, Shima N, Mizuno A, Higashio K, Takahashi N, Suda T, Martin TJ, Gillespie MT: **Transforming growth factor β affects osteoclast differentiation via direct and indirect actions.** *J Bone Miner Res* 2001, 16:1787-1794.
55. Kaji K, Katogi R, Azuma Y, Naito A, Inoue JI, Kudo A: **Tumor necrosis factor-α-induced osteoclastogenesis requires tumor necrosis factor receptor-associated factor 6.** *J Bone Miner Res* 2001, 16:1593-1599.
56. Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, Suda T: **Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells.** *Exp Cell Res* 1999, 247:84-93.
57. Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Woodring J, Pacifici R: **Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-α.** *J Clin Invest* 2000, 106:1229-1237.
58. Roggia C, Gao Y, Cenci S, Weitzmann MN, Toraldo G, Isaia G, Pacifici R: **Up-regulation of TNF-producing T cells in the bone marrow: A key mechanism by which estrogen deficiency induces bone loss in vivo.** *Proc Natl Acad Sci USA* 2001, 98: 13960-13965.
59. Takeuchi E, Tomita T, Toyosaki-Maeda T, Kaneko M, Takano H, Hashimoto H, Sugamoto K, Suzuki R, Ochi T: **Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis.** *Arthritis Rheum* 1999, 42:221-228.
60. Toyosaki-Maeda T, Takano H, Tomita T, Tsuruta Y, Maeda-Tanimura M, Shimaoka Y, Takahashi T, Itoh T, Suzuki R, Ochi T: **Differentiation of monocytes into multinucleated giant bone-resorbing cells: two-step differentiation induced by nurse-like cells and cytokines.** *Arthritis Res* 2001, 3:306-310.

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Identification of a BMP-responsive element in *Id1*, the gene for inhibition of myogenesis

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Abstract

***Background*: Bone morphogenetic protein-2 (BMP-2) stimulates osteoblast differentiation, but inhibits myogenic differentiation in C2C12 myoblasts. BMP-2 induces transcription of *Id1*, an inhibitor for myogenesis, within 1 h in the cells. To examine the molecular mechanism of the action of BMP-2, we analysed a BMP-2-responsive element (BRE) in the 5′ flanking region of the human *Id1* gene.**

***Results*: A GC-rich region between −985 bp and −957 bp of the human *Id1* gene was identified as a BRE. The BRE containing promoter activity was stimulated by BMP-2 or by constitutively active BMP receptors (BMPR-IA and BMPR-IB). The stimulation was blocked by co-transfecting with dominant negative BMPR-IA or Smad7. A unique DNA–protein complex was induced in response to BMP-2 on the BRE. The complex induced by BMP-2 contained Smad1 and Smad4, possibly as a complex of both Smads. BMP-2 failed to stimulate the expression of *Id1* mRNA in Smad4-deficient cells. Over-expression of Smad4, but not Smad1, stimulated the Id1 reporter activity and the expression of endogenous *Id1* mRNA in Smad4-deficient cells.**

***Conclusion*: Signalling of BMP-2 to stimulate the expression of *Id1* would be transduced by BMPR-IA and mediated by Smad1 and Smad4, both of which form a complex on the 29 bp GC-rich element.**

Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, control the proliferation, differentiation and apoptosis of various types of cells (for reviews, see Kingsley 1994; Hogan 1996; Wozney & Rosen 1998; Reddi 2001). BMPs were originally identified as an activity that induces ectopic bone formation when it is implanted into muscular tissue (Urist 1965). BMPs are the active proteins responsible for ectopic bone formation (Wozney *et al.* 1988; Luyten *et al.* 1989; Celeste *et al.* 1990; Sampath *et al.* 1990). Skeletal abnormalities are found in animals and patients with mutations in BMP genes, confirming the physiological importance of BMPs in skeletal development in vertebrates (Kingsley *et al.* 1992; Storm *et al.* 1994; Thomas *et al.* 1996, 1997). Decapentaplegic (Dpp), a BMP homologue in *Drosophila*, also functions as a key morphogen in fly development (for review, see Raftery & Sutherland 1999). Several recombinant BMP proteins have been shown to induce not only ectopic bone formation *in vivo* but also osteoblast differentiation of mesenchymal cells *in vitro* (Wozney *et al.* 1988; Katagiri *et al.* 1990; Sampath *et al.* 1990; Yamaguchi *et al.* 1991; Sampath *et al.* 1992; Asahina *et al.* 1993; Katagiri *et al.* 1994).

Signalling by TGF-β superfamily members including BMPs is initiated following their binding to their respective membrane receptors. Two types of serine/threonine kinase receptors, types I and II, are required for the signal transduction (for reviews, see Sakou 1998; Kawabata *et al.* 1998; Miyazono 1999; Massague 2000; Massague & Chen 2000; Wrana 2000). Among them, BMPR-IA, BMPR-IB and ALK2 are classified as the type I receptors, and BMPR-II, ActR-II and ActR-IIB are the type II receptors. The kinase activity of the ligand-bound type II receptor phosphorylates the type I receptor to activate its

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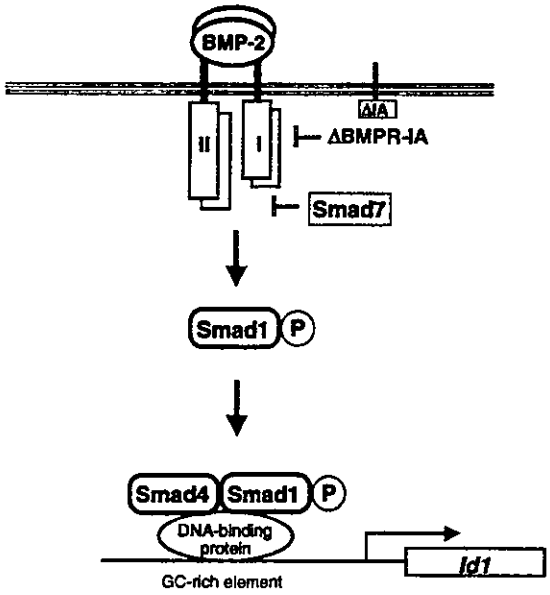

Summary Figure

kinase activity (Wrana *et al.* 1994). Over-expression of the kinase domain-truncated BMP type I receptors blocks the signal transduction induced by BMPs (Maeno *et al.* 1994; Suzuki *et al.* 1994; Namiki *et al.* 1997). In contrast, substitution mutations of the BMP type I receptors at the conserved residue in the GS domain activate the downstream signal transduction without ligand-binding or phosphorylation by the type II receptors (Wieser *et al.* 1995; Akiyama *et al.* 1997; Fujii *et al.* 1999). The activated type I receptors phosphorylate Smad proteins as substrates in the cytoplasm. The type I receptors also activate a signalling pathway through mitogen activated protein (MAP) kinases (Yamaguchi *et al.* 1995).

In the intracellular signalling activated by the type I receptors of BMPs, Smad proteins have been found to play critical roles in osteoblast differentiation induced by BMPs (Yamamoto *et al.* 1997; Nishimura *et al.* 1998; Fujii *et al.* 1999). Eight Smad proteins have been identified in mammals. These Smads are classified into three subgroups: R-Smads, Co-Smads and I-Smads, according to their structure and function (for reviews, see Kawabata *et al.* 1998; Sakou 1998; Miyazono 1999; Massague 2000; Massague & Chen 2000; Wrana 2000; Shi 2001). The R-Smads consist of Smad1, Smad2, Smad3, Smad5 and Smad8. They are directly phosphorylated at the carboxy terminal by the type I receptors. BMP receptors phosphorylate Smad1, Smad5 and Smad8, while the TGF-β and activin receptors phosphorylate Smad2 and Smad3. Thus, the differences in biological activities between BMPs and TGF-β/activin appear to be due to the different usage of the specific R-Smads by the type I receptors. The phosphorylated R-Smads form complexes with Co-Smad, Smad4, then move into the nucleus, bind to the regulatory regions of the target genes, and control their expression. In contrast, I-Smads such as Smad6 and Smad7 inhibit signal transduction of the TGF-β superfamily members by stably interacting with the type I receptors to block phosphorylation of R-Smads (Hayashi *et al.* 1997; Imamura *et al.* 1997; Nakao *et al.* 1997).

To examine the molecular mechanism of ectopic bone formation induced by BMPs, we established an *in vitro* model system using C2C12 myoblasts (Katagiri *et al.* 1994). BMPs inhibit the myogenic differentiation of C2C12 cells, and convert their differentiation pathway into that of osteoblast lineage cells (Katagiri *et al.* 1994). TGF-β1 also inhibits myogenic differentiation, but fails to induce the osteoblast differentiation of C2C12 cells (Katagiri *et al.* 1994). C2C12 cells express BMPR-IA, but no detectable BMPR-IB (Akiyama *et al.* 1997; Namiki *et al.* 1997). Over-expression of the constitutively active (ca)BMPR-IA or caBMPR-IB in C2C12 cells mimicked the effects of the treatment with BMPs (Akiyama *et al.* 1997; Fujii *et al.* 1999). In contrast, over-expression of the kinase domain-truncated BMPR-IA, but not BMPR-IB, blocked the BMP-2 response in a dominant negative fashion in C2C12 cells (Namiki *et al.* 1997). Other studies also reported that the intracellular signalling of BMPs is mainly mediated by the BMP-specific R-Smads such as Smad1 and Smad5, since the over-expression of Smad1 or Smad5 in C2C12 cells induced osteoblast differentiation and inhibited myogenic differentiation (Yamamoto *et al.* 1997; Nishimura *et al.* 1998; Fujii *et al.* 1999). However, the direct target genes of Smad proteins, which are involved in the conversion of the differentiation pathway of C2C12 cells by BMPs, are still not known.

Myogenic differentiation is controlled by the MyoD family transcription factors that have a conserved basic helix-loop-helix (bHLH) structure (for reviews, see Tapscott & Weintraub 1990; Ludolph & Konieczny 1995; Molkentin & Olson 1996; Arnold & Winter 1998; Perry & Rudnicki 2000; Buckingham 2001). The Id family proteins are capable of forming heterodimers with other bHLH proteins, such as MyoD family proteins, through their HLH domains (Benezra *et al.* 1990). However, the Id proteins lack the DNA-binding activity, because they do not have the basic region, an essential domain for the DNA binding (Benezra *et al.* 1990). Thus it is reasonable that the HLH heterodimers formed with the Id proteins inhibited the transcriptional activity in a dominant-negative mode. Indeed, myogenic differentiation was suppressed when Id1 was over-expressed in

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C2C12 cells (Benezra *et al.* 1990). We previously reported that the expression of *Id1* was stimulated in C2C12 cells within 1 h after treatment with BMP-2 (Katagiri *et al.* 1994). Recently, *Id1* was identified as a typical early responsive gene for BMP treatment in various types of cells in mice and humans (Hollnagel *et al.* 1999; Clement *et al.* 2000). These findings suggest that *Id1* could be a direct target gene in the intracellular signalling pathway of BMPs.

