Identification of Bach2 as a B-cell-specific partner for small Maf proteins that negatively regulate the immunoglobulin heavy chain gene 3′ enhancer

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Maf family transcription factors are important regulators in various differentiation systems. Putative Maf recognition elements (MAREs) are found in the 3′ enhancer region of the immunoglobulin heavy chain (IgH) gene. These elements are bound in B-cell extracts by a heterodimeric protein complex containing both Bach2 and a small Maf protein. Analysis of normal hematopoietic cells revealed that Bach2 is specifically expressed in B cells. Bach2 is abundantly expressed in the early stages of B-cell differentiation and turned off in terminally differentiated cells. Bach2 acts together with MafK as a negative effector of the IgH 3′ enhancer and binds to the co-repressor SMRT (silencing mediator of retinoid and thyroid receptor). Hence the Bach2–small-Maf heterodimer may represent the first example of a B-cell lineage, and of a developmental stage-restricted negative effector of the MARE in the IgH 3′ enhancer region.

Keywords: B cell/immunoglobulin/Maf/transcription factor

Introduction

Maf family transcription factors possess a conserved basic-region leucine zipper (bZip) domain which mediates protein–protein interactions and DNA binding (Nishizawa et al., 1989; Katoaoka et al., 1993). While c-Maf, MafB and NRL contain putative transcription activation domains (Nishizawa et al., 1989; Swaroop et al., 1992; Katoaoka et al., 1994a), MafF, MafK and MafG lack canonical trans-activation domains (Andrews et al., 1993b; Fujiwara et al., 1993; Igarashi et al., 1994, 1995b; Katoaoka et al., 1995; Blank et al., 1997). MafF, MafG and MafK are essentially composed of bZip domains and are collectively referred to as the small Maf family proteins. Various dimeric combinations of Maf family proteins bind in vitro to a DNA sequence motif called T-MARE (TGCT- GA/GTCAGCA) containing a 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE), TGAA/GTCACA (Katoaoka et al., 1994a,b, 1995).

Maf family proteins are emerging as important regulators of cell differentiation in various systems (Blank and Andrews, 1997; Motohashi et al., 1997). The hematopoietic cell-specific transcription factor NF-E2 is a heterodimer formed between the erythroid- and megakaryocyte-specific bZip protein, p45 NF-E2 and one of the small Maf family proteins (Andrews et al., 1993a,b; Ney et al., 1993; Igarashi et al., 1994). Resulting heterodimers bind to a NF-E2 consensus site (TGCTGA/GTCACA), which is related to T-MARE (Andrews et al., 1993a,b; Igarashi et al., 1994). A simple nomenclature of NF-E2 binding sites as Maf recognition elements (MAREs, including T-MARE and its derivatives) was recently suggested (Motohashi et al., 1997). Besides generating NF-E2, the expression patterns of the small Maf proteins suggest that they also function in other cell lineages and tissues (Fujiwara et al., 1993; Igarashi et al., 1995b; Motohashi et al., 1996). Fos and several p45-related bZip factors such as Nrf1 (LCR-F1/TCF-11), Nrf2 (ECH), Bach1 and Bach2 have been shown to form heterodimers with the small Maf proteins and bind to the MARE in vitro (Chan et al., 1993; Caterina et al., 1994; Luna et al., 1994; Moi et al., 1994; Itoh et al., 1995; Oyake et al., 1996; Toki et al., 1997; Johnsen et al., 1998). However, the precise role of these potential heterodimeric combinations during development and cell differentiation has remained elusive. Among these bZip proteins, Bach proteins may play mechanistically distinct roles as MARE-binding proteins, since they bear a BTB/POZ domain (Oyake et al., 1996). Several lines of evidence suggest that the BTB domain may be involved in the regulation of chromatin structure (Albagli et al., 1995).

