Glucose regulates MafA transcription factor abundance and insulin gene expression by inhibiting AMP-activated protein kinase in pancreatic β-cells

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ABSTRACT

Insulin mRNA expression in pancreatic islet β-cells is upregulated by extracellular glucose concentration, but the underlying mechanism remains incompletely understood. MafA is a transcriptional activator specifically enriched in β-cells that binds to the insulin gene promoter. Its expression is transcriptionally and post-transcriptionally regulated by glucose. Moreover, AMP-activated protein kinase (AMPK), a regulator of cellular energy homeostasis, is inhibited by high glucose, and this inhibition is essential for the up-regulation of insulin gene expression and glucose-stimulated insulin secretion (GSIS). Here, we mutagenized the insulin promoter and found that the MafA-binding element C1/RIPE3b is required for glucose- or AMPK-induced alterations in insulin gene promoter activity. Under high glucose conditions, pharmacological activation of AMPK in isolated mouse islets or MIN6 cells by metformin or 5-aminoimidazole-4-carboxamide riboside (AICAR) decreased MafA protein levels and mRNA expression of insulin and GSIS-related genes (i.e. glut2 and sur1). Overexpression of constitutively active AMPK also reduced MafA and insulin expression. Conversely, pharmacological AMPK inhibition by dorsomorphin (compound C) or expression of a dominant-negative form of AMPK increased MafA and insulin expression under low glucose conditions. However, AMPK activation or inhibition did not change the expression levels of the β-cell-enriched transcription factors Pdx1 and Beta2/NeuroD1. AMPK activation accelerated MafA protein degradation that is not dependent on proteasome. We also noted that MafA overexpression prevents metformin-induced decreases in insulin and GSIS-related gene expression. These findings indicate that high glucose concentrations inhibit AMPK, thereby increasing MafA protein levels and activating the insulin promoter.

INTRODUCTION

Pancreatic islet β-cells maintain blood glucose homeostasis by secreting the glucose-lowering hormone insulin in response to a rise in blood glucose levels within minutes. This short-term response of β-cells to glucose named glucose-stimulated insulin secretion (GSIS) requires the normal function of β-cell-enriched gene products such as glut2, sur1, and kir6.2 (1, 2).
Glucose is transported into β-cells through the transmembrane glucose transporter Glut2 and is metabolized by the glycolytic pathway and the Krebs cycle to generate ATP. The rise in ATP concentration triggers the closure of the ATP-sensitive K⁺ channel Sur1/Kir6.2 and subsequent plasma membrane depolarization and Ca²⁺ influx, which ultimately leads to insulin secretion (1, 2).

In addition to GSIS, long-term (for hours) exposure of β-cells to high or low glucose concentrations also influences insulin (preproinsulin) gene expression (3). It has been suggested that the β-cell-enriched transcription factors, Pdx1, Beta2/NeuroD1, and MafA, mediate glucose-regulated expression of insulin mRNA. Pdx1 binds to the cis-regulatory enhancer elements A1, A3, and GG2 within the insulin promoter region (4–7), and Beta2/NeuroD1 and MafA bind to the E1 and C1/RIPE3b elements (8–10), respectively (Fig. 1A). These transcription factors cooperate to establish β-cell-restricted expression of the insulin gene (11, 12). However, the mechanisms underlying glucose regulation of insulin gene expression remain incompletely understood.

MafA, a member of the large Maf family of basic-leucine zipper (bZip) transcriptional activators, was identified as the β-cell-specific factor binding to the C1/RIPE3b element (11–13). The C1/RIPE3b element is one of the cis-regulatory elements mediating β-cell-restricted and glucose-regulated expression of insulin (14, 15). MafA activates not only the transcription of insulin but also of other genes involved in β-cell-specific functions, particularly GSIS, thereby playing an important role in the maturation of β-cells (16–18). During mouse embryonic development, MafA expression is restricted to insulin-positive immature β-cells in the pancreas, but it remains low and the cells lack the ability for GSIS (19–21). Upon terminal differentiation, β-cells acquire this ability in parallel with elevated MafA expression. Ablation of mafa gene in mice does not affect β-cell differentiation but leads to age-related glucose intolerance (16). During the progression of type 2 diabetes in human patients or animal models, such as the db/db mouse, β-cells gradually lose their insulin expression and GSIS ability. This dysfunction of β-cells, called β-cell failure, is preceded by reduced expression of MafA, Pdx1, and Nkx6.1 transcription factors (22, 23), but transgenic expression of MafA in β-cells partially rescues β-cell function in mice (24).

The expression of MafA is regulated by multiple extracellular stimuli. High glucose not only up-regulates mafa mRNA but also induces MafA protein by blocking degradation (9, 25). MafA is phosphorylated at multiple sites in β-cells, and phosphorylation events at Ser49, Thr53, Thr57, Ser61 (by GSK3), and Ser65 (by yet unidentified kinase) are prerequisites for MafA degradation under low glucose conditions (25). Oxidative stress also stimulates human MafA protein degradation, mediated through phosphorylation at Thr134 (corresponding to Thr131 in mouse MafA) and p38 mitogen-activated protein kinase (26). Proteasome activator PA28γ also stimulates MafA degradation (27), while aldosterone reduces mafa mRNA and MafA protein through JNK- and p38 MAPK-dependent pathways, respectively, to induce β-cell failure (28). However, the pathway or mechanism whereby glucose regulates MafA remains unknown.