In the present study we characterized and identified a 29 bp GC-rich element as a BMP responsive element (BRE) in the 5′ region of the human *Id1* gene. Electrophoresis mobility shift assay (EMSA) showed that this element was recognized by Smad1 and Smad4 in response to BMP-2. BMP-2 failed to stimulate *Id1* expression in Smad4-deficient cells, but the transient over-expression of Smad4 rescued the BMP-2 response in these cells. These results suggest that BMP-2 induced *Id1* expression is mediated by the binding of Smad1 and Smad4 to the BRE in the *Id1* gene.

Results

Identification of a 29 bp region in the Id1 gene as a BMP-responsive element

To identify a BMP-responsive element (BRE) in the human *Id1* gene, we constructed a series of luc reporter plasmids from Id2.1-luc, which carries a 2.1 kb fragment of the 5′ flanking region of the human Id-1 gene (Fig. 1A). Although Id2.1-luc, Id1.3-luc, Id1.2-luc and Id1.0-luc responded similarly to BMP-2 in C2C12 cells, Id0.8-luc completely lost the ability to respond to BMP-2 (Fig. 1A). The region between −1.0 kb to −0.8 kb

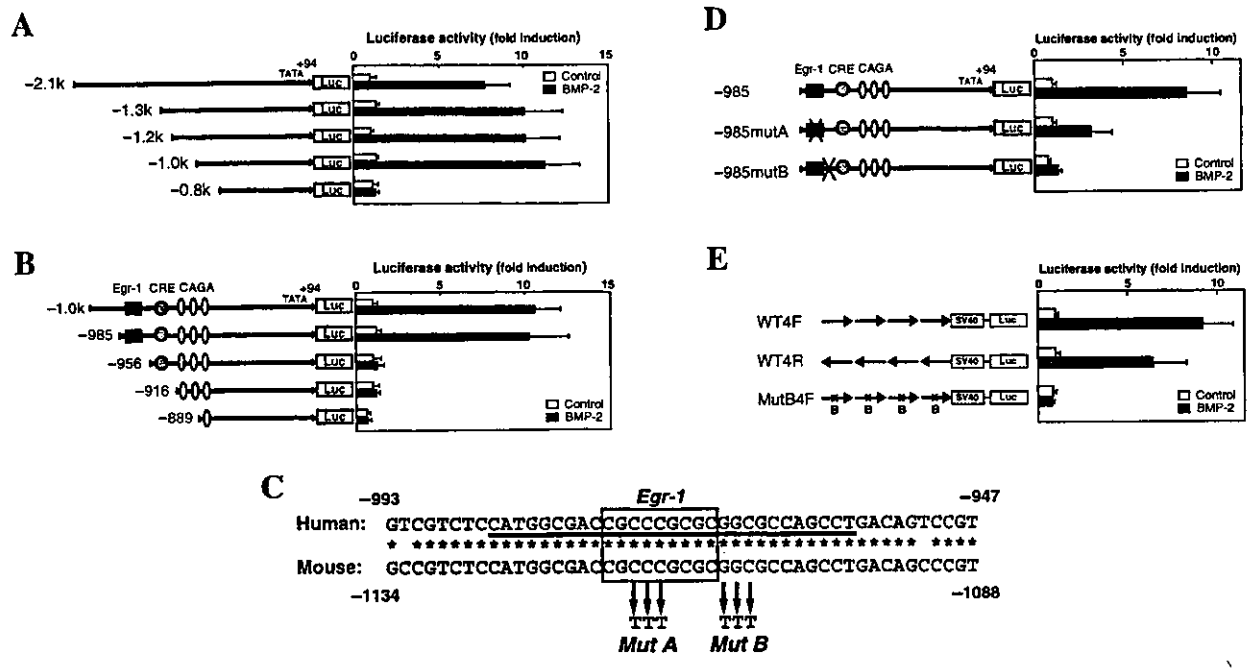

Figure 1 Identification of a BRE in the human *Id1* gene. (A) Deletion analysis of the 5′ region of the human *Id1* gene in luciferase reporter constructs. Deletion constructs were generated from Id2.1-luc. C2C12 cells were transfected with one of the reporter constructs and treated overnight with or without BMP-2. (B) Identification of BRE between −985 bp and −957 bp of the human *Id1* gene. Constructs generated by PCR using Id2.1-luc as a template were transfected in C2C12 cells, and their response to BMP-2 was then examined. Consensus elements for Egr-1 (Egr-1), CREB (CRE) and Smads (CAGA) are indicated. (C) Comparison of the human sequence around the BRE, with the corresponding region of the mouse gene. The BRE identified in the human *Id1* gene is underlined. Identical nucleotides between the human and mouse *Id1* genes are indicated by asterisks. Positions of substitutional mutations used in this study are shown as MutA and MutB. The consensus sequence of Egr-1 is boxed. (D) MutB sufficiently blocked the response to BMP-2 in Id985-luc reporter. Mutations mutA and mutB were introduced in Id985-luc to generate Id985mutA-luc and Id985mutB-luc, respectively. The luciferase activities were determined in C2C12 cells in the presence and absence of BMP-2. (E) Characterization of the GC-rich 29 bp between −985 bp and −957 bp of the human *Id1* gene as a BMP-responsive enhancer. Reporter constructs carrying four copies of the 29 bp element with wild-type or mutB sequence in forward (IdWT4F-luc and IdmutB4F-luc) or reverse orientation (IdWT4R-luc) were transfected in C2C12 cells, and luciferase activity was determined in the presence or absence of BMP-2. Data are means ± s.d. (n = 3).

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contained several potential regulatory elements which may be recognized by transcription factors such as Egr-1, CREB and Smads (Fig. 1B). We further generated an additional series of deletion plasmids and examined their responsiveness to BMP-2. Although Id985-luc responded to BMP-2 similarly to Id1.0-luc, neither Id956-luc, Id916-luc, nor Id889-luc responded to BMP-2 (Fig. 1B). The 29 bp region between −985 and −957 of the human *Id1* gene showed 100% homology with the corresponding region of the mouse *Id1* gene (Fig. 1C). This region contains a consensus sequence of Egr-1 which has been identified as a responsive element for the expression of *Id1* in response to serum (Tournay & Benezra 1996). To clarify whether Egr-1 is involved in the *Id1* expression induced by BMP-2 as an enhancer, we constructed Id985mutA-luc and Id985mutB-luc, in which substitutional mutations had been introduced at the inside and the outside of the Egr-1 consensus, respectively (Fig. 1C,D). Id985mutA-luc partially responded to BMP-2, but Id985mutB-luc almost completely lost the ability to respond to BMP-2 (Fig. 1D). To further characterize the ability of the 29 bp region to respond to BMP-2, we put four copies of the 29 bp fragment with the wild-type or mutB sequence in tandem in the forward (IdWT4F-luc and IdmutB4F-luc) or reverse orientation (IdWT4R-luc) in front of the SV40 promoter (Fig. 1E). Both IdWT4F-luc and IdWT4R-luc were activated in response to BMP-2, but IdmutB4F-luc was not (Fig. 1E).

BMPR-IA transduces intracellular signals to activate *Id1* gene expression in C2C12 cells

Using Id1.0-luc, we examined signalling pathways to activate *Id1* expression in response to BMP-2 in C2C12 cells. In agreement with our previous findings (Katagiri *et al.* 1994), TGF-β1 slightly suppressed the Id1.0-luc in C2C12 cells, whereas BMP-2 greatly stimulated it in them (Fig. 2A). Similar results were obtained in

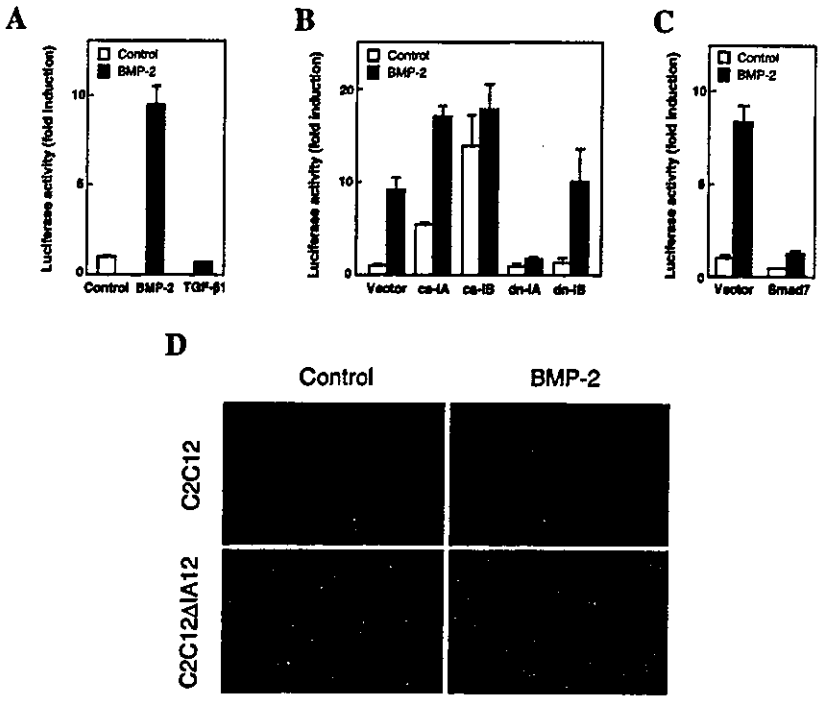

Figure 2 Characterization of signalling pathways to activate Id1 transcription. (A) BMP-2 specifically activates Id1.0-luc. C2C12 cells were transfected with Id1.0-luc, and then treated with BMP-2 or TGF-β1. Luciferase activity was determined after overnight incubation. Data are means ± s.d. (n = 3). (B) BMPR-IA mediates BMP-2 signals in C2C12 cells. C2C12 cells were co-transfected with Id1.0-luc and one of the constitutive active (c) or the kinase domain-truncated (dn) BMPR-IA (IA) or BMPR-IB (IB), and then incubated with or without BMP-2. After an overnight incubation, luciferase activity was determined. Data are means ± s.d. (n = 3). (C) Smad7 blocks the BMP-2-induced response of Id1.0-luc. C2C12 cells were co-transfected with Id1.0-luc and a Smad7 expression construct. After overnight treatment with BMP-2, the luciferase activity was determined. Data are means ± s.d. (n = 3). (D) Id985-EGFPd2 is activated by BMP-2 in C2C12 cells but not in C2C12 cells stably expressing a kinase domain-truncated BMPR-IA (C2C12ΔIA12). Id985-EGFPd2 was transfected in C2C12 and C2C12ΔIA12 cells, and then the cells were incubated for 4 h with and without BMP-2. Cells were monitored using a fluorescence microscope.

