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Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs

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Abstract

Fine-tuning of insulin secretion from pancreatic β-cells participates in blood glucose homeostasis. Defects in this process can lead to chronic hyperglycemia and diabetes mellitus. Several proteins controlling insulin exocytosis have been identified, but the mechanisms regulating their expression remain poorly understood. Here, we show that two non-coding microRNAs, miR124a and miR96, modulate the expression of proteins involved in insulin exocytosis and affect secretion of the β-cell line MIN6B1. miR124a increases the levels of SNAP25, Rab3A and synapsin-1A and decreases those of Rab27A and Noc2. Inhibition of Rab27A expression is mediated by direct binding to the 3′-untranslated region of Rab27A mRNA. The effect on the other genes is indirect and linked to changes in mRNA levels. Overexpression of miR124a leads to exaggerated hormone release under basal conditions and a reduction in glucose-induced secretion. miR96 increases mRNA and protein levels of granuphilin, a negative modulator of insulin exocytosis, and decreases the expression of Noc2, resulting in lower capacity of MIN6B1 cells to respond to secretagogues. Our data identify miR124a and miR96 as novel regulators of the expression of proteins playing a critical role in insulin exocytosis and in the release of other hormones and neurotransmitters.

Keywords: exocytosis; insulin secretion; microRNA; neurotransmitter release; pancreatic β-cell.

Introduction

Insulin secretion from pancreatic β-cells is an essential requirement for blood glucose homeostasis. The release of inappropriate amounts of this hormone leads to severe changes in blood glucose levels that favor the development of diabetes mellitus. The insulin secretory pathway is complex and involves a number of steps that allow vesicular transport of the hormone from rough endoplasmic reticulum to the Golgi apparatus and its delivery to the cell periphery. Although all these events occur in a highly coordinated fashion, the minute-to-minute control of insulin release is mainly accomplished through fine-tuning of exocytosis, the final step of the secretory pathway. During the last decade several, essential components of the molecular machinery that drives insulin exocytosis have been identified. Interestingly, β-cell exocytosis was found to rely on a group of proteins playing a key role in neurotransmitter release, including the SNAREs syntaxin-1, SNAP25 and VAMP2, the SNARE-interacting proteins Munc18-1, Munc13-1, complexin I and the Ras-like GTPase Rab3A (Lang, 1999; Gerber and Sudhof, 2002; Rorsman and Renstrom, 2003). Indeed, despite endodermal origin, pancreatic β-cells display a gene expression pattern largely overlapping that of neuronal cells, probably reflecting physiological similarities in secretory functions and electrical coupling. However, insulin secretion also requires the contribution of non-neuronal proteins such as granuphilin, Noc2 and Rab27A that are selectively enriched in endocrine cells (Matsumoto et al., 2004; Gomi et al., 2005; Kasai et al., 2005). Appropriate balance between neuron-specific and endocrine-specific components appears to be required for optimal secretory functions of β-cells. Indeed, ectopic expression of REST/NRSF (response element silencing transcription factor), a neuronal repressor whose absence in mature β-cells and neurons allows the expression of neuronal phenotypic traits, leads to impairment of glucose-induced insulin secretion (Abderrahmani et al., 2004).

Chronic exposure to elevated levels of glucose and saturated free fatty acids has a deleterious impact on β-cell secretory functions and is thought to promote the development of diabetes (Prentki and Nolan, 2006). Accumulating evidence indicates that defective insulin release may not only be linked to altered expression of metabolic enzymes and glucose signaling, but could also result from differences in the level of critical components of the secretory machinery of β-cells. Indeed, the expression of genes required for insulin exocytosis has been found to be altered in both diabetes animal models (Nagamatsu et al., 1999; Gaisano et al., 2002) and pancreatic islets of diabetic patients (Ostenson et al., 2006). Unfortunately, the molecular mechanisms governing the expression of the key genes involved in insulin exocytosis are poorly understood. We recently demonstrated that induction of the transcriptional repressor ICER in response to prolonged exposure to supraphysiological glucose concentrations led to a strong reduction in the expression of the GTPases Rab3A, Rab27A and in two of their effectors, granuphilin and Noc2 (Abderrahmani et al., 2006). However, changes in the level and/or activity of other molecules regulating gene expression are very likely to contribute to β-cell secretory dysfunction.