MARE-like elements are found in the regulatory regions of an increasing number of genes (Katoaoka et al., 1994b). Of particular interest, we noticed that TREs within the 3′ enhancer region of the immunoglobulin heavy chain (IgH) gene (Pettersson et al., 1990; Matthias and Baltimore, 1993; Madisen and Groudine, 1994) resemble MAREs. Since immunoglobulin genes are regulated by B-cell-specific transcription factors (Fitzsimmons and Hagman, 1996; Arulampalam et al., 1997), the presence of MARE-like elements in the IgH enhancer region suggests the presence of B-cell-specific MARE effector proteins. In this study, we examined MARE-binding activity in B cells. The results presented here indicate that Bach2 functions as a B-cell- and developmental-stage-specific partner for a small Maf protein and that the heterodimer interacts with the MAREs. Interestingly, co-expression of Bach2 and MafK repressed activity of the IgH 3′ enhancer.
B-cell-specific-Maf DNA-binding complex

Fig. 1. Structure of mouse IgH locus. (A) Schematic representation of mouse IgH gene after recombination of the variable region. Boxes and circles indicate exons and enhancers, respectively. Cα3′E and HS3 contain identical putative MAREs. (B) Comparison of the putative MAREs in the IgH Cα3′E and HS3 with NF-E2-type MARE and TRE.

Hence, the Bach2–small-Maf heterodimer may represent the first example of a cell-lineage-restricted negative effector of MAREs, and might be involved in the repression of immunoglobulin genes at earlier stages of B-cell differentiation.

Results

Bach2 binds to MARE in IgH 3′ enhancer regions

Several TREs have been identified in the 3′ enhancer/locus control region (LCR) of the immunoglobulin heavy chain (IgH) gene (Pettersson et al., 1990; Lieberson et al., 1991; Matthias and Baltimore, 1993; Madisen and Groudine, 1994; Grant et al., 1995; Chauveau and Cogne, 1996). At least two of them, located in the Cα3′E and HS3, are identical to the MARE consensus sequence (Figure 1). It should be noted that Cα3′E is composed of an inverted repeat of HS3 (Chauveau and Cogne, 1996), and hence it contains an identical MARE. To examine whether Maf dimers bind to these elements in B cells, we carried out electrophoretic mobility shift assays (EMSA) using nuclear extracts from various B cell lines (Figure 2). The DNA probe which we used is a 29 bp fragment that was derived from IgH HS3 and contains one putative MARE.

As shown in Figure 2A, EMSA and competition assays revealed the presence of only one specific DNA-binding protein complex in the nuclear extracts prepared from the pro-B cell line 63–12 and from the mature B cell line BAL17 (Figure 2A, lanes 2–5). An oligonucleotide containing mutations in the MARE failed to compete with the bound complex (Figure 2A, lanes 6 and 7). Furthermore, the mutated DNA did not generate a corresponding protein–DNA complex when it was radiolabeled and incubated with the nuclear extracts (Figure 2A, lanes 8 and 9). These results established specific binding of the complex to the MARE. This MARE-binding complex was not detected using nuclear extracts from the plasmacytoma cell line J558L (Figure 2B, lane 4), indicating its stage-specific activity. To reveal constituents of the complex, we utilized antibodies that recognize various factors that could bind to a MARE. Formation of the specific MARE-binding complex was inhibited by anti-Bach2 as well as by anti-small-Maf antisera (Figure 2B, lanes 5, 6, 12 and 13). On the other hand, preimmune sera did not show any effect on complex formation (Figure 2C, lanes 6–7). Furthermore, anti-Fos antibodies, which react with c-Fos, FosB, Fra-1 and Fra-2, or anti-Jun antibodies, which recognize c-Jun, JunB and JunD, did not have any effects (Figure 2B, lanes 15–16; data not shown). Finally, involvement of Bach2 in the MARE-binding complex was confirmed using an anti-Bach2 monoclonal antibody (Figure 2C): addition of the Bach2 monoclonal antibody supershifted the MARE-binding complex. These results estab-
lished that the TRE within the 3′ enhancers Ca3′E and HS3 of the IgH gene is actually a MARE and bound by a heterodimer of Bach2 and one or another of the small Maf proteins in B-cell extracts.

**Expression of Bach2 in hematopoietic cells**

We have reported previously that expression of bach2 mRNA in mice is restricted to the brain and spleen. To determine the possible role played by Bach2 during hematopoiesis, we isolated total RNA samples from bone marrow, thymus and spleen of adult mice as well as from fetal livers (13.5 days post-coitus embryos), and performed RNA blotting analysis (Figure 3A). Hematopoietic cells in the adult bone marrow revealed a 2.5-fold higher level of bach2 mRNA expression compared with that of other hematopoietic tissues or brain.