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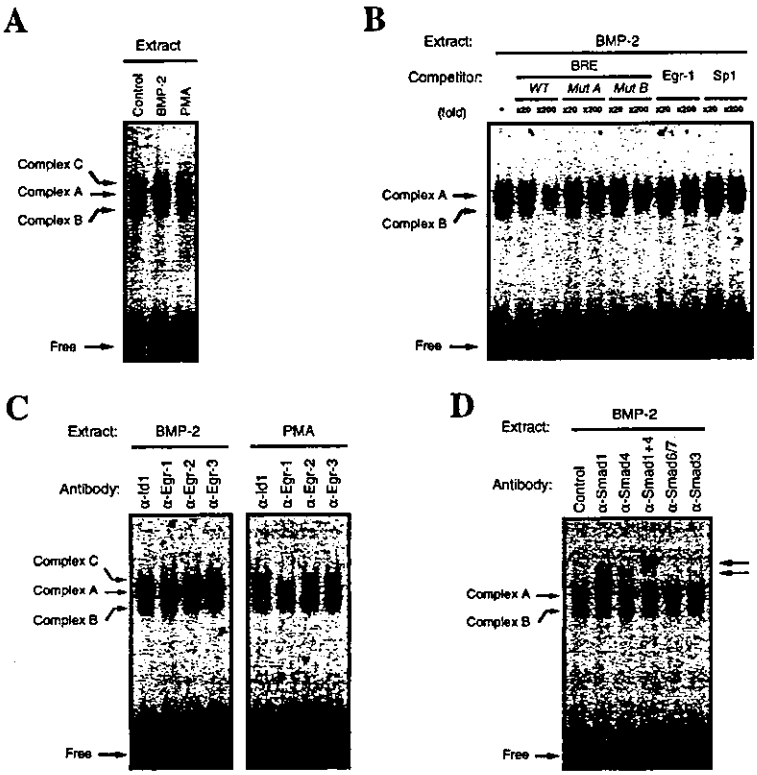

Figure 3 Characterization of transcription factors bound to the GC-rich 29 bp BRE of the Id1 gene in EMSA. (A) BMP-2 specifically induces Complex A. Nuclear extracts were prepared from C2C12 cells treated with and without BMP-2 or PMA. EMSA was performed using the BRE as a probe. (B) Competition analysis of Complex A induced by BMP-2. Various cold oligo nucleotides were added together with the BRE probe to the mixtures. (C) Complex C but not Complex A contains Egr-1. Antibodies against Egr-1, Egr-2, Egr-3 and Id1 (unrelated control) were added to the nuclear extracts prepared from C2C12 cells treated with BMP-2. (D) Complex A contains both Smad1 and Smad4. Antibodies against Smad1, Smad3, Smad4 or Smad6/7 were added to the nuclear extracts prepared from C2C12 cells treated with BMP-2. Smad1+4; Antibodies for Smad1 and Smad4 were added simultaneously to the mixture.

C3H10T1/2 cells (data not shown). When Id1.0-luc was co-transfected with the constitutively active BMP type I receptors, caBMPR-IA or caBMPR-IB, both receptors significantly stimulated Id1.0-luc, even in the absence of BMP-2 in C2C12 cells (Fig. 2B). The stimulatory activity of caBMPR-IB appeared slightly higher than that of caBMPR-IA. In contrast, the activation of Id1.0-luc by BMP-2 was blocked by co-transfecting with the dominant negative BMP receptor, dnBMPR-IA, but not with dnBMPR-IB (Fig. 2B). Co-transfection with Smad7, which blocks Smad-dependent signalling pathways, also blocked the activation of Id1.0-luc by BMP-2 (Fig. 2C). To visualize the induction of *Id1* in living cells, we constructed Id985-EGFPd2 by replacing the coding sequence of luciferase in Id985-luc with an *EGFPd2* cDNA. We have reported that the responsiveness to BMP-2 in C2C12 cells was blocked by overexpressing dnBMPR-IA, but not dnBMPR-IB (Namiki *et al.* 1997). The subclonal cell line C2C12ΔIA12, was established from the C2C12 cultures stably transfected with the dnBMPR-IA (Namiki *et al.* 1997). When C2C12 and C2C12ΔIA12 cells were transfected with Id985-EGFPd2 and treated with BMP-2, an increased number of EGFP-positive cells appeared in the C2C12 cell cultures within 4 h, but not in the C2C12ΔIA12 cell cultures (Fig. 2D). BMP-2 failed to increase the number of the EGFP-positive cells in C2C12 cultures transfected with Id985mutB-EGFPd2 (data not shown).

Smad1 and Smad4 bind to the 29 bp BRE of the *Id1* gene

Next, we characterized transcription factors bound to the 29 bp BRE of the *Id1* gene in EMSA. BMP-2 induced DNA-binding protein(s) in nuclear extracts of C2C12 cells within 1 h, which formed Complex A with the 29 bp BRE probe (Fig. 3A). In contrast, PMA, a potent stimulator of Egr-1, induced Complex C, a slower migrating DNA-protein, but not Complex A (Fig. 3A). In competition experiments, a 20-fold excess amount of cold oligo DNA with the wild-type sequence partially competed with Complex A induced by BMP-2, and a 200-fold excess almost completely competed with it (Fig. 3B). MutA oligo also partially competed with Complex A at a 200-fold excess, but neither mutB, Egr-1, nor Sp1 consensus oligo DNA competed with Complex A (Fig. 3B). Complex B was a sequence with nonspecific binding, since it was competed by all of the cold oligo DNAs examined. The addition of anti-Egr-1 antibody to the reaction mixtures specifically abolished Complex C induced by PMA, but not Complex A induced by BMP-2 (Fig. 3C). In contrast, antibodies

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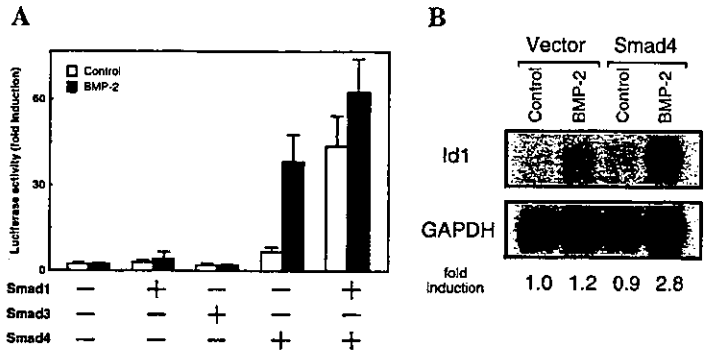

Figure 4 Smad4 is required for the expression of *Id1* in response to BMP-2 in MDA-MB468 cells. (A) Transient transfection with Smad4, but not Smad1 or Smad3, stimulates the expression of *Id1* in response to BMP-2 in MDA-MB468 cells. Co-transfection with Smad1 and Smad4 stimulates the expression of *Id1*, even in the absence of BMP-2 in MDA-MB468 cells. Cells were transfected with a fixed amount of DNA with and without Smad1, Smad3 or Smad4, and then incubated in the presence or absence of BMP-2. Their luciferase activities were determined after overnight incubation. Data are means + s.d. ($n = 3$). (B) Transient over-expression of Smad4 in MDA-MB468 cells induces the expression of endogenous *Id1* mRNA in response to BMP-2. Cells were transfected with Smad4 and then treated with BMP-2 for 1 h. Expression of *Id1* and *GAPDH* mRNA was determined by Northern blotting. Expression levels of *Id1* were normalized to the levels of *GAPDH*.

against Egr-2, Egr-3, Sp1 or p300 affected neither the amounts nor mobility of Complexes A and C in EMSA (Fig. 3C and data not shown). In contrast, addition of anti-Smad1 or anti-Smad4 antibody to the BMP-2-treated nuclear extracts reduced the amount of Complex A and induced supershifted bands (Fig. 3D). Moreover, when both the anti-Smad1 and anti-Smad4 antibodies were added together, a slower migrating double supershifted band appeared (Fig. 3D). In this condition, no other supershifted bands appeared. The addition of anti-Smad6/7 or anti-Smad3 antibodies did not induce a supershift of Complex A (Fig. 3D).

A Smad4-dependent signalling controls the expression of *Id1* induced by BMP-2

We further examined the role of Smads in the expression of *Id1* induced by BMP-2 using human MDA-MB468 breast cancer cells, in which the Smad4 locus has been deleted (Schutte *et al.* 1996). BMP-2 failed to activate IdWT4F-luc even in MDA-MB468 cells after co-transfecting with Smad1 or Smad3 (Fig. 4A). However, co-transfection with Smad4 stimulated both basal and BMP-2-induced luc activities in these cells (Fig. 4A). Moreover, simultaneous co-transfection with Smad1 and Smad4 markedly enhanced the luc activity of IdWT4F-luc, even in the absence of BMP-2 in MDA-MB468 cells (Fig. 4A). Similar results were obtained when Id1.0-luc was used in place of IdWT4F-luc (data not shown).

Furthermore, we examined the expression of the endogenous *Id1* mRNA induced by BMP-2 in MDA-MB468 cells. BMP-2 failed to induce the expression of *Id1* mRNA in MDA-MB468 cells (Fig. 4B). However, transient over-expression of Smad4 in these cells induced the expression of endogenous *Id1* mRNA in response to BMP-2 (Fig. 4B).