Gene expression and cell differentiation are controlled by the concerted action of a large number of transcription factors. However, eukaryotic cells were recently discovered to express hundreds of small (approx. 22 nt) non-coding RNAs called microRNAs (miRNAs) that play

previously unsuspected roles in the regulation of gene expression (Bartel, 2004; Alvarez-Garcia and Miska, 2005). The members of this new class of gene regulators function by partially pairing to sequences in the 3′-untranslated region (3′UTR) of target mRNAs and by inhibiting their translation. The immense regulatory potential of miRNAs is only beginning to be appreciated, but the involvement of these tiny RNA molecules in a variety of physiological processes and in human diseases has already been established. Moreover, there is also evidence of the involvement of miRNAs in the control of pancreatic β-cell functions. Indeed, miR9 and miR375 were found to influence directly or indirectly the expression of proteins involved in exocytosis and to affect insulin secretion (Poy et al., 2004; Plaisance et al., 2006).

Better knowledge of the molecular mechanisms controlling expression of the genes required for insulin exocytosis is needed both to improve our understanding of the differentiation process leading to the acquisition of a secretory phenotype of β-cells and to elucidate the factors affecting insulin secretion in pathophysiological conditions. In this study, we identified two miRNAs governing the expression of key components of the machinery of exocytosis of β-cells and demonstrate that changes in their cellular levels are associated with secretory dysfunctions.

Results

Two miRNAs, miR9 and miR375, have been previously demonstrated to control directly or indirectly the secretory functions of pancreatic β-cells (Poy et al., 2004; Plaisance et al., 2006). To identify other miRNAs potentially involved in the regulation of insulin secretion, we transfected the mouse β-cell line MIN6B1 (Lilla et al., 2003) with RNA duplexes containing the sequences of the mature form of different miRNAs. We selected miRNAs enriched in neurons and/or β-cells (miR7, miR9, miR124a and miR375), as well as miRNAs with a much broader expression pattern (miR15b, miR96, miR146, miR153, miR195, miR210). Some miRNAs have been found to affect insulin biosynthesis (Baroukh et al., 2007). To avoid potentially confounding effects of miRNAs on insulin biosynthesis, the cells were co-transfected with a plasmid leading to constitutive expression of human growth hormone (hGH). When introduced into β-cells, hGH is packaged in dense-core granules and is co-released with insulin, allowing the monitoring of exocytosis from transiently transfected insulin-secreting cells independently from their capacity to produce insulin (Varadi et al., 2002). In addition, since hGH is exclusively secreted from cells overexpressing the miRNAs, this strategy maximizes the possibilities to detect potential secretory defects in the case of suboptimal transfection efficiency. Three days after transfection, hormone secretion was measured under basal conditions (2 mM glucose) and in the presence of 20 mM glucose and cAMP-raising agents, a condition that triggers a strong secretory response in these cells (Lilla et al., 2003). We found that overexpression of miR124a and miR96 causes distinct changes in the cellular secretory properties. Indeed, miR124a led to a significant increase in hormone release at low glucose concentration and to a reduction in the cellular capacity to respond to secretagogues (Figure 1). An increase in miR96 was also associated with a diminished cellular response to glucose and cAMP-raising agents, but this miRNA did not affect basal secretion. As previously reported, overexpression of miR9 (Plaisance et al., 2006) and miR375 (Poy et al., 2004) did not alter basal hormone release, but reduced exocytosis from cells stimulated by secretagogues to an extent comparable to that of miR96 (Figure 1). In contrast, the other miRNAs, including miR7 and miR15b (Figure 1), did not modify the secretory properties of MIN6B1 cells.

In view of these findings we used Western blotting to analyze the expression of a series of proteins known to play a central role in insulin exocytosis (Lang, 1999; Gerber and Sudhof, 2002; Rorsman and Renstrom, 2003). We found that miR124a increases the expression of SNAP25, Rab3A and synapsin-1A, whereas the levels of Rab27A and Noc2 were reduced (Figure 2). The expression of other key components of the machinery of exocytosis of β-cells and of the cytoskeletal proteins tubulin and actin was unaffected. miR96-transfected cells were found to express higher levels of granuphilin, a Rab effector known to negatively modulate insulin exocytosis

