MicroRNA-9 Controls the Expression of Granuphilin/Slp4 and the Secretory Response of Insulin-producing Cells*

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Insulin release from pancreatic β-cells plays an essential role in blood glucose homeostasis. Several proteins controlling insulin exocytosis have been identified, but the factors determining the expression of the components of the secretory machinery of β-cells remain largely unknown. MicroRNAs are newly discovered small non-coding RNAs acting as repressors of gene expression. We found that overexpression of mir-9 in insulin-secreting cells causes a reduction in exocytosis elicited by glucose or potassium. We show that mir-9 acts by diminishing the expression of the transcription factor Onecut-2 and, in turn, by increasing the level of Granuphilin/Slp4, a Rab GTPase effector associated with β-cell secretory granules that exerts a negative control on insulin release. Indeed, electrophoretic mobility shift assays, chromatin immunoprecipitation, and transfection experiments demonstrated that Onecut-2 is able to bind to the granuphilin promoter and to repress its transcriptional activity. Moreover, we show that silencing of Onecut-2 by RNA interference increases Granuphilin expression and mimics the effect of mir-9 on stimulus-induced exocytosis. Our data provide evidence that in insulin-producing cells adequate levels of mir-9 are mandatory for maintaining appropriate Granuphilin levels and optimal secretory capacity.

The insulin in the circulation of adult mammals is produced exclusively by pancreatic β-cells, making them the central regulators of glucose homeostasis. Alterations in β-cell secretory function can cause hyperglycemia and lead to diabetes mellitus. During the last few years a number of key components of the machinery controlling insulin exocytosis have been identified. These components include different SNARE proteins, Rab3 and Rab27 GTPases, and their effectors Noc2 and Granuphilin/Slp4 (1–3). Alteration in the function or in the expression level of these proteins results in insulin secretory defects both in vitro (4–8) and in vivo (9–11). Despite recent progress in understanding the molecular mechanisms regulating the final events in the secretory pathway of β-cells, the factors determining the expression of the proteins involved in insulin exocytosis are largely unknown.

MicroRNAs (miRNAs) are newly discovered regulators of gene expression that act by targeting the 3′-untranslated region (3′-UTR) of mRNA sequences and by preventing the productive translation of the messengers (12–15). miRNAs have been implicated in many processes in invertebrates, including cell proliferation, apoptosis (16–18), fat metabolism (17), and neuronal patterning (19). Because of their spatial and temporal expression pattern and their conservation across species (20–22), miRNAs are believed to play similar roles in all animal cells. In mammals, only for a few miRNAs a specific function has been assigned. A subset of miRNAs has been shown to be involved in metabolic regulation: mir-143 participates in human adipocyte differentiation (23), and the levels of mir-375, a pancreatic islet-specific miRNA, influence insulin secretion in pancreatic β-cells (24). In this study we investigated whether miRNAs can regulate directly or indirectly the expression of the major components of the exocytotic machinery of β-cells. Because the secretory process of β-cells and neurons relies on several common components, we focused our attention on miRNAs selectively expressed in neurons and insulin-secreting cells.

We found that raising the level of mir-9, an miRNA expressed in neurons, in β-cells, and in the rat β-cell line INS-1E results in drastic impairment of glucose-stimulated insulin release. This perturbation of the secretory functions was associated with an increase in the level of Granuphilin/Slp4, a Rab3/Rab27 effector playing a negative modulatory role on insulin exocytosis (6, 25). Using a combination of approaches we identified the Onecut2 (OC2) transcription factor as a target of mir-9 and demonstrated that this factor exhibits a repressor activity on Granuphilin expression. Silencing of OC2 mimicked the effects of mir-9 on stimulus-induced exocytosis and on Granuphilin expression. Conversely, overexpression of OC2 shift assay; si-GFP, synthetic siRNA; mir-9, synthetic miRNA; sil-OC2, synthesis of siRNAs targeted against rat oc2 mRNA; sil-GFP, synthesis of siRNAs targeted against gfp mRNA; graluc, human granuphilin promoter.