Effects of specific inhibitors for MAPK, PI3K and *de novo* protein synthesis on the Id1 expression induced by BMP-2

Recently, it was reported that BMP-2 activated not only Smad proteins but also mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) in some types of cells (Iwasaki *et al.* 1999; Vinals *et al.* 2002). The contribution of these signalling pathways to the *Id1* expression induced by BMP-2 was examined using specific inhibitors for the kinases; SB203580 for p38 MAPK, PD98059 for MEK, and wortmannin and LY294002 for PI3K. These inhibitors affected neither the both basal nor the BMP-2-induced levels of the *Id1* mRNA in Northern blot analysis (Fig. 5A). In contrast, when C2C12 cells were pre-treated with cycloheximide to block the *de novo* protein synthesis, the basal level of *Id1* mRNA was increased to a similar level induced by BMP-2, but no further stimulation of *Id1* mRNA was observed, even in the presence of BMP-2 (Fig. 5B).

Discussion

In the present study, a responsive element for BMP-2 was identified in the human *Id1* gene. We previously

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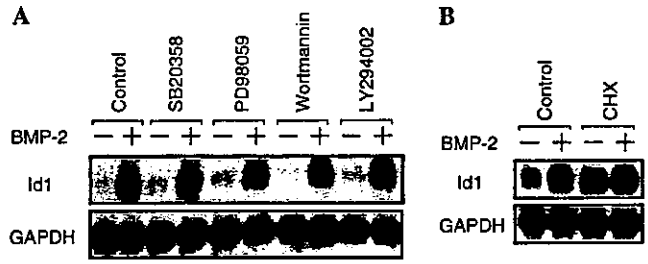

Figure 5 Effects of specific inhibitors for MAPK, PI3K and *de novo* protein synthesis on the levels of Id1 mRNA induced by BMP-2. C2C12 cells were pre-treated for 1 h with and without 10 μM SB203580, 50 μM PD98059, 0.1 μM wortmannin, or 20 μM LY294002 (A), and 10 μg/mL of cycloheximide (CHX) (B). The cells were then cultured for an additional 1 h with fresh media containing each inhibitor in the presence or absence of 300 ng/mL of BMP-2. Expression of *Id1* and *GAPDH* mRNAs was determined by Northern blotting using 20 μg of total RNA.

reported that BMP-2 induced the expression of *Id1* mRNA within 1 h after BMP-2 treatment in C2C12 cells (Katagiri *et al.* 1994). Other studies also identified *Id1* as an early responsive gene induced by BMP-2 in various types of mouse and human cells (Hollnagel *et al.* 1999; Clement *et al.* 2000). To elucidate the molecular mechanisms underlying BMP-2-induced *Id1* expression, we cloned and analysed the 5′ region of the human *Id1* gene. We found that a GC-rich region between −985 bp and −957 bp of the human *Id1* gene was the responsive element to BMP-2. This 29 bp element showed 100% homology between human and mouse *Id1* genes, suggesting that this element has a critical role in the expression of *Id1* in mammals. We previously reported that the over-expression of either BMPR-IA or BMPR-IB with the constitutively active mutation induced the osteoblast differentiation in C2C12 cells (Akiyama *et al.* 1997). In contrast, the only BMPR-IA lacking a kinase domain inhibited the responsiveness to BMP-2 in C2C12 cells (Namiki *et al.* 1997). In agreement with our previous findings, the stimulation of the Id1 reporter activity by BMP-2 was blocked by dnBMPR-IA, but not by dnBMPR-IB. These results suggest that BMPR-IA has a much higher affinity for BMP-2 than BMPR-IB when these receptors are expressed on the surface of C2C12 cells. This possibility is supported by the findings of Ebisawa *et al.* (1999), who demonstrated, using affinity cross-linking followed by the immunoprecipitation, that BMP-2 only binds to BMPR-IA on the surface of C2C12 cells.

In EMSA, using the 29 bp region in the *Id1* gene as the BRE probe, we identified both Smad1 and Smad4 in a DNA-protein complex induced by BMP-2. Interestingly, when antibodies against Smad1 and Smad4 were added simultaneously to the complex, a double supershifted band was observed. In this condition, however, we could not see any other supershifted bands corresponding to that observed by adding each individual antibody against Smad1 or Smad4. These results suggest that Smad1 and Smad4 bind to the element together, possibly as a complex, rather than individually. Transient over-expression of Smad4 in Smad4-deficient MDA-MB468 cells recovered the expression of not only the reporter gene but also endogenous *Id1* mRNA in the presence of BMP-2. Taken together, these results suggest that a complex of Smad1 and Smad4 mediates the transcription of *Id1* in response to BMP-2 by binding to the GC-rich 29 bp element. The Id1 protein induced by Smad1/Smad4 would be involved, at least in part, in the BMP-2-dependent conversion of the differentiation pathway of C2C12 myoblasts into that of osteoblast lineage cells. This possibility is under investigation in our laboratories.

It has been reported that BMP-2 activates not only Smad proteins but also MAPK and PI3K to induce the respective intracellular signals (Iwasaki *et al.* 1999; Vinals *et al.* 2002). However, none of the specific inhibitors for MAPK or PI3K used in this study showed significant effects on the *Id1* mRNA expression in C2C12 cells in the presence or absence of BMP-2. In addition, these inhibitors showed only small effects on the differentiation of C2C12 cells into mature muscle cells and osteoblasts in the absence and presence of BMP-2, respectively (data not shown). Although we cannot rule out the possibility that MAPK and/or PI3K are involved in the effects of BMP-2, the contribution of these signalling pathways would be minor at least in the expression of *Id1* in C2C12 cells.

Smad proteins are capable of binding to several different DNA sequences *in vitro*. It has been reported that Mad, a *Drosophila* homologue of mammalian Smad1, binds to a GC-rich sequence of the target genes in

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Drosophila (Kim *et al.* 1997; Xu *et al.* 1998). Recently, a similar GC-rich sequence was identified in a regulatory region of the mouse Smad6 gene, and this element could be recognized by the BMP-specific Smads such as Smad1 and Smad5 (Ishida *et al.* 2000). It was reported that the MH1 domains of Smads also bind to the CAGA sequence (Dennler *et al.* 1998; Jonk *et al.* 1998; Shi *et al.* 1998). However, this sequence appears to have a lower ligand specificity, since it was recognized by both BMP- and TGF-β/activin-specific Smads, together with Smad4. Indeed, there were three copies of the CAGA sequence within the BMP-responsive region between −1.0 kb and −0.8 kb of the human *Id1* gene, but these sequences were not necessary for the BMP-2-induced *Id1* expression. Moreover, the treatment of C2C12 cells with TGF-β1 or the transient over-expression of Smad3 did not activate expression of *Id1*. In contrast, the GC-rich element between −985 bp and −957 bp of the human *Id1* gene was critical and sufficient for the response to BMP-2. This element was recognized by Smad1 and Smad4, but not Smad3. Taken together, it can be concluded that this GC-rich element, rather than the CAGA sequence, behaves as the BRE in the *Id1* gene. Further studies are necessary to determine the exact binding sequence of Smad1 and Smad4 to form a DNA-protein complex.

It was reported that the *Id1* expression induced by serum is mediated by the binding of Egr-1 to a consensus sequence within the GC-rich region of the 29 bp BRE (Tournay & Benezra 1996). In contrast to serum, BMP-2 induced Complex A rather than Complex C in the EMSA containing Smad1/Smad4 and Egr-1, respectively. Mutational analysis also showed that a 3′ portion of the Egr-1 consensus sequence in the 29 bp element is more critical than the consensus itself in the expression of *Id1* induced by BMP-2. Moreover, both competition and supershifting experiments in EMSA showed that the DNA-protein complex induced by BMP-2 contained no detectable Egr-1 protein. In our preliminary experiments, PMA, a potent inducer of Egr-1 in various types of cells, induced Egr-1, but failed to stimulate the *Id1* mRNA expression in C2C12 cells. These results suggest that Egr-1 is not involved in the expression of *Id1* in response to BMP-2.

Pre-treatment of C2C12 cells with cycloheximide increased the basal levels of Id1 mRNA, but no further stimulation was observed, even in the presence of BMP-2. In addition, DNA-protein complexes were not detected in EMSA, when bacterially expressed full length Smad1 and Smad4 were incubated with the 29 bp BRE as a probe (data not shown). These results suggest that, although a Smad protein itself binds to a specific DNA sequence with a low affinity, it forms a transcriptional complex with other DNA-binding protein(s) to enhance the ability to regulate the target gene expression. The DNA-binding proteins may have a short half life and be continuously degraded through the *de novo* protein synthesis pathway as a rate-limiting transcriptional factor in the expression of Id1 mRNA. It has also been suggested that the role of Smad proteins in Id1 expression is to form a complex with such an unstable transcriptional factor to stabilize them on the promoter. A number of transcription factors have been identified as partners for Smads. However, transcription factors, which are associated with Smad1 and Smad4 to enhance the expression of *Id1* in response to BMP-2, are not known at present. Although P300/CBP was reported to stimulate the transcriptional activity of Smads (Feng *et al.* 1998; Janknecht *et al.* 1998; Nishihara *et al.* 1998; Pouponnot *et al.* 1998; Topper *et al.* 1998), we failed to detect them in a supershift experiment in EMSA. OAZ, a zinc finger protein, was reported as a DNA-binding factor that is associated with Smad1 and Smad4 in response to BMP-2 (Hata *et al.* 2000). The transient over-expression of OAZ stimulated the *Xvent-2* reporter expression in mammalian cells including C2C12 and C3H10T1/2 cell lines (Hata *et al.* 2000). However, OAZ does not appear to be involved in the expression of *Id1* in response to BMP-2, since OAZ is undetectable in C2C12 cells in their basal condition (Hata *et al.* 2000). Further studies are necessary to characterize the transcription factors in the DNA-protein complex induced by BMP-2.

While we were preparing this manuscript, Lopez-Rovira *et al.* (2002) and Korchynski & ten Dijke (2002) independently reported a BMP-2 responsive element and a BMP-6 responsive element, respectively, in the *Id1* gene. In agreement with our findings, they showed the importance of the GC-rich element overlapping with the BRE—which was identified in the present study—for the BMP-dependent stimulation of the *Id1* gene through Smad1/Smad5 and Smad4. Although the CAGA sequences were not essential for the BMP response in our study, they reported that these elements were also required for the BMP response (Korchynski & ten Dijke 2002; Lopez-Rovira *et al.* 2002). This discrepancy has to be clarified by future experiments. Competitive displacement experiments in EMSA also suggested that Smad1 and Smad4 bind to the GC-rich region (Korchynski & ten Dijke 2002; Lopez-Rovira *et al.* 2002). We were able to localize the binding region of the Smad1 and Smad4 complex into a 29-bp GC-rich region in EMSA. Furthermore, the present report shows for the first time that the transient over-expression of

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Smad4 can rescue endogenous *Id1* expression in response to BMP-2 in Smad4-deficient cells.