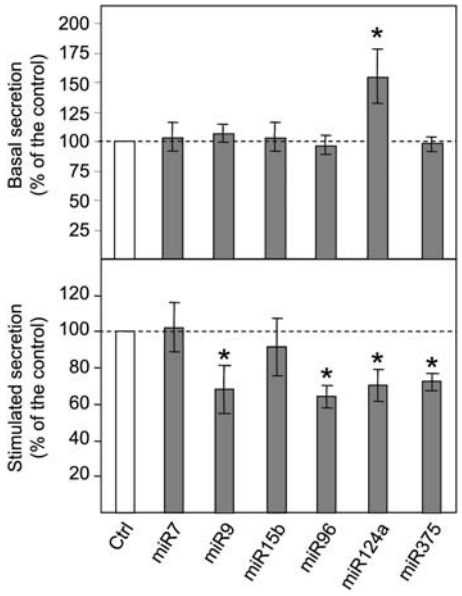

Figure 1 Effect of different miRNAs on basal and stimulated secretion.
MIN6B1 cells were transiently co-transfected with an hGH-encoding plasmid and with oligonucleotide duplexes containing the mature sequence of the miRNAs indicated. Control cells were co-transfected with an unrelated oligonucleotide duplex (see materials and methods). Three days later the cells were incubated either with 2 mM glucose (basal, upper panel) or 20 mM glucose, 10 μM forskolin and 100 μM IBMX (stimulated, lower panel). The fractions of hGH released in the medium or remaining in the cells at the end of the incubation period were determined by ELISA. The results are given as a percentage of the values obtained for control cells and correspond to the mean±SEM of four or five independent experiments performed in triplicate for each miRNA. Over the 45-min incubation period, control cells secreted 6.5% of their hGH content under basal conditions and 68% in the presence of the stimuli. *Significantly different from control cells ($p<0.05$ by ANOVA).

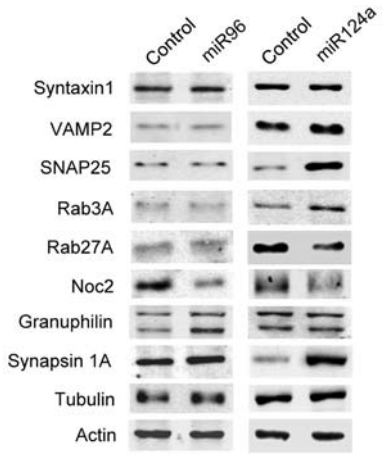

Figure 2 Expression of key components of the exocytotic machinery in cells overexpressing miR124a or miR96.
MIN6B1 cells were transiently transfected with oligonucleotide duplexes corresponding to the mature sequence of miR124a or miR96 or with a control RNA duplex. Three days later the cells were homogenized and the expression of the indicated components of the insulin secretory machinery was analyzed by Western blotting. The Figure shows representative images of at least three independent experiments.

(Coppola et al., 2002; Gomi et al., 2005), and lower amounts of Noc2, an essential component of the β-cell secretory machinery (Cheviet et al., 2004; Matsumoto et al., 2004). Levels of all the other components of the β-cell secretory machinery tested were unchanged (Figure 2). The effect of miR124a on the exocytotic proteins was clearly distinct from those of miR9 and miR375 (Figure 3). In contrast, the effect of miR96 was partially similar to that of miR9 (Figure 3).

The mechanisms underlying the effect of the two miRNAs on the proteins of the exocytotic machinery was then investigated in more detail. miRNAs are generally believed to function as translational repressors (Bartel, 2004; Alvarez-Garcia and Miska, 2005), and SNAP25, Rab3A and synapsin-1A are not among the predicted targets of miR124a (John et al., 2004; Krek et al., 2005; Lewis et al., 2005). Moreover, the increase in SNAP25, synapsin-1A and Rab3A proteins correlates with an increase in the corresponding mRNAs (Figure 4). Taken together, these observations suggest that miR124a action is indirect and probably mediated by changes in gene transcription. In contrast to the other components, the inhibition of Rab27A and Noc2 expression would in principle be compatible with a direct effect of miR124a on mRNA translation. Indeed, putative miR124a recognition sites have been identified in the 3′UTR of Rab27A and Noc2 mRNAs (John et al., 2004; Krek et al., 2005; Lewis et al., 2005). To verify these computational predictions, the sequences corresponding to the putative miR124a recognition sites in Rab27A and Noc2 mRNAs were cloned in the 3′UTR of a luciferase reporter plasmid. Co-transfection in MIN6B1 cells revealed that overexpression of miR124a decreases the amount of luciferase produced from the construct bearing the recognition sequence located in the Rab27A mRNA (Figure 5A). The effect was even more pronounced with a luciferase construct including a larger portion of the Rab27A 3′UTR that encompasses two putative miR124a binding sites (Figure 5A). Overexpression of another miRNA (miR15b) had no effect on both of these constructs (data not shown). miR124a was unable to modulate the expression of a luciferase plasmid containing the 3′UTR of an unrelated gene (OC2) (Figure 5A). These observations indicate that miR124a exerts its action on Rab27A by directly binding to the GTPase mRNA and by inhibiting its translation. In agreement with this assumption, despite a strong decrease in Rab27A protein levels, there was no significant effect of miR124a on Rab27A mRNA content (Figure 4). In contrast to the results obtained with Rab27A constructs, the luciferase activity generated by the plasmid containing the putative target sequence identified in the 3′UTR of Noc2 mRNA was not affected by the overexpression of miR124a (Figure 5B). Moreover, the decrease in Noc2 protein elicited by miR124a was accompanied