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3 The abbreviations used are: SNARE, soluble NSF attachment protein receptors; miRNA, microRNA; UTR, untranslated region; OC2, Onecut2; siRNA, small interference RNA; GFP, green fluorescent protein; RT, reverse transcription; hGH, human growth hormone; EMSA, electrophoretic mobility

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We found that raising the level of mir-9, an miRNA expressed in neurons, in β-cells, and in the rat β-cell line INS-1E results in drastic impairment of glucose-stimulated insulin release. This perturbation of the secretory functions was associated with an increase in the level of Granuphilin/Slp4, a Rab3/Rab27 effector playing a negative modulatory role on insulin exocytosis (6, 25). Using a combination of approaches we identified the Onecut2 (OC2) transcription factor as a target of mir-9 and demonstrated that this factor exhibits a repressor activity on Granuphilin expression. Silencing of OC2 mimicked the effects of mir-9 on stimulus-induced exocytosis and on Granuphilin expression. Conversely, overexpression of OC2 shift assay; si-GFP, synthetic siRNA; mir-9, synthetic miRNA; sil-OC2, synthesis of siRNAs targeted against rat oc2 mRNA; sil-GFP, synthesis of siRNAs targeted against gfp mRNA; graluc, human granuphilin promoter.
rep懋ed Granuphilin expression. Taken together, our data show that appropriate levels of mir-9 are mandatory for preserving optimal stimulus-induced exocytosis in insulin-secreting cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction and siRNA Duplexes—The expression vector directing the synthesis of mir-9 was prepared by introducing oligonucleotides corresponding to the precursor sequence of mir-9 in front of the H1 RNA promoter of the pSuper vector (Oligoengine, Seattle, WA). The oligonucleotide sequences used were as follows: sense, 5’-ATCTATATTTTTAGATTTATCTCGAGTTTATGAGTGGTATTTGCTCTTCTATAAAAGCTAGATACCGAAAGTTTTTTATAA-3’, and antisense, 5’-AGCTTTAAAATTTCCGGGTATCTAGCTTTTAGAAGACCAATACATCATACAGTAGATACACAAAGATAGCAGT-3’. A pSuper expression vector directing the synthesis of mir-30 (26) was kindly provided by Dr. B. R. Cullen, Duke University. To design target-specific siRNAs against OC2 and GFP, sequences from the coding region of mouse and rat oc2 and gfp were selected using the siRNA Target Finder tool from Ambion (www.ambion.com/techlib/misc/siRNA_finder.html). The specificity of the siRNAs was verified by a search of the sequences deposited at the GenBankTM with the basic local alignment search tool (BLAST). The selected oligonucleotides were cloned in the BglII and HindIII sites of pSuper (27). Sequences of oligonucleotides corresponding to the precursor sequences from the coding region of mouse and rat (26) was kindly provided by Dr. B. R. Cullen, Duke University. To generate the 3’-UTR-OC2-luc construct, the 3’-UTR segment of the rat oc2 gene was amplified by PCR in front of the pSuper-INS-1E genomic DNA and inserted in the multiple cloning site of the pSuper vector (Ambion, Austin, TX). 2 µg of small RNA extract was separated on 15% denaturing polyacrylamide gels and transferred to a Hybond-N+ membrane (Amersham Biosciences). The oligonucleotide sequences used to prepare the Northern blotting probes were as follows: sense, 5’-GATCCCCCGAGTAGCATCACCATTTCCTCTGGAATGTGATCGTACATCCCGGGG-3’; and antisense, 5’-AGCCAGCTAGCTCAGGAGGCTCAGTACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATC-3’. The PCR reaction was performed using the Expand PCR system (Roche Applied Science), following supplier conditions. The PCR product was cut with KpnI and XhoII and cloned into the corresponding sites of the luciferase reporter plasmid pGL3-Basic (Promega). Synthetic miRNA (mir-9) and siRNA (si-GFP) duplexes were obtained from Dharmacon (Lafayette, CO). The sequences of mir-9 were the following: sense, 5’-UCUUUGGUAUCUGCUUGAUAAATT3’; antisense, 5’-UCAUACUGCUAGAACCCAAAGTTA-3’. The sequences of si-GFP were: sense, 5’-GCUACCCUGAGAGUC-AGCTT3’; antisense, 5’-GAACUUACGGGUCAGCTT3’. The pXJ42-OC2 contains a fully coding human OC2 cDNA cloned in the Xhol and EcoRI sites of pXJ42 (28).