In conclusion, we have identified a 29-bp BRE from the human *Id1* gene. This GC-rich element was recognized by Smad1 and Smad4, and it was sufficient for the response to BMP-2. We also successfully generated an EGFP reporter construct driven under the control of the BRE to visualize the BMP-dependent signalling in living cells. The BRE sequence, as well as the reporter constructs characterized in this study, would be useful for an understanding of the molecular mechanism of BMP-dependent gene expression.

Experimental procedures

Construction of reporter plasmids

To construct a luciferase reporter plasmid, a 2.1-kb fragment of the 5′ region of the human *Id1* gene was cloned by a standard PCR method using Platinum *Pfx* DNA polymerase (Invitrogen, Groningen, the Netherlands) with human genomic DNA as a template. It was then subcloned between the *Xho*I and *Hin*dIII (Takara Shuzo Co., Shiga, Japan) recognition sites of pGL3-Basic (Promega, Madison, WI, USA). The primer sequences were as follows; 5′-ttctcgagTGAAAAAGATACAGAAGTTGA-3′ (hId1–5Xho) and 5′-ttaagcttTCTTGGCGACTGGCTGAAACA-3′ (hId1–3Hd) (lower cases indicate flanking sequences). Id1.3-luc, Id1.2-luc, Id1.0-luc and Id0.8-luc were made by digesting Id2.1-luc at the *Eco*RI, *Stu*I, *Pst*I and *Pvu*II sites, respectively. Id985-luc, Id956-luc, Id916-luc, Id889-luc, Id985mutA-luc and Id985mutB-luc were generated by PCR using each specific upper primer and the hId1–3Hd primer with Id2.1-luc as a template. The primer sequences were as follows; 5′-ttctcgagCATGGCGACCGCCCGCGCGG-3′ (Id985), 5′-ttctcgagGACAGTCCGTCCGGGTTTTA-3′ (Id956), 5′-ttctcgagCCTGGCGTCTAACGGTCTGA-3′ (Id916), 5′-ttctcgagGTTCAGACGCTGACACAGAC-3′ (Id889), 5′-ttctcgagCATGGCGACCGTTTGCGCGG-3′ (Id985mutA), and 5′-ttctcgagCATGGCGACCGCCCGCGCTTTGCCA-3′ (Id985mutB) (lower case and underline indicate flanking sequences and mutations, respectively). To generate IdWT4F-luc, IdWT4R-luc and IdmutB4F-luc, the specific oligo DNAs of the 29 bp BRE with the wild-type or the mutB sequence flanked by six nucleotides were annealed, digested with *Xho*I and *Sal*I, purified from agarose gels, and subcloned into pGL3-Promoter (Promega). The sequences of the oligo DNAs were as follows; 5′-tcgagCATGGCGACCGCCCGCGCGGCGCCAGCCTg-3′ (IdWT-S), 5′-tcgacAGGCTGGCGCCGCGCGGGCGGTCGCCATGc-3′ (IdWT-AS), 5′-tcgagCATGGCGACCGCCCGCGCTTTGCCAGCCTg-3′ (IdmutB-S) and 5′-tcgacAGGCTGGCAAAGCGCGGGCGGTCGCCATGc-3′ (IdmutB-AS) (lower cases and underlines indicate flanking sequences and mutations, respectively). PCR products were confirmed by DNA sequencing using a GeneRapid DNA sequencer (Amersham Pharmacia Biotech, Buckinghamshire, UK). Id985-EGFPd2 and Id985mutB-EGFPd2 were generated by replacing the coding regions of luciferase with a destabilized *EGFP* cDNA of pd2EGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA) in Id985-luc and Id985mutB-luc, respectively.

Construction of Smad expression vectors

Complementary DNAs for the mouse Smad1, Smad3, Smad4 and Smad7 coding regions, except for the first Met residues, were obtained by an RT-PCR technique using Platinum *Pfx* DNA polymerase (Invitrogen) as previously described (Yanai *et al.* 2001). Total RNAs prepared from C2C12 myoblasts or mouse calvaria of the ddY strain were used for the RT-PCR. The primer sequences were as follows; 5′-ttaagcttAATGTGACCAGCTTGTTTTC-3′ (mS1–5Hd), 5′-tttctagaGACGGAAGCCACAGGTCTTT-3′ (mS1–3Xb), 5′-ttaagcttTCGTCCATCCTGCCCTTCAC-3′ (mS3–5Hd) and 5′-tttctagaCCCGCTCCCTTTACTCCTA-3′ (mS3–3Xb), 5′-aaaagcttGACAATATGTCTATAACAAA-3′ (mS4–5Hd), 5′-aatctagaAATGGTTAGGGCGTCCGTGG-3′ (mS4–3Xb), 5′-ttaagcttTTCAGGACCAAACGATCT-3′ (mS7–5Hd) and 5′-tttctagaTGTCCTCTTCTCCCCACC-3′ (mS7–3Xb) (lower cases indicate flanking sequences). PCR products were digested with *Hin*dIII and *Xba*I (Takara Shuzo Co.), purified from agarose gels, and subcloned into pFLAG-CMV-2 (Sigma-Aldrich, St Louis, MO, USA). Each cDNA was confirmed by DNA sequencing.

Cell culture, transfection and luciferase assay

Mouse C2C12 myoblasts and human MDA-MB468 breast cancer cells were obtained from the American Type Culture Collection, and mouse C3H10T1/2 (10T1/2) fibroblasts from the RIKEN Cell Bank (Tsukuba Science City, Ibaraki, Japan). C2C12 cells and its subclonal cell line, C2C12ΔIA12 cells, which are stably expressing a kinase domain-truncated BMPR-IA (Namiki *et al.* 1997), and 10T1/2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 15% foetal bovine serum (FBS) (Sigma Chemical Co.). MDA-MB468 cells were maintained in Leibovitz's L-15 medium containing 10% FBS. Cells were inoculated at 1.5×10^4 cells/well in 96-well plates with growth media 1 day before transfection by Lipofectamine or Lipofectamine 2000 (Invitrogen). For normalization, pRL-SV40 plasmid (Promega) was co-transfected with luc reporter plasmids. The amount of each plasmid used for the transfection was fixed at 200 ng/well by appropriate empty plasmids. After an overnight incubation, the culture media were replaced with fresh media containing 2.5% FBS in the presence or absence of 300 ng/mL of BMP-2 (provided by Yamanouchi Pharmaceuticals, Tokyo, Japan), and then cultured for an additional 24 h. Luciferase activities in the cell extracts were determined using a dual luciferase assay kit (Toyo Ink, Tokyo, Japan) using a luminometer.

Both Id985-EGFPd2 and Id985mutB-EGFPd2 were transfected in C2C12 and C2C12ΔIA12 cells as described above, in place of the luciferase reporter plasmids. One day after transfection, the cells were incubated with fresh media containing 2.5% FBS in the presence or absence of 300 ng/mL of BMP-2.

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Expression levels of EGFP in the cultures were monitored under a fluorescence microscope (Olympus, Tokyo, Japan).

Electrophoresis mobility shift assay

EMSA was performed essentially as previously described (Katagiri *et al.* 1997). C2C12 cells were incubated for 1 h, with or without 300 ng/mL of BMP-2 or 1 μM PMA, and then nuclear extracts were prepared from the cells by the method of Schreiber *et al.* 1989) with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The double strand BRE probe was labelled with T4 polynucleotide kinase and [γ^{32}P]ATP (New England Nuclear, Boston, MA) using a labelling kit (Takara Shuzo Co.). For the competition experiments, the cold double strand oligos were added to the mixtures with the labelled probe. The sequences of oligo DNAs we used were as follows; 5′-CATGGCGACCGCCCGCGCGGCGCCAGCCT-3′ (wild-type BRE), 5′-CATGGCGACCGTTTGCGCGGCGCCAGCCT-3′ (BRE mutA), 5′-CATGGCGACCGCCCGCGCTTTGCCAGCCT-3′ (BRE mutB), 5′-GGATCCAGGGGGGGCGAGCGGGGGCGA-3′ (Egr-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 5′-ATTCGATCGGGGCGGGGGCAGC-3′ (Sp1; Santa Cruz Biotechnology). Six micrograms of the nuclear extracts were incubated with a labelled BRE probe with the wild-type sequence. For the supershift experiments, antibodies were added prior to the addition of the probe, and incubated for 15 min at room temperature. Antibodies against Smad1 (T-20), Smad3 (I-20), Smad4 (B-8), Smad6/Smad7 (N-19), Id1 (C-20), Egr-1 (C-19), Egr-2 (C-14), Egr-3 (C-24) and p300 (N-15) were obtained from Santa Cruz Biotechnology. The reaction mixture was loaded on to 5% polyacrylamide gel in 0.5 ×TBE (44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA) and resolved by electrophoresis.

Northern blot analysis

Northern blot analysis was performed as previously described (Katagiri *et al.* 1994). In brief, 20 μg of total RNA prepared with Trizol (Invitrogen) was resolved by electrophoresis in a 1.2% agarose-formaldehyde gel, and transferred on to a Hybond-N membrane (Amersham International, Amersham, UK). The membrane was sequentially hybridized with [α^{32}P]-labelled cDNA probes for mouse *Id1* and *GAPDH*. All of the inhibitors for MAPK (SB303580 and PD98059) and PI3K (wortmannin and LY294002) were purchased from Calbiochem, Darmstadt, Germany.

Acknowledgements

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References

Akiyama, S., Katagiri, T., Namiki, M., *et al.* (1997) Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. *Exp. Cell Res.* **235**, 362–369.

Arnold, H.-H. & Winter, B. (1998) Muscle differentiation: more complexity to the network of myogenic regulators. *Curr. Opin. Genet. Dev.* **8**, 539–544.

Asahina, I., Sampath, T.K., Nishimura, I. & Hauschka, P.V. (1993) Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. *J. Cell Biol.* **123**, 921–933.

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L. & Weintraub, H. (1990) The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49–59.

Buckingham, M. (2001) Skeletal muscle formation in vertebrates. *Curr. Opin. Genet. Dev.* **11**, 440–448.

Celeste, A., Iannazzi, J.A., Taylor, R.C., *et al.* (1990) Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA* **87**, 9843–9847.

Clement, J.H., Marr, N., Meissner, A., *et al.* (2000) Bone morphogenetic protein 2 (BMP-2) induces sequential changes of Id gene expression in the breast cancer cell line MCF-7. *J. Cancer Res. Clin. Oncol.* **126**, 271–279.