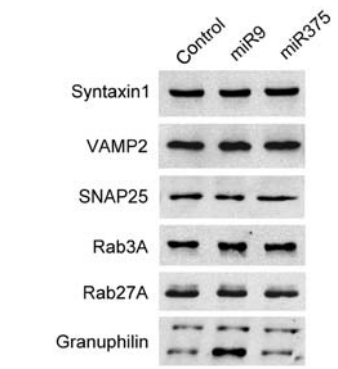

Figure 3 Expression of key components of the exocytotic machinery in cells overexpressing miR9 or miR375.
MIN6B1 cells transfected with oligonucleotide duplexes corresponding to the mature sequence of miR9, miR375 or with a control RNA duplex were homogenized and the expression of the indicated components of the insulin secretory machinery was analyzed by Western blotting. The Figure shows representative images of at least three independent experiments.

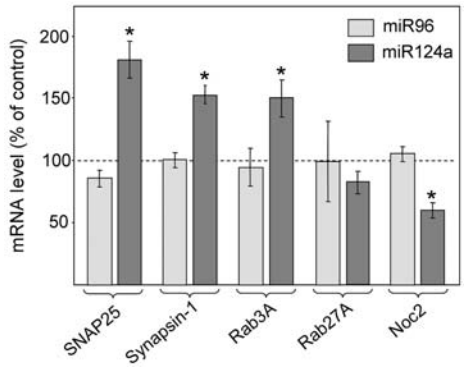

Figure 4 mRNA levels of different components of the exocytotic machinery in cells overexpressing miR124a and miR96.
MIN6B1 cells were transfected with a control RNA duplex or with an RNA duplex containing the mature sequence of miR124a or miR96. Three days later, total RNA was extracted and the mRNA levels of the indicated components of the exocytotic machinery were determined by RT-PCR analysis. The level of each mRNA in cells overexpressing miR124a and miR96 is given as a percentage of the mRNA content in control cells. The values significantly different from controls ($p<0.05$) are indicated by asterisks.

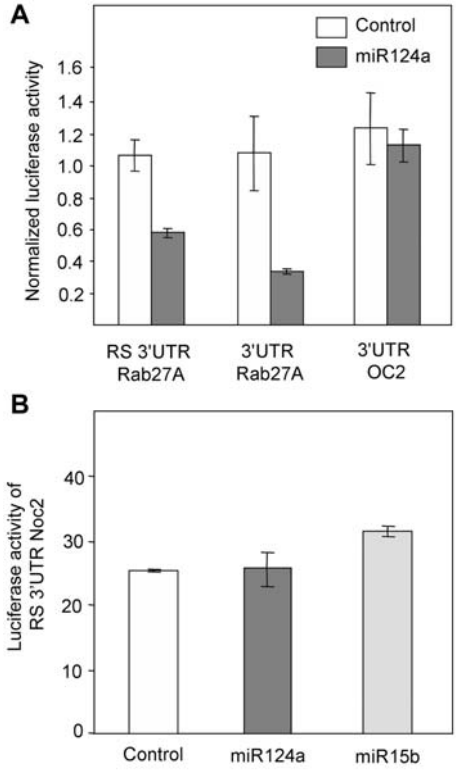

Figure 5 Rab27a is a direct target of miR124a, but not Noc2. (A) MIN6B1 cells were transfected with a luciferase reporter construct containing one of the miR124a recognition sites present in Rab27A mRNA (RS 3′UTR Rab27A), a larger portion of the 3′UTR of Rab27A containing putative recognition sites (3′UTR Rab27A) or the entire 3′UTR of OC2 (3′UTR OC2). Each of the reporter constructs was co-transfected either with a control RNA duplex (open bars) or with an RNA duplex corresponding to the mature sequence of miR124a (gray bars). The luciferase activity produced from the different reporter constructs was measured 2 days later. The results show one representative experiment out of three. (B) Cells were transiently transfected with a luciferase reporter construct containing the putative miR124a recognition sequence located in the 3′UTR of Noc2 (RS 3′UTR Noc2), together with either a control RNA duplex or with oligonucleotide duplexes corresponding to the mature sequences of miR124a and miR15b.

