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Mouse MafA, homologue of zebrafish somite Maf 1, contributes to the specific transcriptional activity through the insulin promoter

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Abstract

Large Maf transcription factors, which are members of the basic leucine zipper (b-Zip) superfamily, have been reported to be involved in embryonic development and cell differentiation. Previously, we isolated a novel zebrafish large Maf cDNA, somite Maf1 (SMaf1), which possesses transactivational activity within its N-terminus domain. To elucidate SMaf1 function in mammals, we tried to isolate the mouse homologue of zebrafish SMaf1. We isolated the mouse homologue of zebrafish SMaf1, which is the same molecule as the recently reported MafA. MafA mRNA was detected in formed somites, head neural tube, and liver cells in the embryos. In the adult mouse, MafA transcript was amplified in the brain, lung, spleen, and kidney by RT-PCR. MafA mRNA was also detectable in β-cell line. Next, we analyzed the transcriptional activity of MafA using rat insulin promoters I and II (RIPI and II), since a part of RIP sequence was similar to the Maf recognition element (MARE) and MafA was expressed in pancreatic β-cells. MafA was able to activate transcription from RIPII, but not RIPI, in a dose dependent manner and the activity was dependent on RIPE3b/C1 sequences. In addition, the amount of MafA protein was regulated by glucose concentration. These results indicate that MafA is the homologue of zebrafish SMaf1 and acts as a transcriptional activator of the insulin gene promoter through the RIPE3b element.

Keywords: Maf; b-Zip; Transcription factor; Insulin

The Maf transcription factors are basic-leucine zipper (b-Zip) family proteins and are homologues of the v-Maf oncoprotein, which was isolated as a transforming component of the avian musculoaponeurotic fibrosarcoma virus, AS42 [1,2]. Maf family proteins are divided into two subgroups, the large Maf and the small Maf proteins. The large Maf proteins, MafA/L-Maf/SMaf1 [3–5], MafB [6], c-Maf [7], and Nrl [8], contain an acidic domain in their N-termini that acts as a transcriptional activation site. By contrast, the small Maf proteins, MafK, MafF [9], and MafG [10], lack such an acidic domain.

Several experiments have demonstrated that the large Mafs are key factors involved in cellular differentiation. MafA/L-Maf/SMaf1 takes part in lens differentiation, induces crystallin expression in chicken embryos [4], and is expressed in the somite during zebrafish development [5]. The MafB member has been identified as the affected gene in mice carrying the *kreisler* (*kr*) mutation [11], and the gene product is required for segmentation of the hindbrain by controlling *Hoxb-3* expression [12]. In addition, MafB down-regulates the expression of the transferrin receptor and inhibits erythroid differentiation in myelomonocytic cells [13]. Furthermore, ENU induced *kreisler* mutant (*kreisler*^{ENU}) displayed inadequate podocyte development in the kidney and caused nephrosis [14]. It has been reported that c-Maf activates the transcription of L7 gene expression in developing

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Purkinje cells [15] and controls the tissue-specific expression of interleukin-4 (IL-4) in $CD4^+$ T helper 2 cells [16]. In addition, severe defects occur in the lens fiber cells of *c-maf* gene knock-out mice [17] and mutation of the *c-maf* gene is the cause of abnormal human ocular development [18]. Nrl, the last member of the large Mafs, is well known as the gene responsible for human Retinitis Pigmentosa [19]. Nrl contributes to the maturation and establishment of neuronal cells [20] and regulates the expression of the rhodopsin gene in the retina [21]. This accumulated information indicates that the large Maf proteins play indispensable regulatory roles in cellular differentiation, morphogenesis, and several distinct developmental processes.

It has been reported that large Mafs bind to the target DNA sequence by their basic domain and form either homodimers or heterodimers with other Mafs, Jun, or Fos [6,22,23] through their leucine-zipper domains. Maf dimers bind to TRE (TPA-responsive element)-type Maf recognition elements, T-MARE (TGCTGACTCAG CA), or CRE (cAMP-responsive element)-type MARE, C-MARE (TGCTGACGTCAGCA) [22,24]. The chicken L-Maf was shown to recognize the core site (TGCTGAC, −108 to −102) within the αCE2 sequence (CTCCGCATTTCTGCTGACCAC, −119 to −99), which was defined as the lens-specific enhancer element in the chicken αA-crystallin promoter [25]. The hoxb-3 r5 enhancer sequences (TGTCATCCCCCTAAGTCAG CAGTTAC and CCAAATTTGCAGACACCTACAT TCTTGGC) are recognized by MafB [12]. The mouse IL-4 promoter (CTCATTTTCCCTTCCTTTCAGC AACTTTAACTC) [16] is associated with c-Maf and the rhodopsin promoter (TGCTGATTCAGCA) is the target site of Nrl [26]. These known Maf target sequences are conserved at least half sequences of MARE, indicating that MARE is the adequate consensus sequence of Maf target elements in vivo.

We previously isolated novel large Mafs named SMaf1 and SMaf2 transcription factors [5], in addition to previously isolated MafB/Val and c-Maf factors from zebrafish [27,28]. At the amino acid level, SMaf1 is classified as MafA/L-Maf-type large Maf. SMaf1 showed transcriptional activation activity and the mRNAs were detected in the paraxial mesoderm cells after somite boundary formation. Our investigation of zebrafish large Mafs indicated that SMafs might play important roles in somitogenesis. Very recently, it has been reported that human and mouse MafA are expressed in the pancreatic β cells of the islet of Langerhans and are the potential regulators of insulin gene expression [29,30]. To elucidate the detailed function of MafA/L-Maf/SMaf1 in mammals, we isolated mouse MafA, the homologue of zebrafish SMaf1, and analyzed its expression profile. Furthermore, we analyzed the transcriptional activity of MafA from several insulin promoter constructs as compared with other large Mafs. Our data indicate that MafA is the potential transcriptional regulator of the mammalian insulin gene.

Materials and methods

Isolation of the mouse homologue of zebrafish SMaf1. The coding sequence of the mouse homologue of zebrafish SMaf1 was amplified from genomic DNA extracted from the tail of C57BL/6J mouse, using sense (5′-TTCTCCGACGACCACCTG) and antisense (5′-CTTCTCG TA(C/T)TTCTCCTTG) oligonucleotide primers. These primers correspond to peptide sequences FSDDQL and KEKYEK, respectively, which are conserved between human EST (BE676631), chicken L-Maf and zebrafish SMaf1. Polymerase chain reaction was performed with 1 cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, then 1 cycle at 72 °C for 3 min in 20 μl of reaction mixture. The PCR product was cloned into pCRII-TOPO vector (Invitrogen) and sequenced. The genomic and cDNA clones were isolated from a 129/SvJ genomic library (Stratagene) and 10.5 dpc mouse embryo cDNA library (Stratagene), respectively, using the PCR fragment as probe. Approximately 8.64×10^5 genomic and 2.4×10^6 cDNA phages were screened, and positive clones were subcloned into pBluescript II SK+ vector (Stratagene), and then the nucleotide sequences were determined.