Cell Culture, Transient Transfection, and Luciferase Assays—The insulin-secreting cell line INS-1E was cultured as previously described (29) in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 IU/ml penicillin, 50 µg/ml streptomycin, 0.1 mM sodium pyruvate, and 0.001% mercaptoethanol. Transient transfections of plasmids were performed using the Effectene transfection kit (Qiagen, Valencia, CA) with a DNA/Effectene ratio of 1/25. Experiments involving transient transfections of miRNAs or siRNAs were carried out with Lipofectamine 2000 (Invitrogen) using 100 nM RNA duplexes. Luciferase activities were measured 2 days after transfection with the dual-luciferase reporter assay system (Promega). For 3’-UTR-OC2-luc and mir-9-sensor or for granuphilin plasmids, co-transfections with the Firefly luciferase SV40 pGL3 promoter (Promega) or with the Renilla luciferase pRLCMV (pRLrenilla), respectively, were used for normalization.

miRNA Extraction and Northern Blotting—Extraction of small RNAs from rat tissues (newborn brain and adult spleen) and cell lines (INS-1E, MIN6 B1, NIH3T3, and HeLa) was performed using the mirVana miRNA isolation kit (Ambion, Austin, TX). 2 µg of small RNA extract was separated on 15% denaturing polyacrylamide gels and transferred to a Hybrid-N+ membrane (Amersham Biosciences). The oligonucleotide sequences used to prepare the Northern blotting probes were as follows: sense, 5’-TCTTGTGGAATATCCTAGCTGATGCTAGCTCTGCTTC-3’; and antisense, 5’-AGCTGTTCGAGCAGCGCTCGACGCTCGTGATCTGCAGTAC-3’. The 3’-UTR-mature sequence in the antisense orientation with [α-32P]UTP (Amersham Biosciences) by in vitro transcription (Maxiscript kit, Ambion). The U6 level was used as the internal control.

Preparation of Rat Islets—Rat islets were isolated from the pancreas of male Sprague-Dawley rats weighing 250–350 g by ductal injection of collagenase. The purification of islets was conducted as described previously (30).

Conventional RT-PCR Analysis and Real-time PCR—Extraction of total RNA was performed using the RNeasy kit (Ambion). Reverse transcription and conventional PCR analysis were carried out as previously described (31). Real-time PCR assays were carried out on a Bio-Rad MyiQ Single-Color Real-
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Time PCR Detection System (Bio-Rad) using the Bio-Rad iQ SYBR Green Supermix, with 100 nm primers and 1 μl of template per 20 μl of PCR and an annealing temperature of 59 °C. Melting curve analyses were performed on all PCRs to rule out nonspecific amplification. Reactions were performed in triplicates. The primer sequences for PCR are available upon request.

Secretion Experiments—For the assessment of the secretory capacity, INS-1E cells (3 × 10⁵) plated in 24-wells dishes were transiently co-transfected with plasmids or RNA duplexes leading to the expression of miRNAs or siRNAs and with a construct encoding the human growth hormone (hGH). Three days later the cells were washed and preincubated for 30 min in Krebs-Ringer/bicarbonate-Hepes buffer (KRBH: 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 1.5 mM CaCl₂, 2 mM NaHCO₃, 10 mM Hepes, and 0.1% bovine serum albumin) containing 2 mM glucose (basal conditions). The medium was then removed, and the cells were incubated for 45 min in the same buffer (basal conditions) or in a buffer containing 20 mM glucose, 10 μM forskolin, and 100 μM isobutylmethylxanthine or 24 mM KCl (stimulatory conditions). Exocytosis from transfected cells was assessed by measuring by enzyme-linked immunosorbent assay (Roche Diagnostics) the amount of hGH released into the medium during the incubation period.