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S. & Gauthier, J.-M. (1998) Direct binding of Smad3 and Smad4 to critical TGF β-inducible elements in the promoter of human plasminogen inhibitor-type 1 gene. *EMBO J.* **17**, 3091–3100.

Ebisawa, T., Tada, K., Kitajima, I., *et al.* (1999) Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. *J. Cell Sci.* **112**, 3519–3527.

Feng, X.-H., Zhang, Y., Wu, R.-Y. & Derynck, R. (1998) The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for Smad3 in TGF-β-induced transcriptional activation. *Genes Dev.* **12**, 2153–2163.

Fujii, M., Takeda, K., Imamura, T., *et al.* (1999) Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Mol. Biol. Cell* **10**, 3801–3813.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A. & Massague, J. (2000) OAZ. uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**, 229–240.

Hayashi, H., Abdollah, S., Qiu, Y., *et al.* (1997) The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Genes Dev.* **89**, 1165–1173.

Hogan, B.L.M. (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580–1594.

Hollnagel, A., Oehlmann, V., Heymer, J., Rüther, U. & Nordheim, A. (1999) *Id* genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. *J. Biol. Chem.* **274**, 19838–19845.

Imamura, T., Takase, M., Nishihara, A., *et al.* (1997) Smad6 inhibits signaling by the TGF-β superfamily. *Nature* **389**, 622–626.

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Ishida, W., Hamamoto, T., Kusanagi, K., *et al.* (2000) Smad6 is a Smad1/5-induced Smad inhibitor: characterization of bone morphogenetic protein-responsive element in the mouse *Smad6* promoter. *J. Biol. Chem.* **275**, 6075–6079.

Iwasaki, S., Iguchi, M., Watanabe, K., Hoshino, R., Tsujimoto, M. & Kohno, M. (1999) Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. *J. Biol. Chem.* **274**, 26503–26510.

Janknecht, R., Wells, N.J. & Hunter, T. (1998) TGF-β-stimulated cooperation of Smad proteins with the coactivators CBP/p300. *Genes Dev.* **12**, 2114–2119.

Jonk, L.J., Itoh, S., Heldin, C.-H., ten Dijke, P. & Kruijer, W. (1998) Identification and functional characterization of a Smad binding element (SBE) in the *JunB* promoter that acts as a transforming growth factor-b, activin, and bone morphogenetic protein-inducible enhancer. *J. Biol. Chem.* **273**, 21145–21152.

Katagiri, T., Akiyama, S., Namiki, M., *et al.* (1997) Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. *Exp. Cell Res.* **230**, 342–351.

Katagiri, T., Yamaguchi, A., Ikeda, T., *et al.* (1990) The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. *Biochem. Biophys. Res. Commun.* **172**, 295–299.

Katagiri, T., Yamaguchi, A., Komaki, M., *et al.* (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J. Cell Biol.* **127**, 1755–1766.

Kawabata, M., Imamura, T. & Miyazono, K. (1998) Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev.* **9**, 49–61.

Kim, J., Johnson, K., Chen, H.J., Carroll, S. & Laughton, A. (1997) *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. *Nature* **388**, 304–308.

Kingsley, D.M. (1994) The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8**, 133–146.

Kingsley, D.M., Bland, A.E., Grubber, J.M., *et al.* (1992) The mouse short ear skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF β superfamily. *Cell* **71**, 399–410.

Korchynski, O. & ten Dijke, P. (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific responses elements in the Id1 promoter. *J. Biol. Chem.* **277**, 4883–4891.

Lopez-Rovira, T., Chalaux, E., Massague, J., Rosa, J.L. & Ventura, F. (2002) Direct binding of Smad1 and Smad4 to two distinct motifs mediated bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J. Biol. Chem.* **277**, 3176–3185.

Ludolph, D.C. & Konieczny, S.F. (1995) Transcription factor families: muscling in on the myogenic program. *FASEB J.* **9**, 1595–1604.

Luyten, F.P., Cunningham, N.S., Ma, S., *et al.* (1989) Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J. Biol. Chem.* **264**, 13377–13380.

Maeno, M., Ong, R.C., Suzuki, A., Ueno, N. & Kung, H.-F. (1994) A truncated bone morphogenetic protein 4 receptor alters the fate of ventral mesoderm to dorsal mesoderm: roles of animal pole tissue in the development of ventral mesoderm. *Proc. Natl. Acad. Sci. USA* **91**, 10260–10264.

Massague, J. (2000) How cells read TGF-β signals. *Nat. Rev. Mol. Cell. Biol.* **1**, 169–178.

Massague, J. & Chen, Y.-G. (2000) Controlling TGF-β signaling. *Genes Dev.* **14**, 627–644.

Miyazono, K. (1999) Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins. *Bone* **25**, 91–93.

Molkentin, J.D. & Olson, E. (1996) Combinational control of muscle development by basic helix-loop-helix and MADS-box transcription factors. *Proc. Natl. Acad. Sci. USA* **93**, 9366–9373.

Nakao, A., Afrakhte, M., Moren, A., *et al.* (1997) Identification of Smad7, a TGF β-inducible antagonist of TGF-β signaling. *Nature* **389**, 631–635.

Namiki, M., Akiyama, S., Katagiri, T., *et al.* (1997) A kinase domain-truncated type I receptor blocks bone morphogenetic protein-2-induced signal transduction in C2C12 myoblasts. *J. Biol. Chem.* **272**, 22046–22052.

Nishihara, A., Hanai, J.I., Okamoto, N., *et al.* (1998) Role of p300, a transcriptional coactivator, in signaling of TGF-β. *Genes Cells* **3**, 613–623.

Nishimura, R., Kato, Y., Chen, D., Harris, S.E., Mundy, G.R. & Yoneda, T. (1998) Smad5 and DPC4 are key Molecules in mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12. *J. Biol. Chem.* **273**, 1872–1879.

Perry, R.L.S. & Rudnicki, M.A. (2000) Molecular mechanisms regulating myogenic determination and differentiation. *Front. Biosci.* **5**, d750–767.

Pouponnot, C., Jayaraman, L. & Massague, J. (1998) Physical and functional interaction of SMADs and p300/CBP. *J. Biol. Chem.* **273**, 22865–22868.

Raftery, L. & Sutherland, D.J. (1999) TGF-β family signal transduction in *Drosophila* development. *Dev. Biol.* **210**, 251–268.

Reddi, A.H. (2001) Bone morphogenetic proteins: from basic to clinical applications. *J. Bone Joint Surg. Am.* **83A**(Suppl. 1), S1–S6.

Sakou, T. (1998) Bone morphogenetic proteins; from basic studies to clinical approaches. *Bone* **22**, 591–603.

Sampath, T.K., Coughlin, J.E., Whetstone, R.M., *et al.* (1990) Bovine osteogenic protein is composed of dimmers of OP-1 and BMP-2A, two members of the transforming growth factor-β superfamily. *J. Biol. Chem.* **265**, 13198–13205.

Sampath, T.K., Maliakal, J.C., Hauschka, P.V., *et al.* (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J. Biol. Chem.* **267**, 20352–20362.

© Blackwell Science Limited

Schreiber, E., Matthias, P., Muller, M.M. & Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucl. Acids Res.* **17**, 6419.

Schutte, M., Hruban, R.H., Hedrick, L., *et al.* (1996) DPC4 gene in various tumor types. *Cancer Res.* **56**, 2527–2530.

Shi, Y. (2001) Structural insights on Smad function in TGF-β signaling. *Bioessay* **23**, 223–232.

Shi, Y., Wang, Y.-F., Jayaraman, L., Yang, H., Massague, J. & Pavletich, N. (1998) Crystal structure of a Smad1 MH1 domain bound to DNA: insights on DNA-binding in TGF-β signaling. *Cell* **94**, 585–594.

Storm, E.E., Huynh, T.V., Copeland, N.G., Jenkins, N.A., Kingsley, D.M. & Lee, S.J. (1994) Limb alterations in brachypodism mice due to mutations in a new member of the TGF β-superfamily. *Nature* **368**, 639–643.

Suzuki, A., Thies, R.S., Yamaji, N., *et al.* (1994) A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255–10259.

Tapscott, S.J. & Weintraub, H. (1990) MyoD and the regulation of myogenesis by helix-loop-helix proteins. *J. Clin. Inv.* **87**, 1133–1138.

Thomas, J.T., Kilpatrick, M.W., Lin, K., *et al.* (1997) Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1. *Nat. Genet.* **17**, 58–64.

Thomas, J.T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J. & Luyten, F.P. (1996) A human chondrodysplasia due to a mutation in a TGF-β superfamily member. *Nat. Genet.* **12**, 315–317.

Topper, J., DiChiara, M.R., Brown, J.D., *et al.* (1998) CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor β transcriptional responses in endothelial cells. *Proc. Natl. Acad. Sci. USA* **95**, 9506–9511.

Tournay, O. & Benezra, R. (1996) Transcription of the dominant-negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene Egr-1. *Mol. Cell. Biol.* **16**, 2418–2430.

Urist, M.R. (1965) Bone: formation by autoinduction. *Science* **150**, 893–899.

Vinals, F., Lopez-Rovira, T., Rosa, J.L. & Ventura, F. (2002) Inhibition of PI3K/p70 S6K and p38 MAPK cascades increases osteoblastic differentiation induced by BMP-2. *FEBS Lett.* **510**, 99–104.

Wieser, R., Wrana, J.L. & Massague, J. (1995) GS domain mutations that constitutively active TβR-I, the downstream signaling component in the TGF-β receptor complex. *EMBO J.* **14**, 2199–2208.

Wozney, J.M. & Rosen, V. (1998) Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. *Clin. Orthop.* **346**, 26–37.

Wozney, J.M., Rosen, V., Celeste, A.J., *et al.* (1988) Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528–1534.

Wrana, J. (2000) Regulation of Smad activity. *Cell* **100**, 189–192.

Wrana, J.L., Attisano, L., Weiser, R., Ventura, F. & Massague, J. (1994) Mechanism of activation of the TGF-β receptor. *Nature* **370**, 341–347.

Xu, X., Yin, Z., Hudson, J.B., Ferguson, E.L. & Frasch, M. (1998) Smad proteins act in combination with synergistic and antagonistic regulators to target DPP responses to the *Drosophila* mesoderm. *Genes Dev.* **12**, 2354–2370.

Yamaguchi, A., Katagiri, T., Ikeda, T., *et al.* (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. *J. Cell Biol.* **113**, 681–687.