In situ hybridization. In situ hybridization was performed as described previously [31]. E12.5 mice embryos were fixed overnight in 4% buffered paraformaldehyde (PFA) at 4 °C. Following cryoprotection with 30% sucrose in PBS, 10 μm sections were cut using a cryostat. They were treated with protease K and fixed with 4% PFA. After postfixation, the sections were hybridized with digoxigenin (DIG)-labeled RNA probes (50 ng/ml) in hybridization solution (50% formamide, 5× SSC, pH 4.5, 1% SDS, 50 μg/ml heparin, and 50 μg/ml yeast RNA) at 65 °C for 16 h. The samples were washed with 50% formamide, 5× SSC, and 1% SDS at 65 °C for 30 min, and three times with 50% formamide, 2× SSC at 65 °C for 30 min, and subsequently incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) at 4 °C overnight. After washing with Tris-buffered saline with 0.1% Tween 20 (TBST), hybridization signals were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphatase as chromogen. For in situ hybridization of sectioned mouse embryos, the probes corresponding to the b-Zip coding region (229–359 amino acid) of the mouse *mafA gene* were synthesized using T3 and T7 RNA polymerases with DIG-11-uridin-5′-triphosphate labeling kit (Roche).

RT-PCR analysis of MafA gene expression. The total RNAs of individual tissues, cultured cell-line, and transfected cells were extracted from adult C57BL/6J mouse (27 w), INS-1 cells, and NIH3T3 cells, respectively, using TRIzol Reagent (Invitrogen). After DNase treatment, 1 μg of the total RNA was used for cDNA synthesis using SuperScript First-Strand Synthesis System (Invitrogen). Polymerase chain reaction was performed with 1 cycle at 94 °C for 2 min, followed by 50 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and then 1 cycle of 72 °C for 3 min in 20 μl of reaction mixture. The MafA cDNA fragments (405 bp) were amplified using sense (5′-CACC ACGTGCGCTTGG) and antisense (5′-CAGAAAGAAGTCGGG TG) oligonucleotide primers.

Cell culture and transient transfection assay. MafA, MafB, and c-Maf cDNAs were subcloned into pEFX3-FLAG eukaryotic expression plasmid [4]. The constructs ΔMafA, ΔMafB, and Δc-Maf encode amino acids 229–359 of mouse MafA, amino acids 207–323 of mouse MafB, and amino acids 252–370 of mouse c-Maf, respectively. The reporter plasmids, RIPII-251 (II-251), RIPII-126 (II-251), RIPII-100 (II-251), RIPI-746 (I-746), and RIPI-135 (I-135), were amplified by PCR from rat genomic DNA and cloned into pGL2-Basic vector (Promega). Mutated RIPII-251 (mII-251) was synthesized from II-251 using a QuickChange Site-Directed Mutagenesis Kit (Stratagene). NIH3T3 cells were maintained in Dulbecco's modified Eagle's

medium, supplemented with 10% fetal bovine serum. The cells were transfected by the lipofection method using FuGENE6 Transfection Regent (Roche) and then harvested at 48 h post-transfection. The luciferase assay was performed according to the supplier's protocol using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiencies were routinely normalized by the co-expressed *Renilla reniformis* luciferase activity, which was expressed by pRL-TK (Promega) expression plasmid.

Immunostaining and Immunoblotting. Immunostaining of cultured cells was carried out as previously described [4]. The cells were fixed and incubated with anti-FLAG M2 antibody (Kodak), and then with goat anti-mouse IgG FITC conjugated antibody (Zymed). As much as 10 μg/ml Hoechst (bisBENZIMIDE, Sigma) was added to final wash solution. Immunoblotting was performed as described previously [32]. Lysates of transfected NIH3T3 cells were generated with cell lysis buffer. For electrophoretic gel mobility shift assay (EMSA), Mafs or ΔMafs were synthesized with TNT coupled reticulocyte lysate or TNT coupled wheat germ extract in vitro transcription/translation systems (Promega), respectively, using pEFX3-FLAG-Mafs and pEFX3-FLAG-ΔMaf constructs. As much as 100 μg of extract from NIH3T3 cells or 1 μl of in vitro translated protein lysate was applied to SDS–polyacrylamide gel. To detect the immunoreactive proteins, we used horseradish peroxidase-conjugated anti-FLAG M2 (Sigma) and ECL blotting reagents (Amersham).

Electrophoretic gel mobility shift assay. Maf and truncated ΔMaf proteins were synthesized with in vitro transcription/translation systems (Promega). RIPE3b and RIPE3b-I (RIPE3b of RIPI) oligonucleotides, (5′-GATCCGTTTGGAAACTGCAGCTTCAGCCCCA) and (5′-GATCCGTTTGGAAATTACAGCTTCAGCCCCA), corresponding to the RIP II (−129 to −105) and the RIP I (−141 to −117), respectively, were labeled with [^{32}P-α]dCTP using Klenow DNA-polymerase. An aliquot of 7 ml of in vitro translated proteins was mixed with 2 ng of a radiolabeled oligonucleotide in a reaction mixture containing 10 mM Hepes–KOH (pH 7.9), 100 mM NaCl, 2 mM EDTA, 80 mM DTT, and 50 μg/ml of poly(dI–dC), and analyzed by a 4% polyacrylamide gel in 0.25× TAE (10 mM Tris–HCl (pH 8.0), 10 mM acetate, and 0.25 mM EDTA). The gels were dried up and exposed to X-ray films for 12 h. In the competition assay, the EMSA reaction mixture was first incubated with a radiolabeled probe at 25 °C for 15 min and then unlabeled RIPE3b, RIPE3b-I or mutated RIPE3b oligonucleotide (5′-GATCCGTTTGGAAACT*CAG*GCTTCAGCCCCA) was added to the reaction mixture.

Results

Mouse MafA is a homologue of zebrafish SMaf1

To isolate the mouse gene fragments homologous to zebrafish *Smaf1* encoding the b-Zip domain, we performed PCR analysis using mouse genomic DNA and oligonucleotides corresponding to the conserved region of known SMaf1/L-Maf homologues (Fig. 1A). The resultant PCR products were subcloned and sequenced (Fig. 1B). Half of the clones encoded part of the basic and leucine-zipper domains of mouse SMaf1 homologue. The *Smaf1* genomic DNA and cDNA were obtained by screening a mouse genomic library prepared from 129Sv mice and a mouse cDNA library prepared from 10.5 dpc CD1 mouse embryo, respectively, using the partial mouse SMaf1 clone as probe. In the result, we isolated a mouse SMaf1 genomic clone that is similar to the known large Maf sequences. And the cDNA clone implicated that this mouse SMaf1 gene is expressed in the mouse embryo.