Nuclear and Total Extract Preparation and Western Blotting—For Western blot analysis, the cells were washed once in ice-cold phosphate-buffered saline, nuclear extracts were prepared as described before (31), and total extracts were obtained by precipitation studies were performed essentially as described (33). Precleared cell lysates were incubated overnight at 4 °C with 5 μg of GFP antibody (Molecular Probes) or 4 μg of OC2 antibody. 5 μl of DNA was used for PCR to detect the presence of specific DNA segments with the following primers: OC2-granuphilin: sense, 5'-TGGGGAGGGTATGTTAATAT-3'; and antisense, 5'-CTTCTAGACACTGGAAGCA-3'; rat insulin promoter: sense, 5'-AGGAGGTAGTGGC-3', and antisense, 5'-AAGTAGAGTTTGGACG-3'.

RESULTS

Mir-9 Is Selectively Expressed in Brain and In Insulin-secreting Cells—We first analyzed by Northern blotting the expression of several miRNAs in two insulin-secreting cell lines (the rat β-cell line INS-1E and the mouse β-cell line MIN6 B1) (Fig. 1A), in freshly isolated rat pancreatic islets (Fig. 1B), in two cell lines unrelated to β-cells (NIH3T3 and HeLa), and in two rat tissues (spleen and brain) (Fig. 1A). In agreement with previous reports (34, 35), mir-16, mir-23b, and mir-30 were detected in all RNA extracts, whereas mir-142 expression was restricted to spleen (Fig. 1A). mir-9 was found to be expressed at high level in brain and, to a lower extent, in the two insulin-secreting cell lines, as well as in pancreatic islets (Fig. 1B), but was undetectable in RNA extracts from NIH3T3 and HeLa cells and from spleen.

Assessment of mir-9 Activity in Insulin-secreting Cells—The restricted expression of mir-9 in neuronal and islet tissues prompted us to test for a role of mir-9 in β-cell-specific tasks. To analyze the function of mir-9, we increased the cellular miRNA content by generating a pSuper plasmid (27) containing the sequence of the mir-9 precursor under the control of the H1 promoter (pSup-mir-9). Northern blotting analysis showed that transient transfection of this plasmid in INS-1E cells led to a significant increase in mir-9 expression (Fig. 2A). To evaluate the activity of mir-9, the mature mir-9 sequence was cloned in the antisense orientation in the 3'-untranslated region (3'- UTR) of a luciferase reporter gene. This construct (mir-9-sensor) allows a perfect match between mir-9 and its target sequence located in the 3'-UTR of the luciferase mRNA. Therefore, the expression of the luciferase reporter is determined by the amount of mir-9 present in the cells. As shown in Fig. 2B, the luciferase activity in INS-1E cells transfected with the mir-9-sensor was significantly lower than in cells transfected with a vector lacking the mir-9 target sequence (empty psiCHECK-1). This finding confirms the inhibitory action exerted by endogenous mir-9. Indeed, in HeLa cells that do not express mir-9 (Fig. 1A), the empty psiCHECK-1, and the mir-9-sensor were expressed at the same level (data not shown). As expected, co-transfection of the mir-9-sensor with pSup-mir-9 resulted in a
further decrease in the luciferase activity. In contrast, a pSuper construct leading to the overexpression of mir-30 (pSup-mir-30) (26) did not affect the expression of the luciferase. An alternative approach to increase the cellular content of miRNAs consists in transfecting the cells with a siRNA duplex homologous to the mature sequence of the miRNA (36). Similar to pSup-mir-9, transfection of mir-9 siRNA duplex (mir-9) resulted in a decrease in the luciferase activity encoded by the mir-9 sensor, whereas a control siRNA duplex against GFP (si-GFP) was without effect (Fig. 2B).