To determine the cell-lineage specificity of Bach2 expression in vivo, we fractionated hematopoietic cells from bone marrow, spleen and thymus into various lineages using lineage-specific monoclonal antibodies (mAbs) and made comparisons by RT–PCR (Figure 3B). Only the B220-positive (B220+) B cells isolated from bone marrow showed a high level of Bach2 expression. Mac-1+ cells (mono-macrophage lineage) did not express Bach2. These results clearly established that Bach2 is a B-cell-restricted transcription factor.

**Expression of Bach2 during B-cell differentiation**

The relative expression levels in bone marrow and spleen suggests that Bach2 is expressed during the earlier stages of B-cell differentiation. This possibility has been addressed by fractionating the B220+ cells into two populations, depending on the cell surface expression of IgM. The B220+/IgM+ fraction contains pre- and pro-B cells, whereas the B220+/IgM− fraction contains immature- and mature-B cells (Hardy et al., 1991; Rolink and Melchers, 1991). Using these populations, we examined the Bach2 expression during B-cell differentiation. As shown in Figure 3C, the B220+/IgM+ fraction, which represents an early stage of B-cell lineage development, showed the highest level of expression of bach2 mRNA, whereas the B220+/IgM− bone marrow cells and B220+ spleen cells expressed less bach2 mRNA. The mitogen lipopolysaccharide (LPS) is known to induce proliferation and differentiation of resting B cells. Upon LPS treatment of spleen B cells, Bach2 expression was further down-regulated (to 50%, Figure 3D). Taken together, these results indicated that Bach2 expression decreases during the maturation of B cells.

The relationship between Bach2 expression and B-cell development was further examined using various B cell lines at different stages of B-cell differentiation (Figure 4A). Among the cell lines examined, B31-1 is a stroma-dependent B-cell line and is at the earliest stage (N.Yanai...
Expression of Bach2 in B cell lines. (A) Expression ofbach2 mRNA in various B cell lines was examined by RT–PCR. Cell lines were stromal cell lines TBR31-1 and ST-2, pre-pro-B cell line B31-1, pro-B cell lines 3B9 and 63–12, pre-B cell lines 18–81 and NF55.3, immature-B cell lines WEHI 231 and WEHI 279, mature-B-cell lines CH1 and BAL17, and plasmacytoma cell lines X63/0 and J558L. The PCR products ofβ-actin mRNA are shown at the bottom.

(B) Immunoblotting analysis of the expression of Bach2 protein in the B-cell lines. Twenty-five micrograms of whole-cell extracts prepared from each B-cell line were separated with 7.5% SDS–polyacrylamide gel, transferred onto membranes, and reacted with anti-Bach2 antiserum. Positions of mol. wt markers are shown at the left-hand side.

(C) Immunoblotting analysis of the expression of small Maf proteins in B cell lines. Whole-cell extracts of indicated cell lines as well as MEL cells were examined for the presence of small Maf proteins with anti-small-Maf antiserum. The ~20 kDa antigen is indicated with an arrow. Positions of mol. wt markers are shown on the left-hand side.