Sequence analysis indicated this SMaf1 homologue clone as mouse MafA, which has been reported very recently [30]. The clone encodes a protein containing acidic, basic, and leucine zipper domains conserved

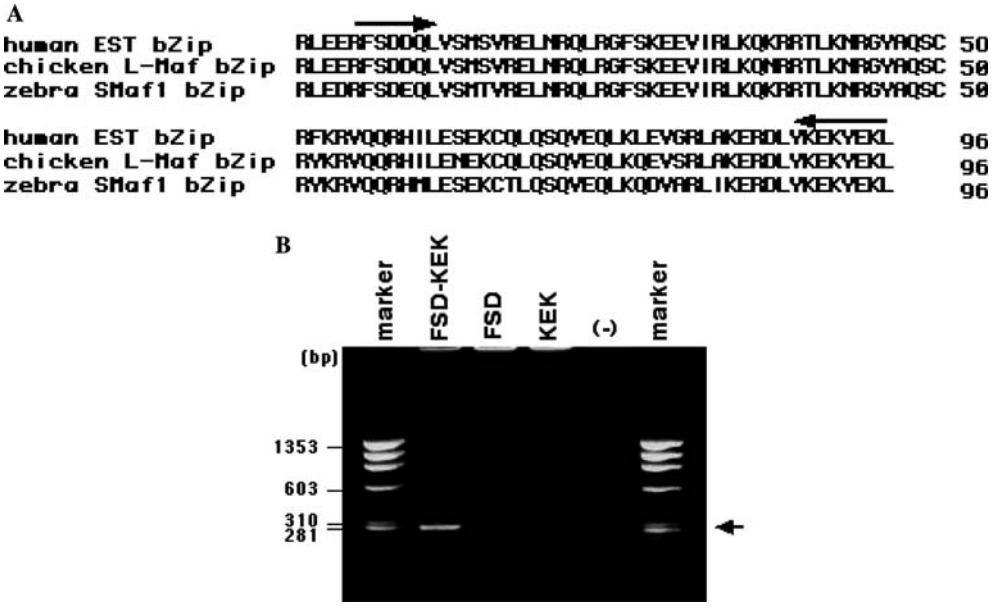

Fig. 1. The mouse homologue of zebrafish SMaf1 exists in the mouse genome. (A) Conserved amino acid sequences of known MafA/L-Maf/SMaf members. The human EST is the data of BE676631. The primer-set was constructed for the b-Zip sequence of L-Maf/SMaf members. Arrows depict the location of sense and antisense primers. (B) DNA fragments derived from mouse genomic *mafA* gene. FSD and KEK lanes show the PCR experiments with a single primer. The arrow indicates the expected PCR product.

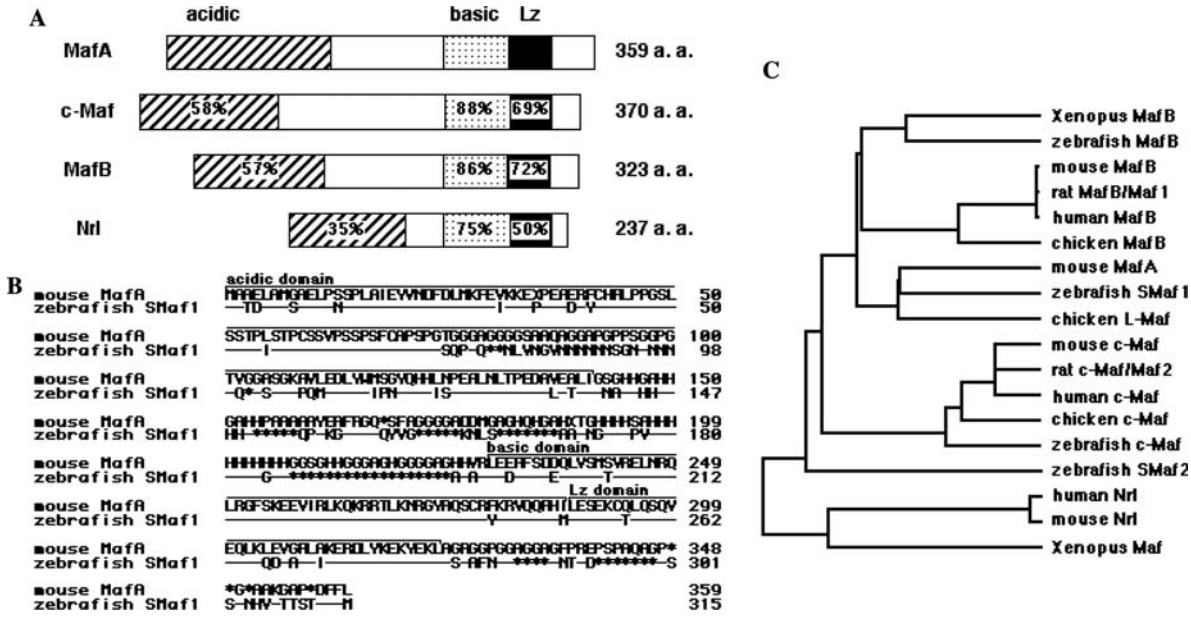

Fig. 2. Mouse MafA is a zebrafish SMaf1 homologue. (A) Schematic representation of MafA and other mouse large Mafs. Hatched boxes, acidic domains; gray-boxes, basic domains; and solid boxes, leucine zipper (Lz) domains. Percentages of amino acid sequences identical to MafA are indicated in each domain box. (B) Sequence alignment of mouse MafA and zebrafish SMaf1. Horizontal bars and asterisks represent identical amino acid residues to mouse MafA and gaps, respectively. The acidic, basic, and Lz domains are indicated. (C) Phylogenetic tree of vertebrate large Mafs. The tree was generated by the UPGMA method using the whole amino acid sequences of the known large Mafs.

amongst the large Maf subfamily, and its basic domain showed high homology (>75%) to that of other mouse large Maf members (Fig. 2A). Mouse MafA cDNA shares 85%, 92%, and 86% amino acid sequence identities with the individual acidic, basic and leucine zipper moieties, respectively, of zebrafish SMaf1 (Fig. 2B). A phylogenetic tree based on the entire amino acid sequences of each Maf protein (Fig. 2C) indicated that the isolated clone belongs to the chicken L-Maf/zebrafish SMaf1 class. According to these observations, we concluded that mouse MafA is a homologue of zebrafish SMaf1. Based on the sequence similarity, the amino acid sequence of mouse MafA is closer to that of zebrafish SMaf1 than that of chicken L-Maf (Fig. 2C).

Expression of mouse MafA

In order to analyze the spatial expression profile of mouse MafA during the fetal period (12.5 dpc), we performed whole mount in situ hybridization using DIG-labeled probe (Fig. 3). MafA mRNA was detected in each formed somite and its expression pattern appeared to be in the myotomal cells (Fig. 3C arrows). The expression pattern of mouse MafA in the somite is similar to that of zebrafish SMaf1. MafA mRNAs were also observed in the head neural tube (Fig. 3E arrows) and liver cells (Fig. 3G arrows). A low level of MafA transcripts was detected in some mesenchyme-like cells.

Next, to reveal the expression of mouse MafA in the adult mouse, we performed RT-PCR using cDNA synthesized from adult C57BL/6J brain, thymus, heart, lung, liver, spleen, pancreas, trunk muscle, and kidney (Fig. 4A). We used pancreas cDNA at three times the volume of the others, in order to obtain equivalent amounts of control HPRT PCR product. In this experiment, the fragments derived from MafA mRNA were detected in the extracts of brain, lung, spleen, pancreas, and kidney. These PCR products were subcloned and the sequences were verified (data not shown). Mouse MafA transcripts were undetectable in the trunk muscle; a similar result was found for zebrafish SMaf1 mRNA, which diminishes as maturation proceeds in the somite cells.