**Overexpression of mir-9 Results in a Decrease in Stimulus-induced Secretion**—To evaluate the role of mir-9 on the secretory capacity, we co-transfected INS-1E cells with pSup-mir-9 and with a plasmid encoding hGH. We have previously shown that hGH is targeted to secretory granules and is co-released with insulin during exocytosis (4). This approach allows a direct assessment of the secretory activity of transiently transfected cells independently of their capacity to produce insulin. Three days after transfection, the cells were incubated under resting conditions (2 mM glucose) or under conditions that stimulate insulin release (20 mM glucose, isobutylmethylxanthine, and forskolin). Increase in mir-9 expression obtained either by transfection of pSup-mir-9 (Fig. 3A) or mir-9 duplex (data not shown) was associated with a diminished secretory capacity of INS-1E cells in response to glucose and cAMP-raising agents. A similar impairment in hormone release was observed when exocytosis was triggered by directly depolarizing the cell membrane with 24 mM potassium (Fig. 3B). The inhibition of exocytosis in miR-9-overexpressing cells was confirmed also when the amount of hGH released during the incubation period was normalized to the amount of hGH produced by the cells under each condition (data not shown). In contrast to the results obtained with mir-9, overexpression of other miRNAs (mir-30, mir-7, or mir-153) did not affect exocytosis in response to secretagogues (data not shown). Together, these data show that appropriate levels of mir-9 are required to maintain optimal stimulus-induced exocytosis.

**Overexpression of mir-9 Up-regulates Granuphilin/Slp4 Expression**—The impairment in KCl-induced exocytosis points to a perturbation in the secretory machinery rather than a defect in stimulus-secretion coupling. This prompted us to evaluate the potential effects of mir-9 on the expression levels of key components of the exocytic machinery. Western blot and real-time quantitative PCR showed a 2- and a 14-fold increase in Granuphilin protein and mRNA levels, respectively.
in cells overexpressing mir-9 (Fig. 4). Under these conditions, the expression levels of other Rab effectors such as RIM2, MyRIP/Slac2c, and Noc2 (Fig. 4A), of the Rab GTPases Rab3a and Rab27a, or of the SNAREs SNAP25, VAMP-2, and Syntaxin-I (data not shown) were unchanged. Overexpression in INS-1E cells of several other miRNAs, including mir-153, mir-375, mir-124a, and mir-7, did not modify Granuphilin expression (data not shown), confirming the specificity of the effect of mir-9 on this important regulator of insulin exocytosis.

The Transcription Factor OC2 Is a mir-9 Target and Can Directly Regulate the Expression of Granuphilin—Because miRNAs are known to function as translational repressors, the observed rise in granuphilin mRNA is unlikely to result from a direct action of mir-9. We reasoned that this effect was due either to an enhanced stability of granuphilin mRNA or to an increase in the activity of the granuphilin promoter. Because many transcription factors are known to be miRNA targets, we favored the second hypothesis. To identify potential targets of mir-9 we inspected the list of gene targets predicted by the TargetScan algorithm (genes.mit.edu/targetscan) (37, 38).
Plasmids that direct the synthesis of siRNAs targeted against rat OC2. RNA interference is triggered by the presence of short double-stranded RNA molecules called small interfering RNAs (siRNAs) that lead to specific silencing of their target. Among these genes, we found the transcription factor OC2, a member of the Onecut family (28, 39). The three potential binding sites for mir-9 located in the 3'-UTR of the rat OC2 gene are shown in Fig. 5A. RT-PCR analysis demonstrated that OC2 can be detected in the insulin-secreting cell line INS-1E and in isolated rat pancreatic islet (Fig. 5B). To test whether OC2 is a mir-9 target, the 3'-UTR region of the OC2 gene was cloned downstream of a luciferase reporter gene. This construct (3'-UTR-OC2-luc) was then co-transfected in INS-1E cells with pSuper-mir-9 (Fig. 6A) or mir-9 duplex (Fig. 6B). Overexpression of mir-9 was found to decrease the luciferase activity of 3'-UTR-OC2-luc by 3-fold compared with cells transfected with an empty construct or with negative controls (pSuper-mir-30 in Fig. 6A and si-GFP in Fig. 6B). This result indicates that mir-9 is able to recognize the 3'-UTR of the OC2 mRNA and to repress its translation. In agreement with this finding, Western blot analysis confirmed a reduced expression of OC2 in INS-1E cells transfected with the mir-9 duplex (Fig. 6C).