Yamaguchi, K., Shirakabe, K., Shibuya, H., *et al.* (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. *Science* **270**, 2008–2011.

Yamamoto, N., Akiyama, S., Katagiri, T., Namiki, M., Kurokawa, T. & Suda, T. (1997) Smad1 and Smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. *Biochem. Biophys. Res. Commun.* **238**, 574–580.

Yanai, T., Katagiri, T., Akiyama, S., *et al.* (2001) Expression of mouse osteocalcin transcripts, OG1 and OG2, is differently regulated in bone tissue and osteoblast cultures. *J. Bone Miner. Metab.* **19**, 345–351.

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Sjögren's Syndrome
Yes Autoreactive Lymphocytes, Why? Virus or Gene?

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Sjögren's Syndrome
Yes Autoreactive Lymphocytes, Why? Virus or Gene?

Key words: clinical features, autoreactive T cells, autoantibody, retrovirus, Epstein-barr virus, microarry analyses, RT-PCR analyses

Sjögren's syndrome (SjS) (1) consists of dry mouth (xerostomia) and dry eyes (xerophthalmia, keratoconjunctivitis sicca). These clinical features of the eyes and mouth are called the sicca syndrome. The lack of secretions also involve the respiratory tract, vagina and skin. The syndrome occurs most commonly in middle-age women. Patients with only eye and oral involvement are classified as primary SjS and those with an associated rheumatic disorder as secondary SjS with rheumatic arthritis (RA) and systemic lupus erythematosus (SLE), polymyositis, scleroderma and periarteritis nodosa. The syndrome also occurs in some patients with chronic active hepatitis, primary biliary cirrhosis. Patients with SjS develop hematopoitic disorders such as pseudolymphoma, lymphosarcoma, giant follicular sarcoma and Waldenstrom's macroglobulinemia (2). Other clinical features of SjS include Raynaud's phenomenon, vasculitis, hypergammaglobulinemic purpura, hyperviscosity syndrome and peripheral neuropathy. Most of the peripheral neuropathy of SjS patients are sensory or autonomic dominant, however a few report of motor dominant neuropathy has been reported (3). Lymphocytic infiltrates involving the renal tubules cause renal tubular acidosis and interstitial pneumonia.

See also p 142.

SjS is characterized by the infiltration of the exocrine glands and other organs with lymphocytes, including CD4 positive αβT cells, and leading to destruction and glandular insufficiency (4). Furthermore SjS is an autoimmune disease characterized by autoantibodies. Rheumatoid factors are found in approximately half of primary SjS patients. It is well known that two antinuclear antibodies, anti SS-A, anti SS-B antibody are associated with SjS (5). Anti SS-A antibody is detected in about 70% of the patients with primary SjS, in about 30% with secondary SjS. Anti SS-B antibody is detected in half with primary SjS and rarely in those with secondary SjS. A 120-kDa organ-specific autoantigen was identified from the salivary gland tissue of an animal model for primary SjS in NFS/sld mutant mice; and it was found to be identical to that of the human cytoskelltal protein α-fodrin (6). As 120-kDa α-fodrin reacts with sera from patients with SjS, anti α-fodrin antibody is a strong candidate as a specific autoantibody for the diagnosis of SjS since it is not detected in serum of patients with other rheumatic diseases. Recent studies of clonality analyses on the TCR repertoire of T cells in several inflamed lesions of SjS patients indicated that these cells are induced by antigen-driven stimulation and sequence analysis of the CDR3 region indicated some conserved amino acid motifs, suggesting that infiltrating T cells recognize relatively limited epitopes on autoantigen (7). These findings support that this disease may be due to autoantigen and autoantibody. However, the etiopathogenesis for autoimmune diseases has not progressed for 30 years. Indeed, the roles of autoreactive T cells to self antigens which may cause an autoimmune disease remain obscure. The reason for the existence of autoreactive lymphocytes and autoantigens should be clarified. Evidence has accumulated on the associations of the etiology and several viruses such as HIV retrovirus, HTLV-1, hepatitis virus and Epstein-Barr virus (EBV). Among these viruses, EBV is a strong candidate for the cause of this disease since the EBV is an ubiquity in humans and the EBV DNA is detected in substantial proportion of epithelial cells and lymphoid cells in salivary glands from patients with SjS (8). The EBV- mediated α-fodrin cleavage may involve the autoantigen SjS (9). We reported the detection of EBV in synovial cells and an abnormal SAP transcript function in RA patients (10, 11). The SAP gene links strongly to EBV specific cytotoxic T cells. The detection of EBV in salivary epithelial cells and the existence of autoantigen may be due to abnormal function or mutation of this gene in patients with SjS. The gene factor as mentioned previously is also important in the initiation of the disease. The particular alleles closely linked to the MHC class II locus increase the risk of developing SjS (12). Recently we examined the gene expressions in the salivary glands of an animal model for SjS (MRL/lpr mice) using a cDNA microarray to identify a set of genes involved in the pathogenesis of organ located dysfunction of the exocrine glands (13). The microarray and RT-PCR analyses of the salivary glands showed that 9 genes (Caspase 3, Cathepsin B, Gnai, Laptm5, Ly-6c, Mel-14, Mpt1, UCP2, Vimentin) were highly associated with the pathogenesis of SjS in humans and mice with SjS. Furthermore, we found that the lysosomal-associated multispanning membrane protein 5 gene (Laptm5) was up-regulated in the salivary glands of an animal model for SjS (NFS/sld mutant mice). These genes identified in our studies using animal models for SjS provide potentially valuable information for elucidation the etiopathogenesis of the disorder.

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References

1) Talal N. Sjögren's syndrome. Curr Opin Immunol 2: 622–624, 1989.
2) Sugai S, Shimizu S, Lpmda S, et al. Lymphoproliferative disorders in Japanese patients with Sjögren's syndrome. Sjögren's syndrome: Clinical and Immunological Aspects. Talal N, Moutsopoulos HM, Kassan SS, Eds. Springer-Verlag, Berlin, 1987: 144–161.
3) Mochizuki H, Kamakura K, Masaki T, Hirata A, Nakamura R, Motoyoshi K. Motor dominant neuropathy in Sjögren's syndrome: report of two cases. Intern Med **41**: 142–146, 2002.
4) Tannenbaum H, Pinkus GS, Anderson LG, Schur PH. Immunologic characterization of the mononuclear cell infiltrates in rheumatoid synovia, in rheumatoid nodules, and in lip biopsies from patients with Sjögren's syndrome. Arthritis Rheum **18**: 305–314, 1975.
5) Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune disease and probes for cell biology. Adv Immunol **44**: 93–151, 1989.
6) Haneji N, Nakamura T, Takio K, et al. Identification of α-fodrin as a candidate autoantigen in primary Sjögren's syndrome. Science **276**: 604–607, 1997.
7) Sumida T, Yonaha F, Maeda T, et al. T cell receptor repertoire of infiltrating T cells in lips of Sjögren's syndrome patients. J Clin Invest **89**: 681–685, 1992.
8) Mariette X, Gozlan D, Clerc M, Bisson M, Morinet F. Detection of Epstein-Barr virus DNA by in situ hybridization and polymerase chain reaction in salivary gland biopsy specimens from patients with Sjögren's syndrome. Am J Med **90**: 286–294, 1991.
9) Inoue H, Tsubota K, Ono M, et al. Possible involvement of EBV-mediated α-fodrin cleavage for organ-specific autoantigen in Sjögren's syndrome. J Immunol **166**: 5801–5809, 2001.
10) Takei M, Mitamura K, Fujiwara S, et al. Detection of Epstein-Barr virus-encoded small RNA1 and latent membrane protein1 in synovial lining cells from rheumatoid arthritis patents. Int Immunol **9**: 739–743, 1997.
11) Takei M, Ishiwata T, Mitamura K, et al. Decreased expression of signaling lymphocytic-activation molecule-associated protein (SAP) transcripts in T cells from patients with rheumatoid arthritis. Int Immunol **13**: 559–565, 2001.
12) Moutsopoulos HM, Mann DL, Johnson AH, Chused TM. Genetic differences between primary and secondary sicca syndrome. N Engl J Med **301**: 761–763, 1979.
13) Shiraiwa H, Takei M, Yoshikawa T, et al. Gene expression analysis in the exocrine glands of NFS/sld and MRL/lpr mice, animal models for primary and secondary Sjögren's syndrome, using a cDNA microarray. Arthritis and Rheum 44 (9), supplement p 251, 2001.

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Identification of candidate genes for Sjögren's syndrome using MRL/lpr mouse model of Sjögren's syndrome and cDNA microarray analysis

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Abstract

Sjögren's syndrome is a chronic autoimmune disease characterized by focal lymphocytic infiltration of lacrimal and salivary glands, but the precise mechanism of this syndrome is poorly understood. To clarify the mechanism of onset and progression of Sjögren's syndrome, it is necessary to identify Sjögren's syndrome-related genes. For this purpose, we used MLR/MpJ-lpr/lpr (MRL/lpr) mouse as a model of human secondary Sjögren's syndrome and analyzed specific mRNA expression pattern in MRL/lpr mouse salivary glands by in-house cDNA microarray. Among arrayed 2304 genes, 13 genes were isolated as highly expressed genes in MRL/lpr mouse salivary gland in comparison with MRL/MpJ-+/+ (MRL/+) mouse tissue. Subsequently, we performed RT-PCR analysis and confirmed the high expression level of nine genes; caspase3, Ly-6C.2, vimentin, Mel-14 antigen, cathepsin B, mpt1, Laptm5, Gnai2 and UCP2. Five of the nine genes have already been identified in patients with Sjögren's syndrome or mice models of the syndrome, but the remaining four genes; mpt1, Laptm5, Gnai2, and UCP2 have not been reported previously as Sjögren's syndrome-related genes. Although, further experiments are necessary to examine the relationship between these four genes and Sjögren's syndrome, our system of mouse model of Sjögren's syndrome combined with in-house cDNA microarray is suitable for the isolation of Sjögren's syndrome-related genes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Sjögren's syndrome; MRL mice; Sialoadenitis; cDNA microarray; Gene expression profile

1. Introduction

Sjögren's syndrome is a chronic autoimmune disease characterized by focal lymphocytic infiltration of lacrimal and salivary glands leading to dry eyes and dry mouth [1]. The infiltrating lymphocytes are mainly $CD4^+$ memory T cells, and most cases show hyperreactivity of autoreactive B lymphocytes and the production of autoantibodies [2,3]. The prevalence of Sjögren's syndrome may approach that of rheumatoid arthritis, which affects between 1 and 3% of the general population. In addition to the primary Sjögren's syndrome, secondary Sjögren's syndrome also occurs in association with other autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus. Although these clinical entities are well recognized, the exact mechanisms of onset and progression of Sjögren's syndrome are poorly understood at present. In fact, there is even no agreement on the diagnostic criteria of this condition [4–6].