Expression of Bach2 during B cell commitment

To determine at which point Bach2 expression commences during the differentiation of B cells, we examined hematopoietic stem cells (c-Kit+/Sca-1+/CD34−/Lin−) and lineage markers negative). A single cell of the stem cell fraction was shown previously to be able to reconstitute bone marrow cells in lethally irradiated mice (Osawi et al., 1996). Expression of Bach2 was compared with those in more differentiated c-Kit+/Lin− cells, which include progenitor cells of various lineages as well as in lineage-markers-positive (Lin+) differentiated cells. As shown in Figure 5A, a significant level ofbach2 mRNA could be detected in the stem cell fraction, whereas the c-Kit+/Lin− cells expressed Bach2 at low levels. Considering the fact that the Lin+ fraction contained differentiated cells of various lineages and that Bach2 expression in this fraction is restricted to the B220+ cells, the expression level in the stem-cell fraction was estimated to be relatively lower than in other B-cell populations.
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Fig. 6. Repression of IgH 3′ enhancer activity by Bach2. (A) Schematic representation of reporter plasmid (line 3) that has IgH promoter (line 1) and a set of IgH 3′ HS1/2, –3 and –4 (Madisen and Groudine, 1994; lines 1 and 2). HS1234 fragments were linked in both orientations relative to the promoter, and both reporter genes gave essentially similar results in experiments described below. HS1234 (–) reporter plasmid carries only IgH promoter. (B) Enhancer activity of HS1234 in different cell background. IgH promoter reporter plasmids with or without the HS1234 mini-enhancer (1 μg) were transfected into the indicated B cell lines. Relative expression was determined by comparing normalized reporter gene levels induced by HS-containing plasmid with that by an enhancerless control (promoter only). Values represent the averages and standard errors of four transfections. (C) Cooperative repression of HS1234 activity by Bach2 and MafK. Bach2- and MafK-expression plasmids were transfected into X63/0 cells in various combinations, as shown below, together with reporter plasmids (0.1 μg) that carried (lanes 5–9) or lacked (lanes 1–4) the HS1234. Amounts of MafK expression plasmid were titrated (0, 0.45 or 0.9 μg) in the presence or absence of Bach2-expression plasmid (0.9 μg). The results are means of four independent experiments, each carried out in duplicate, and standard errors are indicated with thin lines. (D) Cooperative repression by Bach2 and MafK requires their interaction. Effects of co-transfection with MafK (0.9 μg) and wild-type Bach2 (0.1 μg, lanes 3–4) or a Bach2 derivative that lacked the leucine zipper (0.1 μg, lanes 5–6) were compared. (E) HS3 MARE is not the sole target of Bach2/MafK. The MARE within HS3 was deleted from the HS1234 reporter gene, and its response to Bach2 (0.1 μg) and MafK (0.9 μg) expression (lanes 5–8) was compared with the wild-type reporter plasmid (lanes 1–4).

Bach2 is known as one of the earliest markers expressed in the B-cell lineage (Li et al., 1996). To determine the timing of Bach2 induction during B-cell development, we took advantage of the fact that the B31-1 cells, grown on the TBR31-1 stromal cells, are a mixture of B220– and B220+ cells, and that B220– cells give rise to B220+ cells in vitro (N.Yanai and M.Obinata, unpublished observations). Each population was purified and examined for the presence of Bach2 protein by immunoblot analysis. As shown in Figure 5B, expression of Bach2 in the less-differentiated B220+ fraction is more abundant than it is in the B220+ fraction, suggesting that B220+ B31-1 cells, which are already committed to the B-cell lineage, express Bach2 at a high level. Taken together, these results indicate that Bach2 is expressed at low levels in uncommitted hematopoietic stem cells, and that its expression is up-regulated during, or soon after, commitment of stem cells to the B-cell lineage. The commitment to other hematopoietic cell lineages might then lead to the down-regulation of bach2.

Bach2 negatively regulates IgH 3′ enhancer activity

To examine an effect of Bach2 on the IgH 3′ enhancer activity, the HS1, –2, –3 and –4 were cloned in combination as described previously, downstream of the luciferase gene under the control of IgH promoter (Figure 6A). HS3 contains an inverted repeat of the Cox3′E and carries an identical MARE that binds Bach2–small-Maf heterodimer (Figure 1). Hence, the HS1234 reporter plasmid contains at least one functional MARE. The HS1234 mini-enhancer was shown previously to be active in plasmacytoma cells but relatively inactive in pre-B cells (Madisen and Groudine, 1994). Accordingly, the HS1234 mini-enhancer failed to activate IgH promoter-driven reporter gene expression in pro-B- and pre-B-cell lines (Figure 6B).
contrast, the ability of the HS1234 to enhance transcription from the IgH promoter in plasmacytoma cells was evident, as shown in Figure 6B (compare lanes 9 and 10). Its stimulating activity was lower in mature B cells than in plasmacytoma cells (compare lanes 8 and 10).

To examine the regulatory role of Bach2 in B-cell differentiation, we carried out co-transfection experiments (Figure 6C). In this experiment, we used 0.1 µg of reporter plasmid (1 µg was used in Figure 6B). Co-transfection of Bach2-expression plasmid into the plasmacytoma cells repressed reporter gene activity driven by the HS1234 (Figure 6C, lane 6). Expression of MafK alone resulted in only weak inhibition of the reporter gene activity (Figure 6C, lane 9). Interestingly, co-transfection of both Bach2- and MafK-expression plasmids resulted in more efficient repression that virtually abolished the effect of the HS1234 enhancer (Figure 6C, lanes 7 and 8), indicating that Bach2 and MafK cooperatively repressed gene expression. Neither of them showed significant repression of reporter gene in the absence of the HS1234, indicating that effects of Bach2 and MafK were mediated by HS1234.