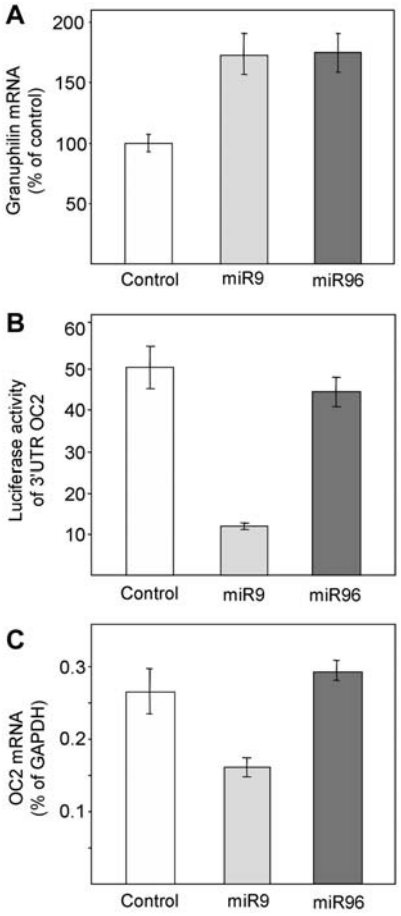

Figure 6 miR96 and miR9 control the expression of granuphilin via different mechanisms.
(A) MIN6B1 cells were transfected with a control RNA duplex or with duplexes corresponding to the sequences of miR9 or miR96. Three days later total RNA was extracted and the level of granuphilin mRNA was determined by RT-PCR. The granuphilin mRNA level in control cells was set to 100%. Results are the mean±SEM for one representative experiment out of three. (B) Cells were transfected with a luciferase reporter construct containing the 3′UTR of OC2, together with control RNA, miR9 or miR96. The luciferase activity produced from the reporter construct was measured 3 days later. (C) MIN6B1 cells were transfected with a control RNA duplex or with duplexes corresponding to the sequences of miR9 or miR96. Three days later total RNA was extracted and the level of OC2 mRNA levels was estimated by RT-PCR. Results are expressed as a percentage of GAPDH mRNA, which was used as an internal control.

by a sizable decrease in the mRNA level (Figure 4). From these observations we conclude that Noc2 is unlikely to be a direct target of this miRNA and that the decrease in expression of this Rab effector is most probably mediated by a change in the level of a transcription factor.

As pointed out above, the impact of miR96 overexpression on insulin exocytosis and granuphilin expression is similar to that of miR9 (Figures 1 and 3) (Plaisance et al., 2006). For both miR9 and miR96, the increase in protein amount was correlated with augmentation of granuphilin mRNA, suggesting an enhancement in gene transcription (Figure 6A). In the case of miR9, we previously demonstrated that the increase in granuphilin expression is caused by translational repression of the synthesis of One Cut 2 (OC2), a negative regulator of granuphilin transcription (Plaisance et al., 2006). Although OC2 is not a predicted target of miR96, we investigated whether a mechanism similar to that of miR9 was also involved in this case. To this end, we transiently co-transfected miR96 with a luciferase reporter construct containing the 3′UTR of OC2. As shown in Figure 6B, the luciferase activity produced from this construct in cells overexpressing cells miR96 was not significantly different from that in control cells. In contrast, in agreement with our previously published data, overexpression of miR9 strongly reduced the expression of the luciferase construct. We then used qRT-PCR to test whether miR96 transfection can affect OC2 mRNA levels via an indirect mechanism. We found that the amount of OC2 mRNA present in cells overexpressing miR96 was not significantly different from that in control cells, while miR9 reduced OC2 mRNA levels by approximately 50% (Figure 6C). Taken together, these findings indicate that miR9 and miR96 increase the expression of granuphilin via distinct pathways. As shown in Figure 2, miR96 also

decreased the amount of Noc2 present in MIN6B1 cells. The 3′UTR of Noc2 mRNA does not contain any potential recognition sequence for miR96 (John et al., 2004; Krek et al., 2005; Lewis et al., 2005). We therefore concluded that the effect of miR96 on the expression of this Rab effector is most probably indirect.