To study the pathogenesis of Sjögren's syndrome, several mouse models were generated and extensively studied. Among these models, the MRL/lpr mouse

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bearing the lpr gene with a deletion of Fas antigen, spontaneously develops systemic vasculitis, glomerulonephritis, arthritis and sialoadenitis. High levels of autoantibodies, immune complexes and rheumatoid factor are also observed in this mouse [7,8]. Inflammation of salivary glands in MRL/lpr mouse is widely accepted as a pathogenic model for human secondary Sjögren's syndrome [9]. Although the fundamental molecular abnormality in MRL/lpr mouse directly depends on the lpr gene, the extent of phenotype and timing of onset are strongly influenced by background genes [10–12].

Gene expression analysis provides an important perspective on unknown biological phenomena. The following methods are established and applied for basic and clinical studies; differential display [13], suppression subtractive hybridization [14], cDNA microarray hybridization [15], and serial analysis of gene expression (SAGE) [16]. A microarray system is a powerful tool for analyzing the expression profile of thousands of genes in a wide range of biological systems. Recently microarray analysis has been applied for the research of various clinical disorders such as lymphoma, Huntington's disease and myocardial infarction, and disease-related genes were isolated in some of these disorders [17–21].

In the present study, we isolated the genes that contribute to the progression of Sjögren's syndrome using mRNAs from MRL/lpr mouse salivary gland and in-house cDNA microarray, and identified nine upregulated genes.

2. Material and methods

2.1. Mice and mRNA preparation

Fifteen 15-week-old female MRL/MpJ-lpr/lpr (MRL/lpr) and MRL/MpJ-+/+ (MRL/+) mice were obtained from Japan SLC, Inc. (Hamamatu, Japan), and kept under standard conditions for 1 week. After sacrifice by cervical dislocation, salivary glands were quickly removed, frozen in liquid nitrogen and then stored at −80 °C until use. Total RNA was prepared from salivary glands of 16-week-old MRL/lpr and MRL/+ mice using TRIZOL reagent (Life technologies, Rockville, MD) and subsequent cleanup was carried out by RNeasy Maxi kit (Qiagen, Germany) according to the instructions provided by the manufacturer. Poly(A)+ RNA was isolated using Oligotex-dT30 mRNA purification kit (TaKaRa Shuzo Co., Kyoto, Japan). The experimental protocol was approved by the ethics review committees for animal experimentation of the participating institutions.

2.2. Preparation of cDNA microarray

A cDNA microarray consisting of 2304 cDNA derived from mouse fetus, brain and kidney was prepared as described previously [15–18]. Briefly, 2 mg/ml of PCR products were mixed at 1:1 with 4 mg/ml nitrocellulose in dimethylsulfoxide (DMSO) just before spotting, and then spotted onto carbogiimide-coated glass slides (Nisshinbo, Chiba, Japan) using a robotics (SPBIO-2000, Hitachi Software Engineering Co., Yokohama, Japan). Murine β-actin was also spotted on the same array to serve as an internal control and luciferase genes from *Photinus pyralis* was used as an external control.

2.3. Microarray procedures

cDNA microarray analyses were performed as described previously [18]. Briefly, Cy3 or Cy5-labeled cDNA probe was prepared from 2 μg of mRNA isolated from MRL/lpr or MRl/+ mouse salivary gland. Different fluorescence-labeled cDNA probes were mixed and applied onto a microarray, subsequently incubated at 65 °C overnight under a humidified condition. The fluorescent images of hybridized microarrays were scanned with a fluorescence laser confocal slide scanner (Scan Array 4000, GSI lumonics, Ottawa, Canada). Background subtraction and normalization using entire arrayed genes were carried out for each spot with appropriate software (QuantArray, GSI Lomonics, Ottawa, Canada) according to the protocol provided by the manufacturer. Data of the microarray fluorescence intensity were also obtained using QuantArray. Genes that showed a high fluorescence intensity over 1.5 fold in MRL/lpr were considered as differentially expressed genes. Among these genes we selected the candidate genes that appeared more than five times in eight microarray hybridization analyses.

2.4. Semi-quantitative RT-PCR analysis

The cDNA templates for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis were synthesized from 1 μg of poly(A)+RNA purified from MRL/lpr or MRL/+ mouse salivary gland, using 200 U of Superscript II reverse transcriptase (Life Technologies) and oligo(dT) primer. PCR amplification was performed as described previously [18] with hot start. The primer pairs and the predicted sizes of the amplified PCR products are shown in Table 1. Temperatures and time schedules were: 2 min at 96 °C for initial denaturation, five cycles of 96 °C for 20 s, 72 °C for 2 min, followed by 20 or 25 (for GAPDH 15 or 20) cycles of 96 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. PCR products were separated on 1.5% Nusieve 3:1 agarose gel (Cambrex, Rockland, ME).

Table 1
Primer pairs used in RT-PCR

Gene	Sense primer	Antisense primer	Product size (bp)
Caspase 3	5′-GGA GCT GGA CTG TGG CAT TGA-3′	5′-CAG TTC TTT CGT GAG CAT GGA-3′	322
Ly-6C.2	5′-GCT ACG AGT GCT ATG GAG TGC-3′	5′-GGA AGG ACC ATC AGA GCA AGG-3′	321
Vimentin	5′-CTG TGT CCT CGT CCT CCT ACC-3′	5′-GCA GTT CTA CCT TCT CGT TGG-3′	309
MEL-14 antigen	5′-GGA AAC TAT CAA CAA TCA CAC G-3′	5′-TGC CAG CCA AAT GAG AAA TGC-3′	550
Cathepsin B	5′-AGA CCT GCT TAC TTG CTG TGG-3′	5′-ACT CCA TTC TCT ACT CCC CAG-3′	465
mpt1	5′-GTG TTG TCT CCT TCT GTG ATG-3′	5′-CTC ACT CTT CAC TGT CCA ACT-3′	474
Laptm5	5′-TCC TGC TCA TTG GCG TGC TC-3′	5′-GAG ACA AGG CTT CCT CGT AG-3′	531
Gnai2	5′-GTG CTG GCT GAG GAT GAG GAG-3′	5′-TGA TGA TGA CGT CAG TGA CGG-3′	337
UCP2	5′-GAC CTC CCT TGC CAC TTC AC-3′	5′-GCA TGG AGA GGC TCA GAA AG-3′	305
Saposin	5′-AGG AGG TGG TGG GAA CAT TTG-3′	5′-TTC TCG GTT CCC AGC AGC AG-3′	501
Trt	5′-GGG CAA GAT GGT CAG TAG AAC-3′	5′-GGT AGT CCA GGA GAG CAA CC-3′	359
Lamrl	5′-CAC CTG GGA CCT TCA CTA ACC-3′	5′-GGA TGG GCA CAG AGG GAA CC-3′	468
HSP 70 cognate	5′-AAG AGC ACA GGA AAG GAG AAC-3′	5′-GAA GCC ACC AGG CAT CCC TC-3′	423
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′	5′-TCC ACC ACC CTG TTG CTG TA-3′	452

3. Results

3.1. cDNA microarray analysis

To investigate the gene expression profile of Sjögren's syndrome, we examined mRNAs of the MRL/lpr mouse salivary gland using cDNA microarrays. We arrayed a set of 2304 cDNA clones derived from oligo-capped mouse brain (MNCb), fetus and kidney cDNA library on our cDNA microarray. β-Actin (accession no. X03672) was used for internal control and luciferase gene from *P. pyralis* was used as an external control. Human Cot I DNA was used as a negative control. cDNAs on the microarrays were hybridized with a mixture of Cy5 and Cy3 labeled cDNA probes. Probes were prepared from poly(A)+RNA of MRL/lpr and MRL/+ mouse salivary glands. Fas gene is preserved in MRL/+ mouse and the severity of inflammation in this mouse is much less than that in MRL/lpr mouse. As the most aggressive inflammation in the salivary gland of MRL/lpr mouse occurs at the age of 12–16 weeks [10,22], we compared mRNAs of MRL/lpr and MRL/+ mouse salivary glands at the age of 16 weeks. Representative hybridization signals are shown in Fig. 1, in which the cDNA probe derived from MRL/+ mouse salivary gland is labeled with Cy-3 (red) and the cDNA probe from MRL/lpr mouse salivary gland is labeled with Cy-5 (green). Red and Green colors indicate relative abundance of expression in MRL/+ and MRL/lpr salivary glands, respectively. The yellow color indicates that both of the mRNAs derived from MRL/lpr and MRL/+ mouse salivary glands are equally expressed. Eight individual experiments were performed in total, and in half of the experiments fluorescent dyes for the probes were exchanged. We identified 13 highly expressed genes in the salivary gland of MRL/lpr mouse by cDNA microarray analysis, which were selected as candidates of Sjögren's syndrome-related genes (Table 2).

3.2. Confirmation of microarray findings by RT-PCR analysis

To confirm the high expression of the above 13 genes in Sjögren's syndrome model mouse, we performed RT-PCR analysis using Poly(A)+RNAs from independent salivary glands of MRL/lpr and MRL/+ mice as templates. The primer pairs are listed in Table 1. RT-PCR analysis reproduced the results of cDNA microarray analysis in nine out of 13 genes; high expression of caspase3, Ly-6C.2, vimentin, Mel-14 antigen, cathepsin B, mpt1, Laptm5, Gnai2 and UCP2 was noted but not the remaining four genes; saposin, Trt, laminin receptor 1 and HSP 70 cognate (Fig. 2). Additional PCR amplifications at 22, 27, and 32 cycles reproduced the same results.

4. Discussion

Several mouse strains, NOD, NZB/NZWF1, MRL/lpr are commercially available and used as a model of human Sjögren's syndrome [9,23,24]. MRL/lpr mice spontaneously develop inflammation of the salivary and lacrimal glands, SLE like nephritis, arthritis and vasculitis. Previous immunohistochemical studies have shown that inflammation of the salivary glands in MRL/lpr mouse is quite similar to that seen in human Sjögren's syndrome [25]. Based on these early reports, we selected in the present study the salivary glands of