To verify the interaction of Bach2 and MafK in the observed cooperative transcription repression, we examined a Bach2 derivative that lacked the leucine zipper (Bach2Δzip), and hence could not form a heterodimer with a small Maf protein (Figure 6D). In this experiment, we used 0.1 µg of Bach2-expression plasmid per transfection (0.9 µg was used in Figure 6C). Both wild-type Bach2 and Bach2Δzip repressed the reporter gene activity to 60% in the absence of MafK. However, Bach2Δzip did not exert synergistic transcription repression with MafK, whereas Bach2 did exert such an effect (compare lanes 4 and 6). The results indicated that a heterodimer of Bach2 and MafK was responsible for the observed cooperative repression of transcription. The residual repression activity of Bach2Δzip that was independent of dimer formation may be due to its interaction with other proteins through remaining regions such as the BTB domain. Such an interaction could inhibit the function of other transcription factors that are involved in enhancer activity of the HS1234.

In order to examine roles of the MARE within HS3 in the context of the large cis-regulatory region, we deleted the MARE from the 6.6 kb HS1234 mini-enhancer (AMARE). As shown in Figure 6E, the deletion caused a modest decrease in the reporter gene expression (compare lanes 1 and 5). Co-expression of Bach2 and MafK still showed some synergistic repression of such a reporter gene activity, even though its extent was reduced. Based on these observations, we could draw two conclusions. First, in plasmacytoma cells, the enhancer function of the HS1234 is partly dependent on the MARE within the HS3. Secondly, the HS3 MARE is not the sole target of the Bach2–small-Maf heterodimer. Other functional MAREs appear to be present within the IgH 3’ enhancer regions (see Discussion). The results described in Figure 6D and E, taken together, strongly implicated Bach2 as a negative regulator of IgH 3’ enhancer that functions by forming a heterodimer with small Maf proteins.

**Bach2 binds to a co-repressor**

Since several BTB-domain-containing proteins have been shown to bind to co-repressors like SMRT, and because we had shown that Bach2 functions as a transcription repressor, we examined whether Bach2 interacts with SMRT in a yeast two-hybrid system. Bach2 was fused to the GAL4 DNA binding domain, and the fusion protein was then expressed in yeast cells. The Bach2 fusion protein did not activate the GAL4-dependent HIS3 reporter gene (Figure 7). However, when it was co-expressed together with SMRT–GAL4 activation domain fusion protein, the Bach2–GAL4 fusion activated the reporter gene, resulting in histidine autotrophy. In contrast to the Bach2 fusion, the Bach1 fusion did not show any interaction with SMRT, as evidenced by its failure to support yeast growth in the presence of the SMRT fusion. These results thus indicate that Bach2 but not Bach1 binds specifically to SMRT.

**Discussion**

The results of the RT–PCR assay and EMSA, shown in this study, have established that Bach2 functions in B cells as a major MARE-binding factor. In terms of cell differentiation, B cell is the second hematopoietic cell lineage in which partners of MARE-effectors have been identified, with a precedent being NF-E2 p45 in erythroid cells (Andrews et al., 1993a,b; Igarashi et al., 1994). This is somewhat surprising in view of our previous observation that, among various hematopoietic cell lines tested, Bach2 expression was detected in the monocytic leukemic cell line M1 but not in BaF3 cells, which are supposed to have a pro-B status (Oyake et al., 1996). However, in this study we examined Bach2 expression in primary B cells and in a large set of established B-cell lines, and the results clearly indicate that Bach2 is in fact a B-cell-specific factor. The specific deployment of Bach2 in B cells suggests that Bach2 and its interacting bZip proteins are important regulators of B-cell differentiation.