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase consisting of catalytic α- (α1 or α2) and non-catalytic β- (β1 or β2) and γ- (γ1, γ2, or γ3) subunits (29). AMPK is activated by intracellular ATP depletion and the consequent increase in AMP, and thus functions as an intracellular energy sensor, regulating cellular metabolism (30). AMP allosterically activates AMPK enzyme activity by binding to the γ-subunit and also promotes activating phosphorylation at T172 of the α-subunit (31). Activated AMPK phosphorylates acetyl-CoA carboxylase (ACC) and hydroxymethylglutaryl-CoA reductase, thereby inhibiting fatty acid and cholesterol synthesis, respectively, and prevents ATP consumption (32, 33). Previous studies have demonstrated that AMPK activity in β-cells is inhibited under high glucose conditions and affects GSIS and insulin gene expression (34, 35). We therefore examined whether AMPK regulates MafA and found that AMPK negatively regulates MafA protein stability. We demonstrate here that under high glucose conditions AMPK activity is suppressed, which leads to MafA protein accumulation and subsequent up-regulation of insulin mRNA expression, as well as that of GSIS genes.
including glut2 and sur1.

RESULTS

Glucose regulates insulin gene promoter activity predominantly through the C1/RIPE3b element

To examine the roles of cis-regulatory elements in the insulin promoter in its regulation by glucose, luciferase assays were performed using a human insulin promoter construct (Fig. 1A). The reporter plasmid was transfected into the glucose-responsive insulinoma-derived cell line MIN6 and cultured in a medium containing high (20 mM) or low (2 mM) glucose concentrations. Consistent with previously reported data (9), luciferase reporter activity was higher in a high glucose environment than in a low glucose environment (Fig. 1B). We mutated three major cis elements, GG2, C1/RIPE3b, and E1, which are bound by Pdx1, MafA, and Beta2/NeuroD1 transcription factors, respectively, and assayed them for regulation by glucose (Fig. 1B). Mutation of the GG2 or E1 element (mutGG2 or mutE1) resulted in lower luciferase activity, but a glucose response remained. The promoter construct containing a mutation in the C1/RIPE3b (mutC1) also exhibited decreased activity, but it became completely unresponsive to glucose concentration, indicating that the C1/RIPE3b element is essential for glucose responsiveness of the insulin promoter.

Glucose regulates MafA and insulin expression through AMPK

Previous studies have demonstrated that AMPK activation reduces insulin gene expression and insulin promoter activity in β-cells (35). To activate AMPK, we used two mechanistically distinct activators of AMPK, AICAR and metformin. AICAR is converted to AICA-riboside monophosphate (ZMP) in the cell, and ZMP directly binds to and activates AMPK by mimicking AMP. By contrast, metformin indirectly activates AMPK in part through inhibiting complex I in the mitochondrial respiratory chain and subsequent ATP depletion. Both compounds thus induce activating T172 phosphorylation of the catalytic AMPKα1/α2 subunit. Similar to the previous results, quantitative RT-PCR analysis showed that mRNA expression of insulin (ins-I and ins-II) decreased after treatment of MIN6 cells with metformin or AICAR (Fig. 2A). Among selected β-cell-enriched and GSIS-related genes, glut2 and sur1 were also down-regulated by metformin or AICAR treatment (Fig. 2A).

Luciferase assay using the insulin promoter reporter construct also showed that treatment of the MIN6 cells with metformin or AICAR reduced insulin promoter activity (Fig. 2B), recapitulating the previous results (35). To determine the cis-regulatory elements responsible for the regulation of the insulin promoter by AMPK, we evaluated the expression from luciferase reporter plasmids with mutations in the GG2, C1/RIPE3b, or E1 elements (Fig. 2B). Luciferase activities generated by the mutGG2 or mutE1 promoter constructs were lower after metformin or AICAR treatment. By contrast, mutation of the C1/RIPE3b (mutC1) resulted in a loss of responsiveness to metformin or AICAR, indicating that negative regulation of the insulin promoter by AMPK requires the C1/RIPE3b element. Thus, the C1/RIPE3b element is essential for regulation of the insulin promoter by both glucose and AMPK.

We next examined the expression levels of Pdx1, MafA, and Beta2/NeuroD1 in metformin- or AICAR-treated MIN6 cells (Fig. 2C) and
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primary isolated islets (Fig. 2D), and found that MafA, but not Pdx1 or Beta2/NeuroD1, was decreased upon AMPK activation using these compounds.