Recently, it has been reported that MafA is a transcriptional activator of the human insulin gene [30]. To assess the expression of MafA in pancreatic β cells, where insulin peptide hormone is produced, we tested RT-PCR using cDNA synthesized from the INS-1 cells derived from mouse pancreatic β cells (Fig. 4B). MafA transcripts were certainly detected in the cDNA of INS-1 cells and also slightly recognized in the cDNA of NIH3T3 mouse fibroblast cells (data not shown).

Large Mafs activate the rat insulin promoter II

It is well known that mouse and rat have two insulin genes and that each gene has a specific expression profile [33–36]. To assess the contribution of large Mafs to the transactivation activity of the insulin gene through the rat insulin promoters I and II (RIPI and II), we first

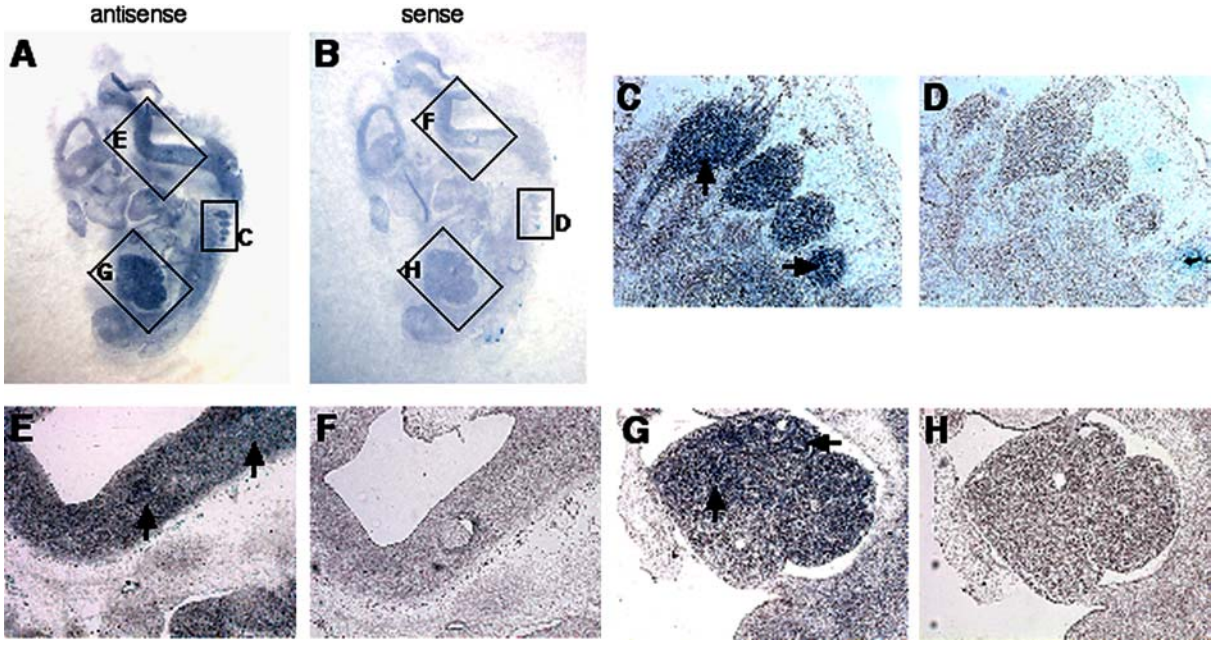

Fig. 3. Expression of Mouse MafA is detected in the mouse fetus. (A) The mouse MafA mRNA expression of longitudinal section with antisense probes. (B) This represents a sense probe control. (C,E,G) and (D,F,H) show higher magnifications of (A,B), respectively, and the frames exhibit each range. Expression of MafA in the somite cells (C,D), the anterior neural tube cells (E,F), and the liver cells (G,H). Each arrow indicates one of the stained cells. A 12.5 dpc mouse fetus was used, sectioned laterally. Anterior to the top (A,B).

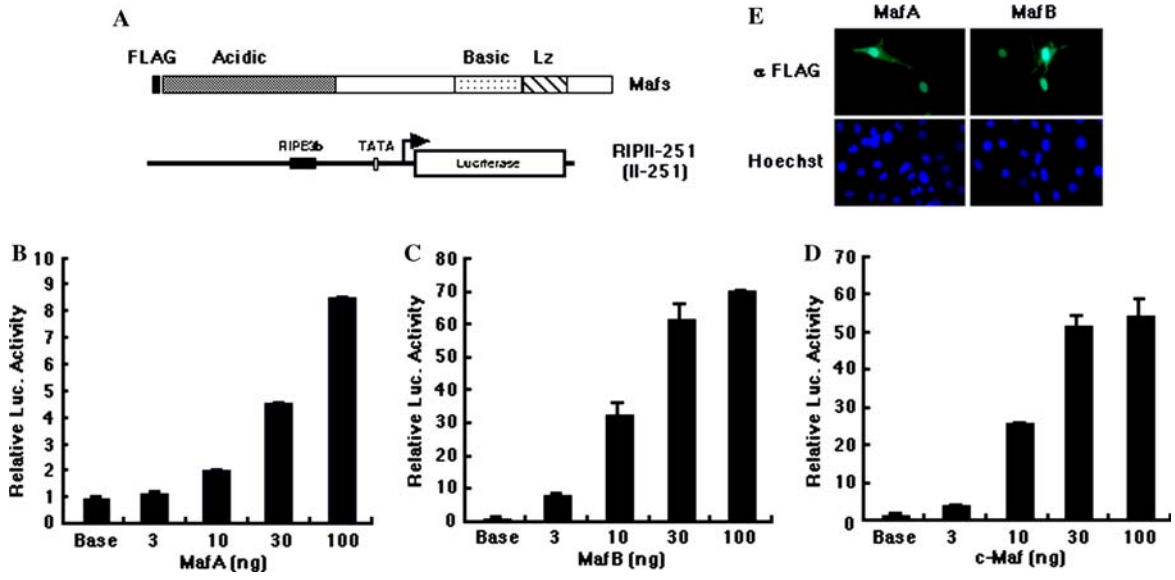

Fig. 5. Large Mafs activate reporter gene expression. (A) Schematic representation of the FLAG-Maf fusion protein and reporter construct (II-251). (B–D) Transactivation activity of large Maf proteins. Rat insulin promoter II (RIPII) reporter plasmid was transfected into NIH3T3 cells with each volume of Maf expression plasmids. (B–D) represent the result of MafA, MafB, and c-Maf, respectively. Luciferase activity in the absence of the effector plasmids was arbitrarily set at 1 and the mean values of triplicate samples are shown. (E) Detection of transfected Maf proteins in NIH3T3 cells by immunostaining. Transfected cells were incubated for 48 h and immunostained with an anti-FLAG Antibody (green). The nuclei were stained with Hoechst (blue).