We can conclude that the partner molecule for Bach2 in B cells is one of the small Maf proteins, for the following reasons. First, antisera raised against MafK abolished formation of the Bach2 DNA-binding complex in EMSA (Figure 2). Secondly, the small Maf-related antigen detected in B cells by immunoblotting analysis was the same as that detected in MEL cells known to express MafK (Igarashi et al., 1995a,b; Figure 4C). However, because the MafK antiserum reacts with recombinant MafF, MafG and MafK proteins (unpublished
observation), we could not determine which of the small Maf proteins actually interacts with Bach2 in B cells. Interestingly, the small Maf antigen was detected also in plasmacytoma cells, which do not express Bach2 (Figure 4C). The switching of MARE effectors from the Bach2–small-Maf heterodimers to other dimers, such as small Maf homodimers, will probably change the pattern of gene expression during B cell differentiation. Because small Maf proteins lack any functional domains other than the bZip domain, occupation of MAREs by small Maf homodimers should keep the element in a silenced status (Engel, 1994), in a way similar to a situation described for the Myc–Max–Mad transcription factor network (Amati and Land, 1994).

The results presented here allow us to articulate several hypotheses for the potential function of Bach2 during differentiation of B cells. Our study shows for the first time the existence of functional MAREs within the IgH 3′ enhancer region. Since this enhancer region, together with the MAREs, are evolutionarily conserved (Mills et al., 1997), important roles would be expected in IgH gene expression. Furthermore, the two MAREs within the 3′ enhancer of the mouse IgH gene are bound by Bach2–small-Maf heterodimers in B-cell extracts (Figure 2). Finally, we show here that Bach2 is expressed at high levels in immature B cells but not in terminally differentiated B cells that express high levels of the IgH gene (plasmacytes; Figure 4). A similar expression profile of Bach2 was also evident in human B-lineage cells (E. Ito, T. Toki and K. Igarashi, unpublished observation). Since Bach2 does not act as a transcriptional activator and rather represses activity of the IgH 3′ enhancer regions (Figure 6), we interpret our findings to show that Bach2 functions by inhibiting premature initiation of robust transcription of the IgH locus in undifferentiated B cells. Consistent with this model, the IgH 3′ enhancer is inactive before terminal differentiation where Bach2 is expressed abundantly, but is very active in plasmacytoma or myeloma cells, in which Bach2 is not expressed (Madisen and Groudine, 1994; this study).

The present results established that Bach2 represses the IgH 3′ enhancer by forming a heterodimer with a small Maf protein, because synergistic repression with MaFK of the enhancer activity was lost upon deletion of the leucine zipper from Bach2 (Figure 6D). However, the MARE within the HS3 is not the only target cis-element. This was indicated by the results that deletion of the HS3 MARE did not completely abolish cooperative transcription repression by Bach2 and MaFK (Figure 6E). In this regard, it should be noted there are several other TREs within HS1, HS2, HS3 and HS4 (Pettersson et al., 1990; Lieberson et al., 1991; Matthias and Baltimore, 1993; Madisen and Groudine, 1994; Grant et al., 1995; Chauveau and Cogne, 1996). Because MARE is closely related to TRE, some of these TREs may actually be a MARE and bind Bach2–small-Maf heterodimer within cells. Indeed, Maf family members can bind to sequences that diverge considerably from the consensus MARE (Ho et al., 1996). We are currently trying to identify other Bach2 target sites within the 3′ enhancer regions.

We examined the effect of the HS3 MARE deletion in pro-B and pre-B cells because there was a possibility that the deletion would relieve repression and activate HS1234 enhancer activity in these early-stage cells. However, the HS3 MARE deletion did not cause activation of the reporter gene expression in these cells (data not shown). As the HS1234 is more than 6 kb in length and contains many cis-elements, its enhancer activity may not reflect function of a singular cis-element. Rather, output may be determined as a combination of activities of positive and negative cis- and trans-elements. Regarding repression, another candidate effector is BASP/Pax5 which is known to repress IgH 3′ enhancer activity in early B cells (Singh and Birshtein, 1993).