To confirm specific role of AMPK in mediating metformin's effect on MafA, we blocked AMPK activity using dorsomorphin (compound C), an ATP-competitive inhibitor of AMPK kinase activity. Simultaneous treatment of MIN6 cells with dorsomorphin inhibited the decrease of MafA by metformin (Fig. 2E). Although dorsomorphin is also known to inhibit type I bone morphogenetic protein (BMP) receptor, this result suggested that metformin decreases MafA through AMPK activation.

To directly test relationship between AMPK activity and MafA abundance, MIN6 cells were transfected with expression plasmids for full-length (FL) or constitutively active (CA) forms of AMPK\(\alpha\)1, and were cultured in high glucose medium. We then analyzed whole cell lysates by immunoblotting and found that both FL and CA forms of AMPK\(\alpha\)1 increased ACC\(\alpha\) phosphorylation and decreased MafA protein levels (Fig. 2F).

To further establish the role of AMPK in the regulation of MafA by glucose, MIN6 cells (Fig. 3A) or primary isolated mouse islets (Fig. 3B) cultured in high or low glucose medium were treated with dorsomorphin. Under high glucose conditions where AMPK activity is low, dorsomorphin treatment further decreased AMPK T172 phosphorylation, AMPK activity (phospho-ACC\(\alpha\)), and increased MafA protein levels. Under low glucose conditions where AMPK activity is high, dorsomorphin reduced AMPK activity and increased MafA protein levels. Therefore, AMPK inhibition counteracted low glucose-induced decrease of MafA.

We next examined the insulin promoter activity in MIN6 cells cultured in high or low glucose medium with or without dorsomorphin by luciferase assay (Fig. 3C), and found that dorsomorphin treatment rescued the promoter activity under low glucose conditions. Quantitative RT-PCR analysis also showed that mRNA expression of insulin (ins-I and ins-II), glut2, and sur1 was restored by dorsomorphin treatment under low glucose conditions (Fig. 3D).

Furthermore, when a kinase-dead dominant-negative (DN) form of AMPK\(\alpha\)1 was expressed in MIN6 cells, MafA protein levels increased even under low glucose conditions (Fig. 3E). However, expression levels of Pdx1 and Beta2/NeuroD1 were not affected.

These observations indicate that low glucose environment decreases MafA protein levels and expression of glucose-responsive genes including ins-I, ins-II, glut2, and sur1, at least in part, by activating AMPK activity.

**AMPK activation destabilizes MafA protein**

Previous studies have showed that low glucose not only down-regulates mafA mRNA levels but also destabilizes MafA protein. Quantitative RT-PCR analysis showed that the mafA mRNA was decreased under low glucose conditions (Fig. 4A), as previously reported (9). However, mafA mRNA was not affected by AMPK activation by metformin or AICAR (Fig. 4B).

To further elucidate the mechanism of regulation of MafA protein abundance by AMPK, the half-life of MafA was determined in MIN6 cells treated with or without metformin (Fig. 4C). The half-life of MafA protein was about 4 h in untreated MIN6 cells cultured in high glucose medium, but it was less than 30 min in metformin-treated cells, indicating that AMPK activation accelerates MafA protein degradation. These results indicate that glucose regulates both mafA mRNA and MafA protein levels, but that AMPK only influences MafA protein levels.

We also examined the effect of AMPK activation or inhibition on exogenous HA-tagged MafA protein expressed under the control of the EF1\(\alpha\) promoter. Similar to endogenous MafA, metformin and AICAR both decreased exogenous HA-MafA protein (Fig. 5A). Dorosomorphin treatment increased HA-MafA levels even under low glucose conditions (Fig. 5B). Co-expression of the FL or CA forms of AMPK\(\alpha\)1 also reduced HA-MafA (Fig. 5C), and AMPK\(\alpha\)1-DN increased HA-MafA under low glucose conditions (Fig. 5D). These data also support a role for AMPK in the regulation of MafA protein stability.

**AMPK facilitates MafA degradation through proteasome-independent mechanism**

Previous studies have demonstrated that multiple phosphorylation events occur at Ser49,
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Thr53, Thr57, Ser61, and Ser65, and play major roles in the proteasome-dependent degradation of MafA (25). Furthermore, oxidative stress promotes proteasome-mediated human MafA degradation through phosphorylation at Thr134 (corresponding to Thr131 in mouse MafA) (26). To determine whether AMPK facilitates MafA degradation through a similar mechanism, MIN6 cells were treated with metformin or the oxidative stress-inducing reagent tert-butylhydroquinone (tBHQ), with or without the proteasome inhibitor MG132. Endogenous MafA protein levels were higher after MG132 treatment, indicating that steady state degradation of MafA in MIN6 cells is proteasome-dependent. Treatment of the cells with tBHQ reduced MafA protein, but this reduction was completely rescued by MG132 treatment, as reported previously (26). By contrast, metformin-mediated MafA reduction was only partially rescued by MG132, indicating that metformin decreases MafA protein through a pathway distinct from oxidative stress and independent of the proteasome.