The possible role of OC2 in the control of Granuphilin expression was then investigated. For this purpose, we took advantage of the RNA interference process to selectively reduce endogenous levels of OC2. RNA interference is triggered by the presence of short double-stranded RNA molecules called small interfering RNAs (siRNAs) that lead to specific silencing of gene expression in a sequence-specific manner (36, 40). Plasmids that direct the synthesis of siRNAs targeted against rat OC2 mRNA (sil-OC2) or, as negative control, against gfp mRNA (sil-GFP) were generated. Semi-quantitative RT-PCR and Western blot analyses showed that introduction of sil-OC2 into INS-1E cells results in a strong reduction in the expression of both endogenous OC2 (Fig. 7A) and transiently co-transfected OC2 (Fig. 7B). These data indicate that expression of this transcription factor can be efficiently reduced using specific siRNAs.

To assess the role of OC2 on granuphilin transcription, the sil-OC2 was co-transfected with a luciferase reporter gene under the control of the human granuphilin promoter (graluc). Silencing of OC2 resulted in a sizeable increase of luciferase activity, whereas the empty vector and sil-GFP had no effect (Fig. 8A). Conversely, co-transfection of graluc with a vector encoding full-length rat OC2 led to a drastic decrease in the activity of the granuphilin promoter (Fig. 8B), indicating that this transcription factor is able to negatively modulate the expression of the Rab effector.

Examination of the 4-kb promoter sequence of graluc revealed the presence of a putative OC2-binding site (between −1567 and −1500 bp) that is conserved between human, rat, and mouse. Nucleotides known to be required for functional integrity of the motif are preserved (28) (Table 1). EMSA experiments were performed to verify the ability of OC2 to bind to this putative binding sequence (Fig. 9). Nuclear extracts from INS1-E cells were incubated with the putative OC2 element...
from the granuphilin promoter as a labeled probe. Slow migrating complexes were observed (Fig. 9A). The use of a 100-fold molar excess of unlabeled OC2 granuphilin element or OC2 motif from hnf3/H9252 promoter (that has been previously shown to specifically bind OC2 (28)) prevented the formation of the three upper complexes (A1, A2, and A3), whereas unspecific sp1-binding oligonucleotides (41) did not (Fig. 9A). Supershift experiments using an antibody against OC2 demonstrate that the A1 complex includes the OC2 protein. This complex was not supershifted by the addition of antibodies against OC1/HNF6 or C/EBPB/H9252 confirming the specificity of the binding (Fig. 9B). A2 and A3 complexes probably contain OC2-like factors that are not recognized by the OC2 antibody and whose identity remains to be determined. To further demonstrate binding of OC2 to the granuphilin promoter we performed chromatin immunoprecipitation experiments in INS1-E cells using a primer set designed to encompass the OC2 element within the rat granuphilin promoter. As a negative control for the binding of OC2, a primer set that encompasses the insulin promoter was used. The results in Fig. 10 confirm that OC2 associates with consensus motif found in the granuphilin promoter.

The effect of OC2 on endogenous granuphilin expression was verified. Cells were transfected with sil-OC2 or sil-GFP plasmids and were evaluated for their ability to increase granuphilin expression in the presence or absence of sil-OC2 by measuring the level of the granuphilin transcript by real-time PCR. As shown in Fig. 11, silencing of OC2 resulted in an increase in granuphilin expression, whereas transfection of the cells with an empty plasmid or with sil-GFP was without effect. Taken together, these data indicate that mir-9 mediates at least part of its effect on granuphilin gene through the OC2 transcription factor.