analyzed the activities of MafA, MafB, and c-Maf in a co-transfection/transactivation assay using RIPII in NIH3T3 fibroblast cells. The effector Mafs were prepared as constructs with FLAG at the N-termini, while the reporter construct RIPII-251 (II-251) was *cis*-linked to a luciferase gene and contained the rat insulin

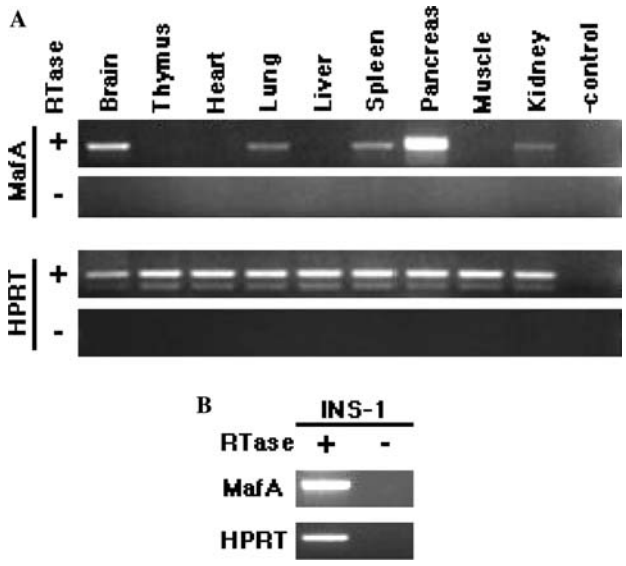

Fig. 4. MafA mRNA is expressed in various organs of adult mice. MafA mRNAs were detected in the cells of brain, lung, spleen, pancreas, kidney, and INS-1 cells (A,B; upper panels). HPRT was used as an internal control (A,B; lower panels). Reverse transcriptase (RTase)—samples were examined to expose DNA contamination of each RNA source.

promoter II (−251 to −1), including the RIPE3b element which is a putative Maf binding site (Fig. 5A). The transcriptional activity of MafA increased with an increasing transfection volume of effector (Fig. 5B). The transcriptional activities of MafB and c-Maf were six to seven times stronger than that of MafA (Fig. 5C). Taken together, these results indicate that the large Mafs possess transcriptional activity for the RIPII. Since c-Maf and MafB knock-out +/− mice did not show dysglycemia, whereas MafA knock-out +/− mouse showed exceptional sugar metabolism (our unpublished observation), further analysis was restricted to MafA and MafB was used as a positive control. To confirm the expression of transfected genes, we performed immunostaining of culture cells transfected with MafA or MafB using anti-FLAG antibody (Fig. 5E). The Maf proteins induced to cells were detected in the nucleus.

RIPE3b is the target site of MafA and MafB

To designate the site of MafA recognition, two reporter constructs were prepared. II-126 and II-100 contain 126 bp (−126 to −1) and 100 bp (−100 to −1) of RIPII, respectively (Fig. 6A). We also constructed ΔMafs, where ΔMafA (amino acid 229–359) and ΔMafB (amino acid 207–323) lack acidic domains, in order to estimate the functional importance of the acidic domain for transactivation (Fig. 6B). The ΔMafs were tagged with N-terminal FLAGs. Whereas MafA and MafB exhibited similar luciferase activities with II-251 and II-126 reporters, II-100 was not activated by either Maf

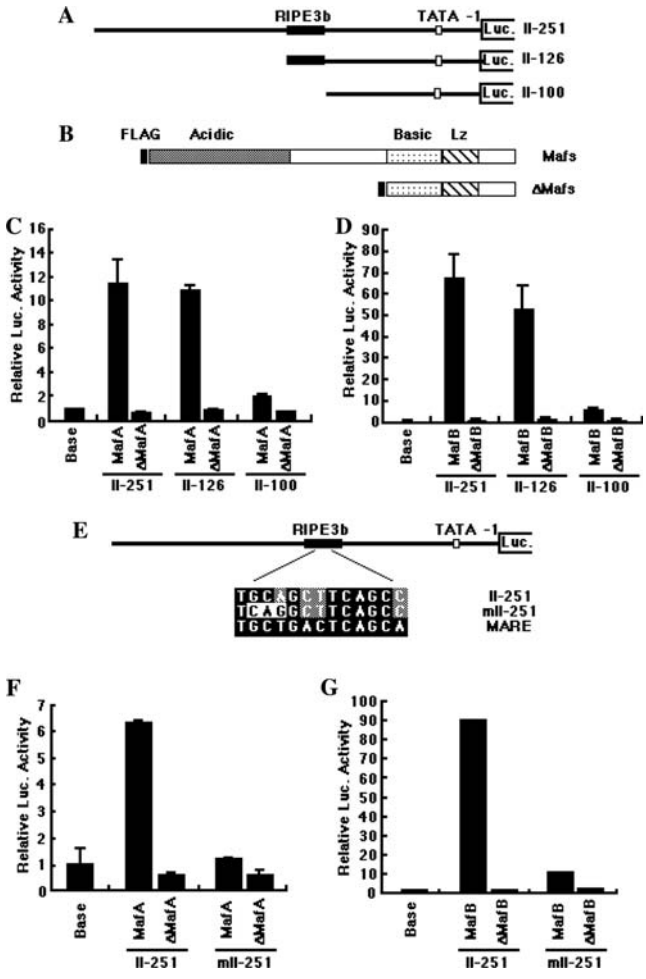

Fig. 6. The RIPE3b element is the target sequence of Mafs. (A) The 5′ end deletion reporters and (B) truncated Maf mutants are shown schematically. (C,D) Results of transient transfection assay using MafA and MafB effector, respectively. The large Maf proteins activated the expression of reporter constructs, II-251 and II-126, but not of II-100. (E) The mutant sequences of RIPII-251 reporter are shown compared with MARE motif. (F,G) The mII-251 reporter was not sufficiently activated with both MafA and MafB. (C–F) Both ΔMafA and ΔMafB, which lacked the acidic domains, did not cause obvious activation.

factor (Figs. 6C and D). These results indicate that the responsible elements for Maf activation are located between −126 and −101 of RIPII. Since this region includes the RIPE3b element, which contains the Maf recognition element (MARE) like sequence [22] (Fig. 6E), we focused on the core site (−119 to −113) of the RIPE3b element as the Maf binding sequence. To elucidate the importance of the sequence, we constructed a mutated RIPE3b (mII-251) reporter based on the II-251 construct, whose mutation abolishes binding of Mafs [5] (Fig. 6E). When MafA or MafB was transfected with mII-251 reporter plasmid, the luciferase activity decreased by 90–95% (Figs. 6F and G). These data indicated that the core sequence (−119 to −113) of the RIPE3b element is the target site for Mafs, and the two

nucleotides GC are crucial for transactivation of RIPII by Mafs. In addition, neither ΔMafA nor ΔMafB activated the transcription of the reporter in any of the experiments (Figs. 6C, D, F, and G). These results suggested that activation of MafA and MafB through the insulin promoter depends on acidic domain function.