To determine the role of MafA phosphorylation in its AMPK-mediated degradation, the phosphoacceptor Ser and Thr residues of MafA were mutated to Ala (Fig 6B). The 5A mutant had five Ala substitutions at Ser49, Thr53, Thr57, Ser61, and Ser65, and the T131A mutant had a single Thr131 to Ala mutation. The ΔN164 mutant lacks the amino-terminal domain. These HA-tagged MafA variants were expressed in MIN6 cells and the cells were treated with or without metformin and/or MG132 (Figs. 6C and 6D). HA-tagged wild-type (WT) MafA protein expression was lower after metformin treatment and this was only partially rescued by MG132, indicating that metformin decreases MafA protein through a pathw ay distinct from oxidative stress and independent of the proteasome.

AMPK decreases insulin expression through MafA down-regulation

We next examined whether ectopic expression of MafA restores the expression levels of β-cell-specific mRNAs in AMPK-activated conditions. MIN6 cells were transfected with an empty expression vector or HA-tagged MafA expression plasmid. The cells were then treated with or without metformin under high glucose condition, followed by analysis of protein expression by immunoblot (Fig. 7A) and mRNA expression by quantitative RT-PCR (Fig. 7B). Immunoblot analysis using anti-HA antibody (detecting exogenous HA-MafA protein) and anti-Maf (detecting both exogenous HA-MafA and endogenous MafA proteins) revealed that transfection of the HA-MafA expression plasmid achieved an excess amount of HA-MafA protein relative to endogenous MafA levels (Fig. 7A). Without metformin, expression of HA-MafA increased the glucose-responsive genes, ins-I, ins-II, glut2, and sur1 (Fig. 7B). The expression levels of these mRNAs were reduced by metformin treatment, but were restored by HA-MafA expression. These results suggest that AMPK activation by pharmacological activator decreases expression of insulin and other
glucose-responsive mRNAs at least in part through down-regulating MafA protein.

DISCUSSION

In this study, we have shown that glucose regulates insulin gene expression and MafA protein abundance by modulating AMPK activity in β-cells. As shown previously, MafA protein levels increase (25) and AMPK activity decrease (34, 35) under high glucose conditions in isolated mouse primary islets and MIN6 insulinoma-derived cells. Under high glucose conditions, pharmacological activation of AMPK by metformin or AICAR treatment, or overexpression of a CA form of AMPK, reduced MafA protein and insulin gene expression. By contrast, pharmacological inhibition of AMPK by dorsomorphin treatment or ectopic expression of a DN form of AMPK increased MafA protein and insulin gene expression under low glucose conditions.

We have shown here that the MafA-binding element, C1/RIPE3b, in the insulin promoter region is critical for the effects of both glucose and AMPK, suggesting that they regulate insulin gene expression mainly through MafA. Previous studies have shown that prolonged AMPK activation attenuates GSIS in β-cells (36, 37). Conversely, MafA is reportedly required for GSIS because it regulates β-cell-specific genes such as glut2 and sur1, in addition to insulin (ins-I and ins-II) (16–18). We further demonstrated that introduction of exogenous MafA rescued expression levels of ins-I, ins-II, glut2, and sur1 mRNAs in MIN6 cells treated with metformin. These findings together suggest that AMPK activation, induced by metformin or low glucose conditions, decreases insulin expression and GSIS by down-regulating MafA.

MafA, Pdx1, and Beta2/NeuroD1 have been identified as glucose-responsive transcriptional activators of insulin gene expression. We have shown here that the expression levels of MafA, but not Pdx1 and Beta2/NeuroD1, were lower under low glucose conditions or after AMPK activation. However, it has been shown that glucose regulates co-factor recruitment and the transcriptional activity of Pdx1 and the subcellular localization of Beta2/NeuroD1 (38–40). Therefore, such activities or behaviors of Pdx1 and Beta2/NeuroD1 might be regulated by AMPK. Our mutational analysis of the insulin promoter does not exclude a possible involvement of Pdx1 and/or Beta2/NeuroD1 in glucose- or AMPK-mediated regulation of the insulin promoter activity because we did not mutate all the binding elements of Pdx1 and Beta2/NeuroD1 within the insulin promoter region to eliminate the influence of these transcriptional activators.

Our gene expression analyses revealed that low glucose reduced mafA mRNA, as reported previously (9), whereas AMPK activation did not. Our observation that AMPK activation decreases MafA protein but not mafA mRNA indicates that AMPK regulation is only a part of the glucose response in β-cells. Expression of mafA mRNA is regulated by many β-cell-enriched transcription factors, including FoxA1, Nkx2.2, and Pdx1, through directly binding to its enhancer regions (41). Thus, glucose might regulate mafA expression by modifying the activities of these transcriptional activators independent of AMPK.