Discussion

Pancreatic β-cells release insulin in response to fluctuations in circulating levels of glucose and other nutrients. The insulin secretory process is finely tuned to ensure the output of an appropriate amount of hormone to meet metabolic demands. Insufficient insulin supply resulting from a decrease in β-cell mass and/or defects in their secretory process leads to chronic hyperglycemia and diabetes mellitus. During the last decade, a number of proteins playing an essential role in insulin secretion have been identified. The expression of several of these proteins in β-cells was found to be altered in diabetes animal models (Nagamatsu et al., 1999; Gaisano et al., 2002) and in diabetic patients (Ostenson et al., 2006). Moreover, different studies have reported changes in the level of some of these components under physiopathological conditions known to favor the development of type 2 diabetes such as chronic hyperglycemia and hyperlipidemia (Busch et al., 2002; Abderrahmani et al., 2006; Kato et al., 2006). Unfortunately, mechanisms controlling the expression of most of the components of the secretory machinery of β-cells are still poorly understood. Detailed knowledge of these mechanisms is essential both to elucidate the causes of defective secretion under disease states and to engineer insulin-secreting cells capable of replacing lost β-cells in diabetic patients (Halban, 2004).

miRNAs, a newly discovered class of gene regulators, were recently shown to be essential for pancreatic β-cell development (Lynn et al., 2007). In this study, we assessed the role of miRNAs in the expression of proteins required for insulin exocytosis. We found that two members of this family of small non-coding RNAs, miR124a and miR96, can affect the expression of several components of the exocytotic machinery and alter hormone release in insulin-secreting cells. It has been shown that miR124a is enriched in neuronal tissues and that its delivery in HeLa cells shifts the gene expression profile towards that of neurons (Lim et al., 2005). Insulin exocytosis relies to a large extent on proteins abundantly expressed in neurons that are required for neurotransmitter release (Lang, 1999; Rorsman and Renstrom, 2003). We found that overexpression of miR124a in MIN6B1 cells increased the level of three such neuronal genes, SNAP25, Rab3A and synapsin-1A. This would in principle improve the cellular secretory properties. However, the miRNA also decreases the level of two central factors in endocrine secretion, Rab27A and Noc2, finally leading to impairment of insulin exocytosis. It was recently shown that miR124a increases during pancreas embryonic development in a time window that is critical for β-cell differentiation (Baroukh et al., 2007). Our data suggest that an increase in miR124a may be needed to drive the expression of neuronal genes required for insulin exocytosis during β-cell differentiation. In view of our observations, the level of miR124a needs to be precisely controlled to avoid detrimental effects on other non-neuronal components of the secretory apparatus.

The mechanisms by which miRNA affects the expression of genes of the exocytotic machinery were partially elucidated in this study. In agreement with previous computational predictions (John et al., 2004; Krek et al., 2005; Lewis et al., 2005), we have experimentally demonstrated that Rab27A is a direct target of miR124a. Rab27A is a Ras-like GTPase that controls intracellular transport and delivery of secretory vesicles to the plasma membrane in endocrine cells, melanocytes and different cells of hematopoietic origin (Fukuda, 2005). Interestingly, Rab27A is undetectable in neurons (Tolmachova et al., 2004). Our data suggest that expression of the GTPase in neurons is probably prevented by the presence of very high levels of miR124a. In this study, we obtained evidence that the effect of miR124a on SNAP25, synapsin-1A, Rab3A and Noc2 is indirect, but we could not define the precise cascade of events leading to the changes in expression of these genes. Comparative sequence analyses suggest that miR124a is part of a network including the transcription factors REST/NRSF and CREB (cAMP response element-binding protein) that coordinates the expression of neuronal genes (Wu and Xie, 2006). Since the genes encoding SNAP25 and synapsin-1A are REST targets (Bruce et al., 2004), increases in the level of these proteins may possibly result from further downregulation of the already low REST activity of β-cells. Another possible mechanism affecting expression of the exocytotic genes may involve the transcription factor HNF3β/Foxa2, which was recently found to be a direct target of miR124a in insulin-secreting cells (Baroukh et al., 2007). Foxa2 controls the expression of several important β-cell genes, including insulin (Kaestner, 2000; Wang et al., 2002). Although it has not been reported that any of the four genes is regulated by Foxa2, miR124a-mediated transcriptional repression of this transcription factor may possibly contribute to the effects on SNAP25, synapsin-1A, Rab3A and Noc2 observed.

In this study, we found that miR124a overexpression led to an increase in hormone secretion at low glucose concentrations and a reduction in the cellular capacity to respond to stimulatory conditions. It has been demonstrated that overexpression of miR124a in insulin-secreting cell lines increases the intracellular Ca^{2+} concentration at resting conditions and diminishes Ca^{2+} elevation upon cell depolarization (Baroukh et al., 2007). These changes in Ca^{2+} signaling were attributed to reduced activity of the ATP-sensitive K^+ channel. In fact, expression of KIR6.2 and SUR1, the genes encoding the subunits of the ATP-sensitive K^+ channel, are controlled by Foxa2 (Lantz et al., 2004). These changes in Ca^{2+} signaling are expected to raise basal insulin secretion and decrease hormone release in the presence of secretagogues and could contribute to the effects of miR124a observed in our study. An alternative explanation for the effect of miR124a on basal secretion could be furnished by the increase in SNAP25, since this SNARE protein inactivates Kv2.1 voltage-dependent K^+ channels (MacDonald et al., 2002). A SNAP25-mediated blockade of