MafA and MafB specifically bind to the RIPE3b element

To confirm that Mafs directly bind to the RIPE3b element, we carried out an electrophoresis gel mobility shift assay (EMSA) using RIPE3b oligonucleotide as probe. Maf and ΔMaf proteins were synthesized using in vitro systems and the proteins were confirmed by immunoblotting with FLAG epitope antibody (Fig. 7A). The results showed that all MafA (lane 3), MafB (lane 7), ΔMafA (lane 13), and ΔMafB (lane 17) proteins could bind to the RIPE3b oligonucleotide (Figs. 7B and C). To examine the DNA binding specificity, unlabeled RIPE3b oligonucleotide or mRIPE3b oligonucleotide was added to the binding reaction as competitor. Addition of unlabeled RIPE3b oligonucleotide, but not mRIPE3b oligonucleotide, reduced the shifted DNA–protein complexes containing Maf or ΔMaf factors in each case. We concluded that MafA and MafB bind to RIPII and recognize the core site of RIPE3b specifically. Our results also demonstrated that the reduced activity observed using acidic domain deletion mutants is not due to a loss in DNA-binding (Figs. 6C, D, F, and G).

MafA and MafB do not activate the RIPI

There is a high similarity between the sequences of RIPI and RIPII. To assess the transcriptional activity of Maf factors with the RIPI, we carried out luciferase assays using an RIPI reporter (Fig. 8A). The luciferase activity of the RIPI (I-746) was not of an adequate level compared with that of RIPII (II-251) with either MafA or MafB (Figs. 8B and C). Further examination was carried out using I-135 reporter (Fig. 8A) to exclude the possibility that the I-746 construct contains negative regulatory elements. The transactivation activity of MafA or MafB was not detected for I-135 (Figs. 1B and C). Taken together, we concluded that both MafA and MafB could activate the RIPII, but not RIPI. The nucleotide difference in the core sequence of RIPE3b between RIPII and RIPI is a possible explanation for this. In the RIPE3b of RIPI (RIPE3b-I), the crucial nucleotide G required for recognition of Maf proteins (see Fig. 6) was substituted with A (Fig. 8D). To confirm this hypothesis, we did EMSA using RIPE3b-I and RIPE3b oligonucleotides as competitors. RIPE3b-I showed significantly less

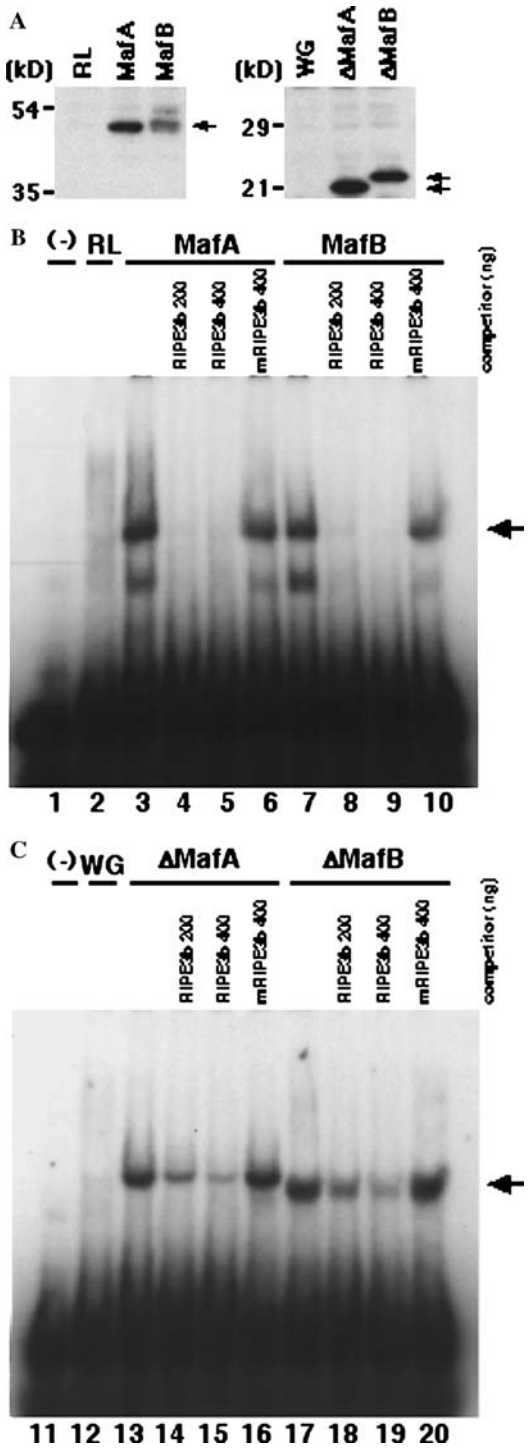

Fig. 7. MafA and MafB bind to RIPE3b. (A) Immunoblotting for the confirmation of Maf and ΔMaf proteins synthesized in vitro. (B,C) DNA binding activity of MafA and MafB (lanes 3–10) or ΔMafA and ΔMafB (lanes 13–20) was analyzed using RIPE3b oligonucleotides as probe. Lanes indicated as (—), RL or WG contained no proteins (lanes 1 and 11), the crude proteins in rabbit reticulocyte lysate (lane 2), and those in wheat germ extract (lane 12), respectively. We used 100- (lanes 4, 8, 14, and 18) or 200- (lanes 5, 9, 15, and 19)-fold excess of RIPE3b oligonucleotides, or 200-fold excess of mRIPE3b oligonucleotides (lanes 6, 10, 16, and 20) as the unlabeled competitors. Arrows denote the positions of the DNA–Mafs complexes.