We have shown here that AMPK activation accelerates MafA protein degradation. Previous studies have demonstrated that MafA degradation depends on its phosphorylation status and the proteasome (25, 26). Consistent with this, oxidative stress-induced MafA degradation was attenuated by treatment of cells with the proteasome inhibitor MG132. However, MG132 treatment did not inhibit AMPK-induced MafA degradation. A previous study using mass spectrometry has identified eighteen phosphorylation sites of MafA in β-cells (42). Among these phosphorylation sites, we mutated functionally relevant sites and found that phosphorylation events at Ser49, Thr53, Thr57, Ser61, Ser65, and Thr131 of MafA are not required for its AMPK-induced degradation. Substitution of the other phosphoacceptor residue in MafA, Ser342, with Ala also did not abrogate the AMPK-induced degradation (unpublished observation). Therefore, MafA protein is destabilized by AMPK through a previously unrecognized mechanism. As far as we have examined, AMPK does not bind MafA directly in a co-immunoprecipitation assay (unpublished observation). Furthermore, none of the previously identified phosphorylation sites of MafA is canonical AMPK target site. We thus favor a model that AMPK destabilizes MafA via an indirect interaction. The mechanism whereby
AMPK promotes MafA degradation remains to be elucidated.

The AMPK activator metformin has long been proposed as an anti-type 2 diabetes drug because it increases glucose utilization in peripheral insulin-sensitive tissues (43, 44). However, it has also been shown that AMPK activation reduces GSIS from β-cells (34, 35). The data presented herein suggest how AMPK activation leads to the inhibition of GSIS in β-cells: AMPK activation decreases MafA abundance and thereby reduces expression of insulin and genes involved in GSIS, such as glut2 and sur1. Elucidation of the precise molecular mechanism of MafA regulation by AMPK should be particularly useful as part of the process of developing better strategies for the treatment of type 2 diabetes.

EXPERIMENTAL PROCEDURES

Plasmids and reagents

The firefly luciferase reporter plasmid pGL4/h-ins-p (WT, mutGG2, mutC1, or mutE1) was constructed by inserting a KpnI-HindIII fragment of the pGL2-based h-ins-p-luc plasmid (WT, mutGG2, mutC1, or mutE1) (11) into pGL4.10 (Promega, San Luis Obispo, CA). The expression plasmid for Renilla luciferase, pEF-Rluc, has been described previously (45). To construct an expression plasmid for full-length mouse AMPKα1 (pHygEF2B/mAMPKα1-FL), the entire open-reading frame of mouse AMPKα1 was amplified by RT-PCR (5'-ttcgcgccgaactagtATGCGCAGACTCAGTTCT-3' and 5'-taaagggaacgccgaTACTGTGCAAGAATTTT A-3') and cloned into the pHygEF2 vector (27) to make pHygEF2/HA-h-mafB plasmid (27) to make pHygEF2/HA-m-mafA (ΔN164).

Metformin, tert-butyldihydroquinone (tBHQ), cycloheximide (CHX) (WAKO, Osaka, Japan), 5-aminoimidazole-4-carboxamide riboside (AICAR), MG132 (Merck Millipore, Darmstadt, Germany), and dorsomorphin (Ark Pham, Arlington Heights, IL) were obtained from commercial suppliers.

Cells and transfection

The mouse insulinoma-derived cell line MIN6 was a generous gift from Dr. Jun-ichi Miyazaki (Osaka University). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose, supplemented with 15% fetal calf serum (FBS) in a humidified atmosphere containing 5% CO2 at 37 °C. For transfection, 1×10⁶ cells were mixed with transfection cocktail (total 3.6 µg of plasmid, 7.2 µl of P3000 reagent, and 4 µl of Lipofectamine 3000 (Invitrogen, Carlsbad, CA)) and were seeded into 24-well plates. When glucose conditions were altered, the medium was changed to DMEM containing high (20 mM) or low (2 mM) glucose supplemented with 0.5% FBS.

Isolation and culture of primary islets

Mouse primary islets of Langerhans were isolated by a standard collagenase digestion method (47) and incubated in RPMI 1640 medium containing 10% FBS. When glucose conditions were altered, the medium was changed to DMEM containing high (20 mM) or low (2 mM) glucose supplemented with 10% FBS. All experiments were performed according to the guidelines for the care and use of laboratory animals of the Yokohama City University. The experiments were approved by the Animal Research Committee of
Yokohama City University (T-A-16-1004).

**Luciferase assay**
MIN6 cells were transfected with 3.6 µg plasmid DNA (0.5 µg of luciferase reporter plasmid, 0.5 µg of pEF-Rluc, and 2.6 µg of expression plasmid) using 4 µl of Lipofectamine 3000 and 7.2 µl of P3000 reagents (Invitrogen). Cells were harvested 48 h after transfection. Firefly and *Renilla* luciferase activities were measured using a dual luciferase assay system (Promega), and firefly luciferase activities were normalized on the basis of *Renilla* luciferase activities. Data represent the mean ± S.E. of five independent experiments. Statistical significance was calculated using Student’s t test.