voltage-dependent outward K^+ currents could in principle prolong action potentials and enhance intracellular Ca^{2+} (MacDonald et al., 2001). However, this phenomenon is unlikely to explain the increase in basal secretion in cells overexpressing miR124a. In fact, overexpression of SNAP25 did not significantly affect basal secretion of MIN6B1 cells. Under basal conditions, MIN6B1 cells overexpressing SNAP25 released 7.5±0.8% of their hormone content compared to the 6.8±0.8% obtained for cells transfected with an empty vector.

Surprisingly, despite the changes in Ca^{2+} homeostasis observed and the fact that Foxa2 is essential for glucose-induced insulin release (Wang et al., 2002), Baroukh et al. (2007) did not detect significant changes in hormone secretion in cells overexpressing miR124a. A possible explanation for these contradictory findings is that variations in miR124a levels may differentially affect the translational repression of miRNA targets. Since the individual changes in gene expression elicited by miRNA can have either beneficial or detrimental effects on secretion, the final outcome may differ according to the level of miR124a overexpression and the underlying cellular context.

In this study we found that miR96 modulated the expression of granuphilin and Noc2, two Rab3A- and Rab27A-interacting proteins with important roles in insulin secretion (Coppola et al., 2002; Cheviet et al., 2004; Matsumoto et al., 2004; Gomi et al., 2005). At present, there is almost no information available on this miRNA and its function is unknown. miR96 is widely expressed, but its level varies considerably in different tissues (Barad et al., 2004). As is the case for miR9, miR96 increases the expression of granuphilin, a protein with potent inhibitory action on insulin exocytosis (Coppola et al., 2002; Gomi et al., 2005; Kato et al., 2006). The two miRNAs affect granuphilin expression via two distinct mechanisms. Thus, whereas miR9 functions by relieving the inhibitory action exerted by OC2 (Plaisance et al., 2006), the action of miR96 is independent of changes in the level of this transcription factor. It has been demonstrated that granuphilin expression is controlled by at least three other transcription factors, SREBP-1 (Kato et al., 2006), MafA (Kato et al., 2006) and ICER (Abderrahmani et al., 2006). SREBP-1 and MafA positively modulate the activity of the granuphilin promoter, whereas ICER acts as a transcriptional repressor. Future studies will have to determine whether the effect of miR96 is mediated through changes in the expression or activity of one of these factors, or if it is due to perturbation of the function of an additional transcriptional regulator yet to be identified. The negative action of miR96 on glucose-induced insulin secretion is probably also linked to a decrease in Noc2 levels. Noc2, in contrast to granuphilin, plays a positive role in exocytosis. Indeed, a decrease in Noc2 content obtained by RNA interference or gene knockout is associated with a defect in insulin secretion (Cheviet et al., 2004; Gomi et al., 2005). The changes in Noc2 would add to the inhibitory effect provoked by higher amounts of granuphilin, further reducing the cellular capacity to respond appropriately to insulin secretagogues.

In conclusion, this study has uncovered two additional miRNAs that can both directly and indirectly control the expression of key components of the machinery governing exocytosis in pancreatic β-cells. SNAP25, Rab3A, Rab27A, granuphilin, Noc2 and synapsin-1A are essential modulators of the exocytotic process in many other cells endowed with specialized secretory functions (Jahn, 2004; Fukuda, 2005). Thus, our findings can contribute to understanding regulation of the exocytotic process not only in insulin-secreting cells, but also in other neuroendocrine secretory systems.

Materials and methods

Cell culture and transient transfections

The insulin-secreting cell line MIN6 clone B1 (Lilla et al., 2003) was cultured in 24- or 6-well dishes at a concentration of 1.3×10^5 cells/cm^2 in DMEM-Glutamax medium supplemented with 15% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 70 μM β-mercaptoethanol. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) using a concentration of 1.33 ng/μl plasmids and/or RNA duplexes. Co-transfection experiments were performed at a plasmid/duplex mass ratio of 1:10.