Stimulus-induced Secretion from INS-1E Cells Is Reduced after Silencing of OC2—Next we investigated the impact of OC2 silencing on the secretory capacity of INS1-E cells. We found...
TABLE 1
Sequence comparison between the putative granuphilin OC2 element and known functional OC2 elements

| Gene   | Species | Sequence                          |
|--------|---------|-----------------------------------|
| HNF3β  |         | GAAAAAAAAATCAATATCGGGCC           |
| PFK-2 GRU |    | ACAAAAAAAATCCATAACTTTCA          |
| granuphilin | R | CATTAAAAATCAAGTTC               |
|         | H | CTTAAAATCCAGTTC                 |
|         | M | CATTAAAAATCAAGTTC               |

* HNF3β, hepatocyte nuclear factor 3β; PFK-2 GRU, sequence in the GRU of the first intron of rat phosphofructo-2-kinase.
* R, rat; H, human; M, mouse. The underlined sequences correspond to the consensus for OC2-binding.

that, in cells co-transfected with sil-OC2 and with the hGH-expressing vector, hormone release elicited by glucose and cAMP-raising agents is reduced by 45% compared with cells transfected with empty pSuper or sil-GFP (Fig. 12A). The ability of cells transfected with sil-OC2 to secrete in response to 24 mM KCl was diminished by 50% compared with control cells (Fig. 12B). These results indicate that appropriate levels of OC2 are required for optimal stimulus-induced secretion and that reduction in OC2 expression consequent to an increase of mir-9 may contribute to the observed defect in exocytosis.

DISCUSSION

Insulin release from pancreatic β-cells is a precisely regulated event permitting tight control of blood glucose levels. Fine-tuning of insulin secretion is insured by the concerted action of several proteins associated with secretory granules and plasma membrane of β-cells. Despite recent progress in understanding the mode of action of these proteins we still ignore the mechanism that determines the expression of the key elements required for insulin exocytosis. In this study, we demonstrate that mir-9, an miRNA whose expression is restricted to brain and pancreatic islets, plays a critical role in the control of the secretory function of insulin-producing cells. Overexpression of mir-9 led to a loss in the efficiency of the cells to respond to stimuli, suggesting an alteration in the function of the secretory machinery rather than a defect in signal transduction. Consistent with this hypothesis, we found that mir-9-overexpressing cells display increased Granuphilin levels. Variations in the secretory activity and in Granuphilin expression were not observed after transfecting other more widely expressed miR-

FIGURE 9. Sequence-specific binding activity of OC2 on granuphilin OC2-element. EMSA with 32P-labeled granuphilin OC2-element using nuclear extracts from INS-1E cells. A, slow migrating complexes (arrows A1, A2, and A3) were detected, compared with free probe migration. The DNA-binding activity with granuphilin OC2-element was competed by adding a 100-fold molar excess of unlabeled wild-type granuphilin OC2-element and of unlabeled wild-type hnf3β OC2-element but not with the consensus sp1 element binding. B, this pattern was supershifted by OC2 antibodies but not with antibodies directed against unrelated proteins such as HNF6 and C/EBPβ transcription factors.

FIGURE 10. Occupancy of the promoter of granuphilin by OC2. Formaldehyde cross-linked chromatin samples from INS1-E cells were used for immunoprecipitation with antibodies against OC2 (anti-OC2) and GFP (anti-GFP) (negative control). The precipitated DNA was analyzed by PCR with specific primers corresponding to rat granuphilin promoter or with primers specific to insulin rat I promoter (negative control for the binding of OC2). DNA aliquots from PCR reactions performed on the extracts before immunoprecipitation (Input) were loaded on the same gel.

FIGURE 11. Analysis of the inhibition of OC2 on granuphilin transcript. INS-1E cells were transiently transfected with the empty pSuper, sil-OC2, or sil-GFP (control). Two days later, total RNA was isolated and real-time PCR was performed to measure the granuphilin and tubulin (internal control) mRNAs level. All values are expressed as percent of empty plasmid and are means ± S.E. *, p < 0.05.