**Immunoblot analysis**
Preparation of MIN6 whole cell extracts and immunoblotting were performed as described previously (9, 25). The antibodies used were anti-c-Maf (M-153), anti-NeuroD (G-20), anti-Pdx1 (H-140), anti-TFIID (TATA-binding protein (TBP): SI-1), anti-AMPKα1/2 (H-300), anti-phospho-AMPKα1/2 (Thr172), anti-ACCα (H-76), anti-phospho-ACCα (Ser78/Ser80; F-2) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-HA-tag (561, MBL, Nagoya, Japan). Anti-c-Maf (M-153) cross-reacts with MafA and was used to detect MafA in MIN6 extracts, as MafA is the major Maf family member expressed in MIN6 cells.

**Quantitative RT-PCR analysis**
Total RNA was prepared from MIN6 cells using ISOGEN (Nippon Gene, Tokyo, Japan) and subjected to reverse transcription using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). The resulting cDNAs were analyzed using a LightCycler 96 system and SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN). The following primer sets were used: mafA, 5’-TTCAGCAAGGGAGGTCAT-3’ and 5’-CCGCCAACCTCTCGTATTTTC-3’; pdx1, 5’-ACCAAGCTACGCGTTGGAAGGCCAGT-3’ and 5’-TGGTTGATCGACTACTGCTACCAGCTCC-3’; and beta2, 5’-GGTTATGAGATCGTACTATGAA-3’ and 5’-CTCGAGAGAACTGACACTCATCTGTC-3’. The primer sets for ins1, ins-2, glut2, sur1, kir6.2, and gapdh genes have been described previously (48, 49). All data were normalized to gapdh expression as an internal standard. Data represent the mean ± S.E. of three independent experiments. Statistical significance was calculated using Student’s t test.

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**Conflict of interest:** The authors declare that they have no conflict of interest with the contents of this article.

**Author contributions:** RI performed most of the experiments and analyzed the data. KK designed the research and wrote the manuscript with RI. All authors reviewed the results and approved the final version of the manuscript.
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FOOTNOTES
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The abbreviations used are: AMPK, AMP-activated protein kinase; GSIS, glucose-stimulated insulin secretion; AICAR, 5-aminoimidazole-4-carboxamide-ribonucleotide; bZip, basic-leucine zipper; ACC, acetyl-CoA carboxylase; tBHQ, tert-butylhydroquinone.

FIGURE LEGENDS

FIGURE 1. Glucose regulates the insulin promoter, MafA abundance, and AMPK activity. (A) Schematic representation of the human insulin promoter reporter constructs. Locations of the binding sites for Pdx1 (A1, A3, and GG2), MafA (C1/RIPE3b), and Beta2.NeuroD1 (E1) are also indicated. (B) The firefly luciferase reporter constructs (0.5 µg) indicated in (A) and a Renilla luciferase expression plasmid pEF-Rluc (0.5 µg) were transfected into MIN6 cells, and the cells were incubated in medium containing high (Hi, 20 mM) or low (Lo, 2 mM) glucose for 16 h. Data are expressed relative to the activity in cells that received pGL4/h-ins-p (WT) at high glucose concentration. Data are mean ± S.E. of five independent experiments. *: P<0.05, n.s.: not significant (Student’s t test). (C, D) MIN6 cells (C) or isolated mouse islets (D) were incubated in high (Hi, 20 mM) or low (Lo, 2 mM) glucose medium for 16 h, and whole cell extracts were analyzed by immunoblotting using the indicated antibodies. TATA-binding protein (TBP) was analyzed as a loading control. Data in (C) and (D) are representative of two independent biological experiments. The intensity of the bands was quantified using ImageJ software and the relative amounts (averages of two independent experiments) are indicated below the bands.

FIGURE 2. AMPK negatively regulates MafA and the insulin promoter. (A) Total RNA isolated from MIN6 cells treated with the AMPK-activating reagents metformin (Metf, 500 µM) or AICAR (200 µM) under high glucose conditions for 16 h were analyzed by quantitative RT-PCR using specific primers. Data are mean ± S.E. of three independent experiments. *: P<0.05, n.s.: not significant (Student’s t test). (B) Luciferase assay. MIN6 cells were transfected with the insulin promoter reporter constructs used in Fig. 1A and B (0.5 µg) and pEF-Rluc (0.5 µg). The cells were then treated with metformin (Metf, 500 µM) or AICAR (200 µM) under high glucose conditions for 16 h. Luciferase activities are presented relative to that of pGL4/h-ins-p (WT) vector under high glucose condition. Data are mean ± S.E. of five independent experiments. *: P<0.05, n.s.: not significant (Student’s t test). (C, D) Whole cell extracts obtained from MIN6 cells (C) or primary mouse islets (D) grown in high glucose medium containing metformin (Metf, 500 µM) or AICAR (200 µM) for 16 h were analyzed by immunoblotting using the indicated antibodies. (E) MIN6 cells grown in high glucose medium were treated with metformin (Metf, 500 µM) with or without the AMPK inhibitor dorsomorphin (Dorso, 10 µM) for 16 h. Cell extracts were then subjected to immunoblot analysis. (F) Expression plasmids for the full-length (FL) or constitutively active (CA) forms of AMPKα1 were transfected into MIN6 cells grown in high (Hi, 20 mM) glucose conditions, and the cell extracts were analyzed by immunoblotting. Data in (C), (D), (E), and (F) are representative of two independent biological experiments. The intensity of the bands was quantified using ImageJ software and the relative amounts (averages of two independent experiments) are indicated below the bands.
Glucose regulation of MafA via AMPK