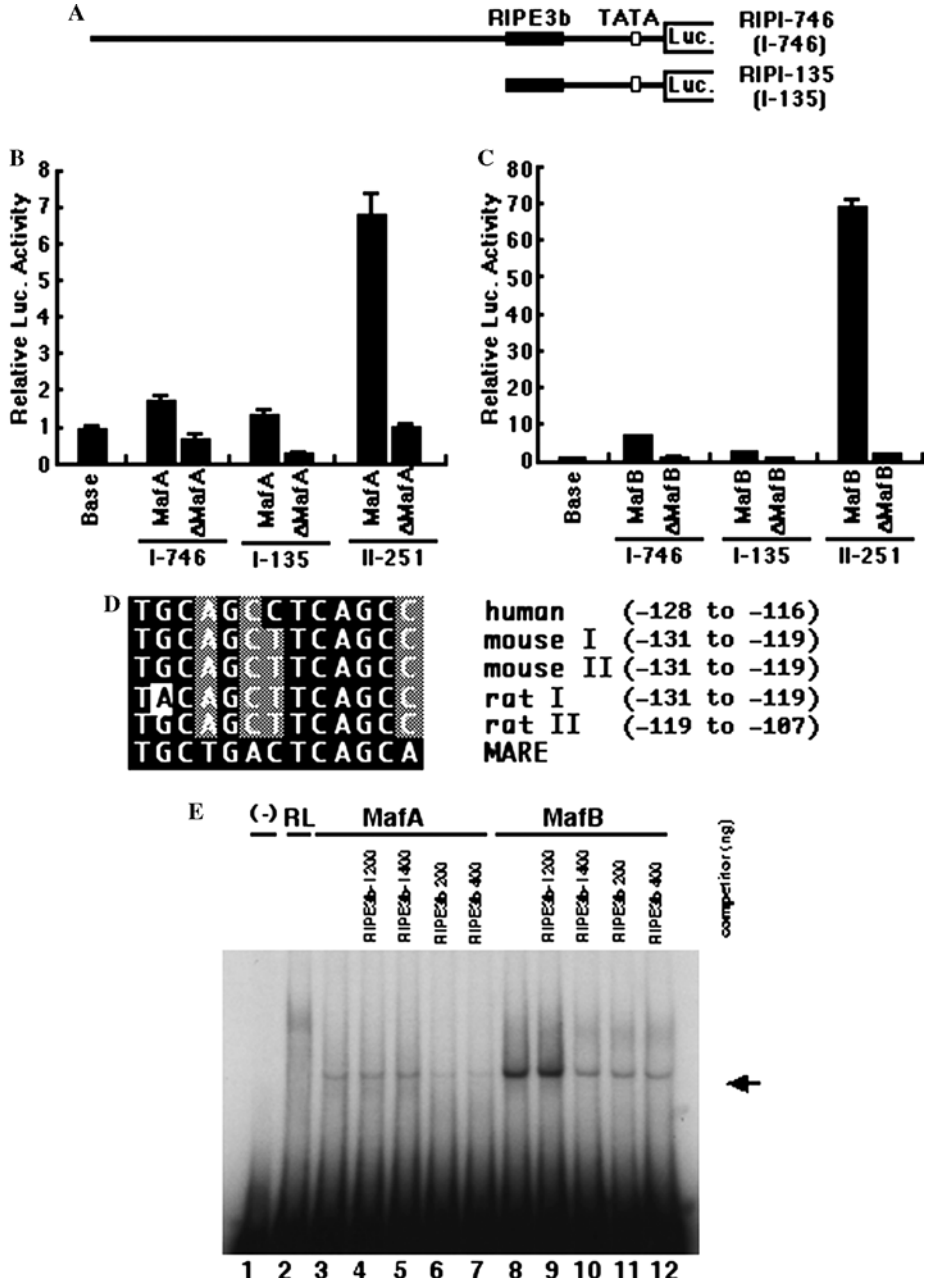

Fig. 8. Rat insulin promoter I (RIPI) reporter is not activated by Mafs. (A) Schematic representation of RIPI reporter constructs. (B,C) Mafs do not possess adequate transcriptional activity for RIPI. (D) Conservation of the nucleotide sequence of insulin promoters and MARE. Focus on the −130th nucleotide, "A," of RIPI. (E) DNA binding activity of MafA and MafB (lanes 3–12) was analyzed using RIPE3b oligonucleotides as probe. Lanes indicated as (—) and RL contained no proteins (lanes 1) and the crude proteins in rabbit reticulocyte lysate (lane 2), respectively. We used 100- (lanes 4 and 9) or 200- (lanes 5 and 10)-fold excess of RIPE3b-I oligonucleotides (RIPE3b of RIPI), or 100- (lanes 6 and 11) or 200- (lanes 7 and 12)-fold excess of RIPE3b oligonucleotides as the unlabeled competitors. Arrows denote the positions of the DNA–Mafs complexes.

competing activity as compared with RIPE3b (Fig. 8E). These data indicate that transcription from RIPI is not under large Maf regulation.

The amount of MafA protein increases by high glucose level

It has been shown that the transcriptional activity of MafA was lower than those of MafB and c-Maf (Figs. 5B–D). To confirm the expression of Maf proteins in transfected NIH3T3 cells, we performed immunoblotting and immunostaining of transfected NIH3T3 cells with anti-FLAG antibody. MafA protein was least detected as compared with other Mafs and ΔMafs (Figs. 9A and C). To assess transcriptional level from transfected MafA plasmids, we carried out RT-PCR analysis (Fig. 9B). MafA transcripts were sufficiently expressed as well as ΔMafA. Since insulin expression was induced