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As such as mir-30 or mir-15b indicating that this effect is specific for mir-9. Granuphilin is a Rab3/Rab27 effector that is associated with dense-core secretory granules of pancreatic β-cells and few other endocrine cells (6, 42, 43). Granuphilin exerts a potent negative modulatory action on insulin exocytosis. Indeed, overexpression of Granuphilin in pancreatic β-cells results in a strong decrease in insulin release evoked by secretagogues (6, 44). Moreover, Granuphilin knock-out mice secrete abnormally elevated amounts of insulin in response to stimuli (25). Thus, maintaining adequate levels of this protein appears to be a critical prerequisite for preserving the fine-tuning of β-cell exocytosis. miRNAs are known to attenuate rather than to increase gene expression. This prompted us to search for factors mediating the effect of mir-9 on Granuphilin expression. Among the mir-9 targets predicted using bioinformatic tools, we focused our attention on two factors involved in gene regulation: the transcription factor OC2 and the mRNA-binding protein PTBP1. Direct experimental testing in insulin-secreting cell lines revealed that only the transcription factor OC2 is a direct target of mir-9. This factor was already shown to be present in whole pancreas during embryonic development and shortly after birth (28, 39). Here we show that relatively low levels of OC2 can also be detected in adult islet and in two well differentiated insulin-secreting cell lines. Silencing of OC2 in INS-1E cells mimicked the effects of mir-9 on stimulus-induced secretion and Granuphilin expression. These results indicate that mir-9 exerts at least part of its action by reducing OC2 expression. EMSA, chromatin immunoprecipitation, and gene reporter experiments showed that OC2 negatively regulates the expression of Granuphilin by binding to a specific OC2 element located in the gene promoter. Indeed, overexpression of OC2 decreased the activity of a luciferase reporter gene under the control of the granuphilin promoter and silencing of OC2 by RNA interference resulted in the opposite effect. Thus, although OC2 was previously characterized as a transcriptional activator, our present data indicate that, like the related factor HNF6 (45), it can also act as a transcriptional repressor and inhibit transcription from some promoters. OC1/HNF6, which plays a pivotal role in the differentiation of pancreatic islets (46–48), displays close homologies with OC2 and has distinct but overlapping DNA binding and transcriptional activity properties, suggesting an eventual role of this transcription factor in Granuphilin expression. However, several studies have failed to detect HNF6 in mature pancreatic β-cells (49, 50) rendering this hypothesis very unlikely. Moreover, EMSA experiments show that antibodies directed against HNF6 do not supershift the protein-OC2-like element DNA complex. More experiments are necessary to characterize the molecular mechanism by which OC2 down-regulates the granuphilin promoter.

Although mir-9 functions as a repressor, we showed here that it is co-expressed in insulin-secreting cells with its target gene oc2. This may be explained by the relative low expression level of mir-9 in INS-1 cells. Moreover, we found that overexpression of OC2 also alters insulin secretion in INS-1E cells (data not shown), suggesting that too high levels of OC2 can be deleterious for β-cell function. Together, these findings support the idea that mir-9 is required to maintain appropriate levels of OC2 in insulin-producing cells. Tight balance between these factors may be necessary to determinate the appropriate level of key components of the machinery of exocytosis. In neurons mir-9 expression is much higher than in pancreatic β-cells. Because neurons do not express Granuphilin, it can be speculated that much higher expression of mir-9 can be toler-
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Figure 13. Summary of the effects of miR-9 on OC2 and Granuphilin expression and on insulin exocytosis. The presence of relatively low amounts of miR-9 allows the expression of OC2. This in turn controls the level of Granuphilin and permits an efficient response to insulin secretagogues. If miR-9 increases, the expression of OC2 drops, and the consequent rise in the level of Granuphilin exerts a negative control on the secretory response of the cells.

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