FIGURE 3. Glucose regulates MafA abundance and insulin expression through AMPK. (A, B) MIN6 cells (A) or isolated mouse islets (B) grown in high (Hi, 20 mM) or low (Lo, 2 mM) glucose medium were treated with the AMPK inhibitor dorsomorphin (Dorso, 10 µM) for 16 h, and whole cell extracts were analyzed by immunoblotting using the indicated antibodies. (C) The insulin promoter reporter plasmid pGL4/h-ins-p (0.5 µg) and a Renilla luciferase expression plasmid pEF-Rluc (0.5 µg) were transfected into MIN6 cells, and the cells were incubated in medium containing high (Hi, 20 mM) or low (Lo, 2 mM) glucose with or without dorsomorphin (Dorso, 10 µM) for 16 h. Data are expressed relative to the activity in cells that received the reporter plasmid at high glucose concentration without dorsomorphin. Data are mean ± S.E. of five independent experiments. *: P<0.05 (Student’s t test). (D) MIN6 cells were grown in high (Hi, 20 mM) or low (Lo, 2 mM) glucose medium and treated with dorsomorphin (Dorso, 10 µM) for 16 h. Total RNA was then isolated and subjected to quantitative RT-PCR analysis. Data are mean ± S.E. of three independent experiments. *: P<0.05 (Student’s t test). (E) The dominant-negative (DN) form of AMPKα1 was overexpressed in MIN6 cells grown under the low glucose conditions and the cell extracts were analyzed by immunoblotting. Data in (A), (B), and (E) are representative of two independent biological experiments. The intensity of the bands was quantified using ImageJ software and the relative amounts (averages of two independent experiments) are indicated below the bands.

FIGURE 4. AMPK promotes MafA protein degradation. (A) Total RNA isolated from MIN6 cells grown in high (Hi, 20 mM) or low (Lo, 2 mM) glucose conditions for 16 h were analyzed by quantitative RT-PCR using specific primers. Data are mean ± S.E. of three independent experiments. *: P<0.05. (B) MIN6 cells grown in high glucose medium were treated with metformin (Metf, 500 µM) or AICAR (200 µM) for 16 h. Total RNA was then isolated and subjected to quantitative RT-PCR analysis. Data are mean ± S.E. of three independent experiments. n.s.: not significant. (C) Degradation rates of endogenous MafA protein. MIN6 cells treated with or without metformin (Metf, 500 µM) for 16 h were incubated with cycloheximide (CHX, 10 µg/ml) for the indicated periods, and cell extracts were analyzed by immunoblotting. TBP was used as a loading control because TBP seems to be a long-lived protein in MIN6 cells. Signal intensities of MafA relative to TBP are shown in the right panel. Data represent the results of three independent experiments. *: P<0.05, **: p<0.01 (Student’s t test).

FIGURE 5. Glucose and AMPK regulates exogenously expressed MafA protein. (A) An expression plasmid containing HA-tagged MafA (HA-MafA) under the control of the EF1α promoter was transfected into MIN6 cells, and the cells were treated with metformin (Metf, 500 µM) or AICAR (200 µM) for 16 h in high glucose medium. The cell extracts were analyzed by immunoblotting using the indicated antibodies. (B) MIN6 cells transfected with the HA-MafA expression plasmid were grown in high (Hi, 20 mM) or low (Lo, 2 mM) glucose medium and treated with dorsomorphin (Dorso, 10 µM) for 16 h. Cell extracts were then subjected to immunoblot analysis. (C) The HA-MafA expression plasmid was co-transfected into MIN6 cells with an expression plasmid for full-length (FL) or constitutively active (CA) AMPKα1. Cell extracts were analyzed by immunoblotting. (D) MIN6 cells were transfected with expression plasmids for HA-MafA and a dominant-negative form (DN) of AMPKα1, and the cells were incubated in medium containing the indicated concentrations of glucose for 16 h. Whole cell extracts were subjected to immunoblot analysis. Data in (A), (B), (C), and (D) are representative of two independent biological experiments. The intensity of the bands was quantified using ImageJ software and the relative amounts (averages of two independent experiments) are indicated below the bands.
AMPK promotes MafA protein degradation independent of the proteasome and multiple phosphorylation events. (A) Oxidative stress, but not AMPK, promotes proteasomal MafA degradation. MIN6 cells were treated with metformin (Metf, 500 µM) or the inducer of oxidative stress tBHQ (5 µM) with or without the proteasome inhibitor MG132 (5 µM) for 16 h. Cell extracts were then subjected to immunoblot analysis. (B) Schematic representations of MafA mutants. The boxed areas represent the evolutionarily conserved domains. bZip: basic-leucine zipper. (C, D) Wild-type (WT) or mutant forms of HA-tagged MafA (5A, T131A, and ΔN164) shown in (B) were expressed in MIN6 cells. Electrophoretic mobility of the 5A mutant is higher than WT MafA because of loss of phosphorylation events at five Ser/Thr residues. Cells were then treated with or without metformin (Metf, 500 µM) and MG132 (5 µM) for 16 h and were analyzed by immunoblotting. Short exposure of the boxed area of the anti-HA blot (D) was also indicated. (E) Wild-type (WT) or mutant forms of HA-tagged MafA (5A, T131A, and ΔN164) were expressed in MIN6 cells. Cells were then grown in high (Hi, 20 mM) or low (Lo, 2 mM) glucose medium for 16 h and were analyzed by immunoblot analysis. Data in (A), (C), (D), and (E) are representative of two independent biological experiments. The intensity of the bands was quantified using ImageJ software and the relative amounts (averages of two independent experiments) are indicated below the bands.