RNA extraction and qRT-PCR assays

Total RNA extraction was performed using RNAqueous isolation kits (Ambion, Austin, TX, USA). Conventional mRNA qRT-PCR assays were carried out as previously described (Abderrahmani et al., 2001) using GAPDH RNA as a control. Real-time PCR assays were performed on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences for mRNA PCR are available from the authors upon request.

miRNA overexpression and plasmid construction

To increase the expression of miRNAs, cells were directly transfected with RNA duplexes (Dharmacon, Lafayette, CO, USA, or Eurogentec, Seraing, Belgium) corresponding to the mature miRNA sequence (see miRNA Registry of the Sanger Institute; http://microrna.sanger.ac.uk). An siRNA duplex directed against GFP (sense, 5′-GAC GUA AAC GGC CAC AAG UUC-3′; antisense, 5′-ACU UGU GGC CGU UUA CGU CGC-3′) with no effect on pancreatic β-cell functions was used as a control. To generate the RS-3′UTR-Rab27a-luc and RS-3′UTR-Noc2-luc constructs, DNA oligonucleotides (Microsynth, Balgach, Switzerland) containing the predicted miR124a recognition site of the Rab27A 3′UTR (NM_023635) or the Noc2 3′UTR (NM_029548) were inserted between the XhoI and EcoRI sites of the psi-CHECK-1 plasmid (Promega, Madison, WI, USA). The multiple cloning site of this plasmid is located in the 3′UTR of the *Renilla* luciferase gene between the stop codon and an artificial polyadenylation site. The oligonucleotide sequences were: RS-3′UTR-Rab27a-luc, sense, 5′-TCG AGG CTG ATG GTC CTG TCG CCT GCC TTA ACA CG-3′; antisense, 5′-AAT TCG TGT TAA GGC AGG CGA CAG GAC CAT CAG CC-3′; RS-3′UTR-Noc2-luc, sense, 5′-TCG AGG ATT TTA AGT TTG CGC GTG TCT TGG GTT G-3′; and antisense, 5′-AAT TCA ACC CAA GAC ACG CGC AAA CTT AAA ATC C-3′. To produce 3′UTR-Rab27A-luc, a 270-bp segment of the 3′UTR of the mouse Rab27A gene containing two potential miR124a recognition sites was amplified by PCR from genomic DNA and inserted by the same way in the psi-CHECK-1 vector. The sequences of the primers were: sense, 5′-ACG CGC TCG AGC TTT GTA TGA TTT AGT CC-3′; and antisense, 5′-AAT CTG AAT TCT CAA GAC CGT GC-3′. The

construction of 3′UTR-OC2-luc was as previously described (Plaisance et al., 2006).

Secretion assay

To assess secretory capacity, MIN6B1 cells (2.5×10^5) plated in 24-well dishes were transiently co-transfected with RNA duplexes and with a construct encoding hGH. Three days later the cells were washed and preincubated for 30 min in Krebs buffer (127 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.1% bovine serum albumin and 25 mM HEPES, pH 7.4) containing 2 mM glucose. The medium was then discarded and the cells were incubated for 45 min either in the same buffer (basal conditions) or in Krebs buffer containing 20 mM glucose, 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX, stimulated condition). After collecting the supernatant, cells were lysed in phospate-buffered saline (PBS) containing 0.5% Triton X-100 to evaluate the hGH content. The amount of hGH in samples was assessed using a hGH ELISA kit (Roche Diagnostics, Rotkreuz, Switzerland).

Western blots and luciferase assays

For Western blot analysis, cells were washed once in PBS and total extracts were obtained by lysing the cells using brief sonication. Protein samples of 30 μg were loaded onto acrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated overnight with primary antibodies against syntaxin-1a and tubulin (Sigma, Buchs, Switzerland), VAMP-2 and Rab3a (Synaptic System, Göttingen, Germany) and SNAP-25 (BD Biosciences, San José, CA, USA). Antibodies against Rab27a, Noc2 and synapsin-1a were kindly provided by Dr. M. Seabra (Imperial College, London, UK), Dr. B. Burgoyne (University of Liverpool, UK) and Dr. B. Riederer (University of Lausanne, Switzerland), respectively. Immunoreactive bands were visualized using either a chemiluminescent substrate (Amersham Biosciences, Piscataway, NJ, USA) after incubation with a secondary horseradish peroxidase-coupled antibody or an Odyssey® infrared imaging system (Li-cor Biosciences, Bad Homburg, Germany) that reveals dye-conjugated secondary antibodies (Rockland, Gilbertsville, PA, USA). Luciferase activity was measured 2 days after transfection with a dual luciferase reporter assay system (Promega). *Renilla* luciferase activity produced from psiCHECK-1, RS-3'UTR-Rab27a-luc, 3'UTR-Rab27a-luc, RS-3'UTR-Noc2-luc and 3'UTR-OC2-luc plasmids was normalized to the activity obtained from a co-transfected SV40 PGL3 promoter plasmid (Promega).

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