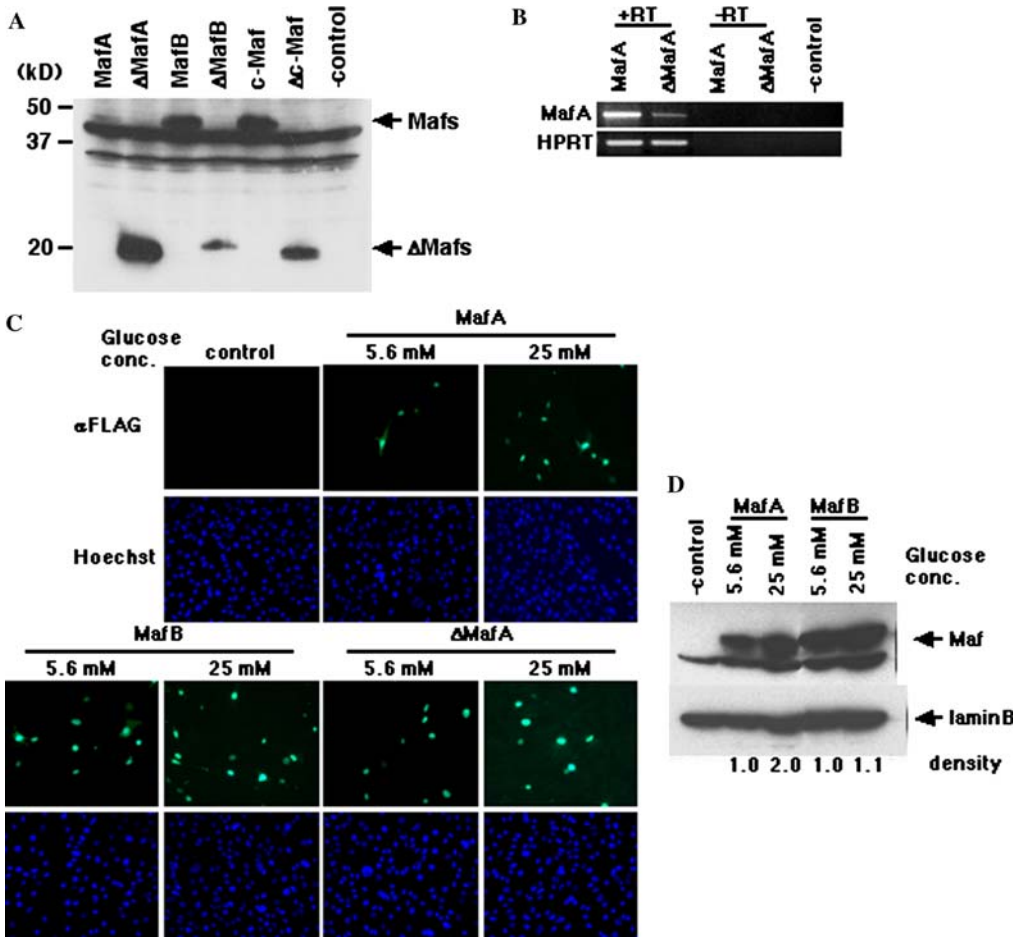

Fig. 9. High glucose state is required for the accumulation of MafA protein. (A) Immunoblot detection of Mafs and ΔMafs. Mafs and ΔMaf expression plasmids were transfected into NIH3T3 cells cultured in normal glucose (5.6 mM) medium. Whole-cell extracts of NIH3T3 were subjected to immunoblot detection by anti-FLAG antibody. (B) Expression levels of MafA and ΔMafA in the transfected cells cultured in normal glucose medium. MafA and ΔMaf mRNAs were detected by RT-PCR analysis. (C) Immunostaining of transfected cells. Maf expression plasmids were transfected into NIH3T3 cells. At 24 h after transfection, normal glucose medium was replaced with high glucose medium (25 mM) for cells in high glucose condition. Then immunostaining at 48 h after transfection was performed. (D) Immunoblot detection of Maf proteins using transfected NIH3T3 cells. The band density was measured by densitometer (Bio-Rad) and normalized with laminB. The relative value is depicted at the bottom.

by high glucose concentration in vivo, we checked the amount of MafA protein in high glucose condition (25 mM). Immunostaining and Western blot analyses clearly showed that MafA protein was more abundant in high glucose condition than in normal glucose condition (5.6 mM), while that of MafB or ΔMafA was not changed by glucose concentration (Figs. 9C and D). These results indicated that high glucose state induced the accumulation of MafA protein. Taken together, we concluded that the amount of MafA protein was regulated by glucose concentration, and the acidic domain of MafA protein might be responsible for this regulation.

Discussion

In this paper, we isolated mouse MafA as the homologue of zebrafish SMaf1. In fact, mouse MafA belongs to the L-Maf/SMaf1 class (Fig. 2C). We showed that mouse MafA transcripts were located in several tissues, using whole mount in situ hybridization (Fig. 3) and RT-PCR analysis (Fig. 4). The MafA mRNA was expressed in mouse somite cells, where the detected level of zebrafish SMaf1 expression was striking [5]. Additionally, the transcripts of zebrafish SMaf1 were also detected in hindbrain cells during zebrafish embryogenesis [5], whereas, in our experiments, the mRNAs of mouse MafA were observed in the developing mouse fetus (12.5 dpc) (Fig. 3). Chicken L-Maf is expressed in the developing lens cells of the chicken embryo, whereas quail MafA, which is almost the same in structure to L-Maf except for one amino acid difference, is expressed in retinal cells [3]. We also detected MafA mRNA in the lens and retina of the mouse embryo (our unpublished observation). These results manifest the various functions of the MafA/L-Maf/SMaf1 class of Maf factors in the vertebrate.

In this study, we indicated that MafA and MafB activate the RIPII and bind to the core site of RIPE3b. Mouse and rat possess two insulin promoters each (insulin promoters I and II), whereas human possesses only one promoter; these insulin promoters have unique transcriptional regulations [33–36]. Two significant differences appeared when the RIPE3b sequences of these insulin promoters were aligned with the MARE sequence (Fig. 8D). One difference was in the human insulin promoter sequence and the other was in the RIPI. Since nucleotide −122 "C" in the human sequence is identical to the one in MARE, although the nucleotide is different from that in other insulin promoters, human RIPE3b seems to be easily activated by Maf transcription factors. On the other hand, nucleotide −130 "A" in RIPI obviously differs from nucleotide "G" in the other insulin promoters and in the MARE sequence. As we have demonstrated in this paper and as others have reported previously [22,24], "G" is crucial for Maf activity. This difference in the RIPI must be the reason why the Mafs did not activate the RIPI reporter in our experiments. Taken together, we conclude that MafA is not a sufficient transactivator for the RIPI. Furthermore, these data indicate that both the mouse and human insulin promoters contain Maf target sites and can be activated by Maf transcription factors. We have revealed that MafA activates the rat insulin promoter II, but not promoter I. It has been reported that the responses of rat insulin genes to stimulation are different [33,34]. Our results imply that, under conditions of stimulation, the difference between rat insulin promoters I and II depends on Maf regulation. It has been reported that mouse insulin promoters I and II are also differentially regulated [35,36]. Since the RIPE3b elements of both genes are identical, other elements except for RIPE3b may be responsible for the regulational difference between mouse insulin promoters I and II.

The RIPE3b/C1 element has been identified as one of the most important elements for pancreatic β-cell specific insulin expression [37–39]. Other important elements are known as E1 and A3 [40–45]. The pancreatic islet restricted transcription factors BETA2/NeuroD and PDX1, which bind to the E1 and A3 elements, have been isolated. Mice deficient in these transcription factors revealed these factors to be indispensable for islet cell development and insulin gene expression [46–49]. In addition, mutations in the BETA2 and PDX1 genes were found in some patients with maturity-onset diabetes of the young (MODY) [50,51]. The RIPE3b has also been shown to be involved in β-cell-specific insulin gene transcription as well as in its glucose-regulated expression. Previous studies have identified a β-cell-restricted RIPE3b-binding factor, called the RIPE3b1 activator, that appears in response to glucose in pancreatic β-cell nuclear extracts [38]. Very recently, two papers have been published stating that the RIPE3b1 activator is MafA [29,30]. Our data confirmed their observations and added new information about RIPE3b recognition by large Maf transcription factors.

It was shown that the transactivation ability of MafA is lower than that of MafB in our system of analysis (Figs. 5,6 and 8). The highest level of transactivation activity of MafA was obtained with 500 ng of effector plasmid (data not shown), and that of MafB was obtained with 100 ng of effector. Since we carried out the transfection assay using 100 ng of effector, MafA activity was not maximal, but that of MafB was. Except for this point, MafA does not contain ample transactivation activity. We also demonstrated that the amount of MafA protein was regulated by glucose concentration (Figs. 9C and D). There are several possible explanations for this result. The most likely possibility is that MafA protein is unstable in the cells, whereas other Mafs are stable in normal glucose condition (Fig. 9A). Recently, it has been published that chicken L-Maf, which is the homologue of mouse MafA, is phosphorylated by FGF/ERK signaling pathways and that phosphorylation of L-Maf induces its degradation [52]. In addition, it has been reported that quail MafA is phosphorylated and its phosphorylation is important for maximum transactivation [53]. These data suggest that mouse MafA must also be phosphorylated and that its phosphorylation may regulate the transcriptional activity and/or stability of MafA by extracellular signaling, glucose. We are now trying to analyze the relationship between the phosphorylation and protein stability of MafA.

In conclusion, we isolated mouse MafA as a homologue of zebrafish SMaf1 and isolated MafA was expressed in several tissues including pancreatic beta cells. MafA was able to activate transcription from RIPII, but not RIPI, through the RIPE3b element. Further analysis concerning the protein modification and stability of MafA may open up new insights into understanding insulin production and one of the causes of diabetes mellitus.

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