AMPK decreases insulin expression through MafA down-regulation. (A, B) MIN6 cells were transfected with an empty expression vector or HA-MafA expression plasmid, and were treated with metformin (Metf, 500 µM) for 16 h in high glucose medium. Whole cell extracts and total RNA were subjected to immunoblot analysis (A) and quantitative RT-PCR analysis (B), respectively. Data (A) is representative of two independent biological experiments. The intensity of the bands was quantified using ImageJ software and the relative amounts (averages of two independent experiments) are indicated below the bands. Data (B) are mean ± S.E. of three independent experiments. *: P<0.05 (Student's t test).
human insulin promoter luciferase (pGL4/h-ins-p)

**A**

**B**

**C**

**D**

**Fig. 1**
Fig. 3

Luciferase Activity

A MIN6 cells

| Glucose | Hi | Lo |
|---------|----|----|
| Dorso   | -  | +  |
| pACCα   | 100| 36 |
| ACCα    | 100| 36 |
| pAMPK   | 100| 36 |
| AMPK    | 100| 36 |
| Beta2   | 100| 36 |
| MafA    | 100| 36 |
| Pdx1    | 100| 36 |
| TBP     | 100| 36 |

(kDa)

B Islets

| Glucose | Hi | Lo |
|---------|----|----|
| Dorso   | -  | +  |
| pACCα   | 100| 36 |
| ACCα    | 100| 36 |
| pAMPK   | 100| 36 |
| AMPK    | 100| 36 |
| Beta2   | 100| 36 |
| MafA    | 100| 36 |
| Pdx1    | 100| 36 |
| TBP     | 100| 36 |

(kDa)

C pGL4/h-ins-p

D Relative mRNA levels

E MIN6 cells

| Glucose | Hi | Lo |
|---------|----|----|
| AMPKα1  | -  | -  |
| DN      | -  | +  |
| pACCα   | 100| 36 |
| ACCα    | 100| 36 |
| Beta2   | 100| 36 |
| MafA    | 100| 36 |
| Pdx1    | 100| 36 |
| TBP     | 100| 36 |

(kDa)
**Fig. 4**

A. Relative mRNA levels of mafA, pdx1, and beta2 under different glucose conditions.

B. Relative mRNA levels of mafA, pdx1, and beta2 under different treatments: - (vehicle), + Metf, + AICAR.

C. Western blot analysis of MafA and TBP under - (vehicle) and + Metf conditions with CHX treatment.
Fig. 5

Panel A: Western blot analysis of ACCα, pACCα, AMPK, pAMPK, HA-MafA, and TBP under different conditions. Metf and AICAR treatment are indicated.

Panel B: Western blot analysis of ACCα, pACCα, AMPK, pAMPK, HA-MafA, and TBP in response to glucose concentration changes (Hi: high, Lo: low).

Panel C: Western blot analysis of AMPKα1, pACCα, ACCα, HA-MafA, and TBP under glucose concentration changes (Hi: high, FL: full length, CA: truncated).

Panel D: Western blot analysis of AMPKα1, pACCα, ACCα, HA-MafA, and TBP under different conditions (Hi: high, Lo: low, DN: dominant negative).
Fig. 7

A

| Metf     | empty | HA-MafA |
|----------|-------|---------|
| -        |       |         |
| -        | 100   | 72      |
| +        | 257   | 123     |
|         | 100   | 97      |
|         | 72    | 98      |
|         | 104   |         |
|         | (657,670) | (657,670) |
| pAMPK    | 72    | 100     |
| AMPK     | 72    | 97      |
| HA-MafA  | 55    | 100     |
| MafA     | 55    | 100     |
| MafA + HA-MafA (long exposure) | 55 | 46 |
| TBP      | 36    | 100     |

B

Relative mRNA levels

- empty
- empty + Metf
- HA-MafA
- HA-MafA + Metf

Gene expression levels:
- ins-1
- ins-II
- glut2
- sur1
- kir6.2
Glucose regulates MafA transcription factor abundance and insulin gene expression by inhibiting AMP-activated protein kinase in pancreatic β-cells
Ryo Iwaoka and Kohsuke Kataoka

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