An interesting possibility is that Bach2 could modulate the activity of the IgH locus through its BTB domain, which is one of the hallmarks of Bach proteins and distinguishes them from other p45-related factors (Oyake et al., 1996). The BTB domain has been implicated in transcription repression by the transcription factors BCL6 and PLZF (Chen et al., 1993; Ye et al., 1993). These proteins interact with co-repressor molecules like SMRT through the BTB domain (Dhordain et al., 1997; Grignani et al., 1998; Lin et al., 1998). Bach2 indeed binds to SMRT in a yeast two hybrid assay (Figure 7), raising the possibility that it recruits the co-repressor to the IgH locus. In the transfection assay, Bach2 repressed the IgH 3′ HS1234 mini-enhancer reporter gene that contains multiple binding sites for various B cell transcription factors. Such an dominant effect of Bach2 might be explained by its interaction with co-repressors like SMRT. We examined effect of histone deacetylase inhibitor trichostatin A, and found that it does not abrogate transcription repression by Bach2 (A. Muto and K. Igarashi, unpublished observation). Because trichostatin A inhibits some, but not all, of histone deacetylase (Carmen et al., 1996), further studies are necessary to interpret the mechanism by which Bach2 represses transcription.

It will also be interesting to analyze whether Bach2 participates in the function of the IgH 3′ enhancer in terms of altering the chromatin structure of the locus, thereby contributing to the LCR activity of this element (Madisen and Groudine, 1994). BTB-domain-containing proteins have previously been shown to be involved in regulating chromatin structure (Albagli et al., 1995). We showed recently that Bach1 is a candidate molecule that binds to the β-globin LCR. Bach1 functions as a novel type of architectural transcription factor that mediates interactions among multiple MAREs within the β-globin LCR depending on the protein interaction through the BTB domain (Igarashi et al., 1998). In this sense, it should be noted that IgH 3′ LCR also contains at least two separated MAREs (Figure 1) and other putative target sites. Hence Bach2 may orchestrate assembly of a regulatory complex on this region by mediating interactions among the cis-elements.

Previous results suggest that stem cells express various genes at low levels that later become up-regulated when the stem cells differentiate into a specific hematopoietic cell lineage (Hu et al., 1997). The commitment and differentiation of stem cells, which express these ‘differentiation markers’ at low levels, toward a particular cell lineage occurs by the induction and repression of specific sets of genes (Hu et al., 1997). From this point of view, the up-regulation of Bach2 during differentiation of stem cells into B220+/IgM− bone marrow cells, as well as its
high expression in B220− B31-1 cells, indicates a potential role of Bach2 in lineage commitment of B cells. Several transcription factor genes have been shown to be essential for proper development of B cells (Fitzsimmons and Hagman, 1996). Among these factors, EBF and BSAP/Pax5 show a very similar expression pattern to that of Bach2 (Barberis et al., 1990; Hagman et al., 1993). Expression of these proteins is restricted to the B cell lineage; they are turned on at an early stage of B cell development and down-regulated upon maturation to plasma cells. Ikaros, another hematopoietic-specific transcription factor, is required for development of both B and T cells, and is expressed in the stem-cell fraction as well (Georgopoulos, 1997). Bach2 may act together with these B- and lymphoid-specific transcription factors, leading to the differentiation of B cells from uncommitted stem cells.

In conclusion, the present study has identified Bach2 as a B cell-specific component of a small Maf heterodimer and implicated Bach2 in the regulation of B cell-specific gene expression. The results thus provide crucial information and together with further studies will enhance our ability to understand how the development of B cells is regulated by members of the bZip family of transcription factors.

Materials and methods

**B-cell cultures**

Pro-B cell lines 63-12 and 38B9 (Alt et al., 1981; Shinkai et al., 1992), pro-B cell lines NFS5.3 and 18-81 (Rosenberg and Baltimore, 1978; Hardy et al., 1986), immature-B cell lines WEHI 231 and WEHI 279 (Warner et al., 1979; Sibley et al., 1980), mature-B cell lines CH1 and BAL17 (Lynes et al., 1978; Kim et al., 1979), and plasmacytoma lines X63/0 (X63-Ag8.653) and J558L (Kearney et al., 1997; Oi et al., 1983) were maintained in Iscove's modified Dulbecco's medium (IMDM, Gibco-BRL) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 2-mercaptoethanol (5×10⁻⁵ M). B31-1 cell line, a bone marrow stromal-cell-(TBR-31–1)-dependent B cell line which contains B220– and B220⁺ cell lineages, was provided by Dr. Stephen Schreiber.

**Spleen cell suspensions**

Spleen cell suspensions were treated with ammonium chloride lysis buffer (pH 7.4) which contained 155 mM NH₄Cl, 10 mM KHCO₃ and 50 mM NaCl. Spleen cell suspensions were then treated with ammonium chloride lysis buffer (pH 7.4) which contained 155 mM NH₄Cl, 10 mM KHCO₃ and 50 mM NaCl. Spleen cell suspensions were then treated with ammonium chloride lysis buffer (pH 7.4) which contained 155 mM NH₄Cl, 10 mM KHCO₃ and 50 mM NaCl. Spleen cell suspensions were then treated with ammonium chloride lysis buffer (pH 7.4) which contained 155 mM NH₄Cl, 10 mM KHCO₃ and 50 mM NaCl. Spleen cell suspensions were then treated with ammonium chloride lysis buffer (pH 7.4) which contained 155 mM NH₄Cl, 10 mM KHCO₃ and 50 mM NaCl.

**EMSAs**

An oligonucleotide probe was from the IgH HS3 (5′-CTGAGCTCTACGCCTCAGGAG-3′) or the derivative-containing mutations (5′-CTGAGCGGACCGCTTCCCTCAGCAGG-3′; mutations are underlined). The double-stranded oligonucleotide was labeled with [γ-³²P]ATP and T4 Polynucleotide Kinase (TOYOBO). Incubation of B cell extracts with the DNA probe was carried out at 37°C for 10 min under the conditions described previously (Kataoka et al., 1994b). Where indicated, antibodies were added to the binding reaction at dilutions of 1/10 or 1/20, with incubation for 30 min before addition of DNA. The reaction products were electrophoresed on 4% polyacrylamide gels.

**Cell staining and sorting**

Fluorescence-conjugated monoclonal antibodies to TER119, B220, Mac-1, Gr-1, Thy-1.2, surface-IgM, CD4, CD8, and CD44 were obtained from PharMingen. Monoclonal antibodies to CD34 and CD5 were as described previously (Osawa et al., 1996). Cells (1×10⁶) in single-cell suspensions, obtained from bone marrow, spleen or thymus of 129/SvJ (6–12 weeks) were incubated with appropriate mAbs on ice for 30 min. Cells were washed with staining medium (SM, phosphate-buffered saline containing 0.05% NaN₃ and 3% fetal bovine serum), and resuspended in SM containing 1 μg/ml of propidium iodide. Cells were sorted using a four-color fluorescence-activated cell sorter (FACS Vantage, Becton Dickinson) and 6×10⁵ to 1×10⁶ cells were collected for each fraction. RNA was prepared from each fraction as described above. cDNA was synthesized by random hexamer priming and PCR was carried out with specific primers for Bach2 (J2020F; 5′-CGGCTGCGAAAGGAAAGCTGAC-3′ and J209R; 5′-CCTGGAATCTGTGAGCTGGA-3′) and β-actin (5′-GGCCATATGGGATGGTGAC-3′ and 5′-GGATTTTTCCTCCTTCTG-3′). The amplification of the genomic bach2 DNA is much larger than that derived from cDNA because of the presence of an intron (unpublished observation). The amount of templates used were normalized for relative levels of β-actin cDNA. All PCRs were carried out in 20 μl reactions using 1.25 U of Ex Taq DNA polymerase (Takara) and 20 pmol of each primer, with a thermal cycler (Perkin Elmer 9600) and a profile of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. At least two different protocols (typically 26 and 30 cycles) were employed to ensure linearity of amplification. Products were resolved on 2% agarose gels, transferred onto ZetaProbe membranes (Bio-Rad) and hybridized with radiolabeled DNA fragments that are specific for each target cDNA.

**Immunoblotting analysis**

Whole cell extracts, prepared from B-cell lines as described previously (Fagano et al., 1992), were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels. Proteins were fractionated by electrophoresis on 12% polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were probed with specific antibodies and visualized by enhanced chemiluminescence.
Transfection assay
Cultured B cell lines were transfected using FuGENE 6 reagent (Boehringer Mannheim) according to the manufacturer’s protocol. Amounts of transfected plasmids are indicated in the figure legends. Preparation of cell lysates and luciferase assays were carried out using TOYO-INKI, following the supplier’s protocol, with a Lumat LB950 luminometer (Berthold). Fire-fly luciferase activity was normalized for transfection efficiency as determined by the control sea pansy luciferase activity.

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