

ICER induced by hyperglycemia represses the expression of genes essential for insulin exocytosis

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The GTPases Rab3a and Rab27a and their effectors Granuphilin/Slp4 and Noc2 are essential regulators of neuroendocrine secretion. Chronic exposure of pancreatic β -cells to supraphysiological glucose levels decreased selectively the expression of these proteins. This glucotoxic effect was mimicked by cAMP-raising agents and blocked by PKA inhibitors. We demonstrate that the transcriptional repressor ICER, which is induced in a PKA-dependent manner by chronic hyperglycemia and cAMP-raising agents, is responsible for the decline of the four genes. ICER overexpression diminished the level of Granuphilin, Noc2, Rab3a and Rab27a by binding to cAMP responsive elements located in the promoters of these genes and inhibited exocytosis of β -cells in response to secretagogues. Moreover, the loss in the expression of the genes of the secretory machinery caused by glucose and cAMP-raising agents was prevented by an antisense construct that reduces ICER levels. We propose that induction of inappropriate ICER levels lead to defects in the secretory process of pancreatic β -cells possibly contributing, in conjunction with other known deleterious effects of hyperglycemia, to defective insulin release in type 2 diabetes.

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Introduction

The release of appropriate amounts of insulin from pancreatic β -cells is an essential prerequisite for the achievement of blood glucose homeostasis. Defects in this process can cause profound metabolic disorders and, eventually, lead to dia-

betes mellitus. Fine-tuning of insulin release necessitates a tight control of the final events directing insulin exocytosis. During the last few years, many of the proteins playing a key role in the regulation of these events have been identified. These proteins include the SNAREs SNAP25, Syntaxin-1 and VAMP-2, several components controlling the assembly of the SNARE complex such as Munc18 and complexin-1 and members of the Synaptotagmin family that couple Ca^{2+} changes to vesicle fusion (for a review see Lang, 1999; Easom, 2000; Rorsman and Renström, 2003). In addition, Rab3a and Rab27a, two members of the Rab GTPase family associated with insulin-containing secretory granules, and their effectors RIM2, MyRIP/Slac2c, Noc2 and Granuphilin/Slp4 were found to be necessary for proper regulation of insulin release (Iezzi *et al.*, 1999; Ozaki *et al.*, 2000; Coppola *et al.*, 2002; Yi *et al.*, 2002; Waselle *et al.*, 2003; Cheviet *et al.*, 2004a). The involvement of Rab3a, Rab27a, Noc2 and Granuphilin in the control of blood glucose levels has been confirmed also *in vivo* (Yaekura *et al.*, 2003; Matsumoto *et al.*, 2004; Gomi *et al.*, 2005; Kasai *et al.*, 2005). Despite the demonstration of their roles in insulin secretion, the mechanisms determining the expression of these key regulators of hormone and neurotransmitter release is poorly understood and the capacity of the secretory machinery of β -cells to adapt to various physiological and pathological conditions has not been explored in detail.

Glucose is the main physiological stimulus of insulin secretion but prolonged exposure of pancreatic β -cell to high glucose levels leads to alterations in the secretory function, including increased insulin release under basal conditions and reduced capacity to respond to secretagogues (Eizirik *et al.*, 1992). Long-term exposure of β -cells to physiologically elevated glucose concentrations is known to have profound impacts on gene expression, but the effect of prolonged hyperglycemia on the expression of the components of the secretory machinery of β -cells has not been investigated in detail. Moreover, the promoters of the key genes involved in insulin exocytosis and the transcription factors susceptible to control their expression have not been characterized.

In this study, we found that incubation of the insulin-secreting cell line INS-1E in the presence of elevated glucose concentrations results in a selective decrease in the expression of Rab3a, Rab27a and of two of their effectors, Noc2 and Granuphilin. The promoters of the four genes involved in exocytosis that are affected by glucose were found to contain consensus elements for the binding of ICER (inducible cAMP early repressor), a transcriptional repressor whose expression is increased under hyperglycemic and hyperlipidemic conditions (Zhou *et al.*, 2003) and in animal models of type 2 diabetes (Inada *et al.*, 1998). Indeed, we found that ICER is a potent inhibitor of Granuphilin, Noc2, Rab3a and Rab27a synthesis. Our data indicate that inappropriate levels of ICER

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can repress the expression of these components of the machinery of insulin exocytosis potentially contributing to the secretory defects observed after chronic exposure of pancreatic β -cells to high glucose concentrations. In view of the wide range of cells in which Rab3a, Rab27a, Noc2 or Granuphilin regulate secretion, we propose that ICER induction may contribute to modulate the function of the exocytotic machinery in other secretory systems and be part of adaptive processes such as synaptic plasticity.

Results

Prolonged exposure of pancreatic β -cells to elevated glucose concentrations is known to lead to secretory defects, including excessive insulin release under basal conditions and diminished secretory capacity in response to stimuli (Eizirik *et al*, 1992). Incubation of the rat β -cell line INS-1E for 2 days in the presence of 20 mM glucose resulted in a similar impairment in the secretory process. Indeed, incubated for 45 min at basal conditions INS-1E cells cultured at 2 mM glucose released $2.1 \pm 0.1\%$ ($n = 3$) of their hormone content, while the corresponding value for the cells cultured at 20 mM glucose was $4.4 \pm 1.2\%$ ($n = 3$). Moreover, high glucose culture reduced hormone release elicited by glucose from $24.2 \pm 1.7\%$ ($n = 3$) to $7.8 \pm 0.6\%$ ($n = 3$) and secretion triggered by depolarizing K^+ concentrations (30 mM) from $17.2 \pm 1.1\%$ ($n = 3$) to $7.2 \pm 0.8\%$ ($n = 3$).

In view of these findings, we determined by Northern blotting the expression level of several key components of the secretory machinery of pancreatic β -cells. We found that after 2 days culture in the presence of 20 mM glucose, the expression of the Rab GTPases Rab3a and Rab27a and of their effectors, Granuphilin and Noc2 was drastically diminished (Figure 1A). As expected, the expression of other control genes such as MIF (Waeber *et al*, 1997; Plaisance *et al*, 2002)

was increased. The decrease in the mRNA level of Rab GTPases and of their effectors was associated with a reduction in the protein content. Indeed, Western blot analysis demonstrated that the expression of Granuphilin, Noc2 and Rab3a is strongly reduced after culture at 20 mM glucose (Figure 1B). Unfortunately, INS-1E cells contain relatively low levels of Rab27a and using our antibodies we were unable to detect the protein by Western blotting (data not shown). However, immunocytochemical analysis confirmed that prolonged exposure to elevated glucose levels diminishes the cellular content of all four proteins (Supplementary Figure 1). The effect of glucose was specific for Rab3a, Rab27a, Granuphilin and Noc2. In fact, the expression of Rab8, another member of the Rab family, was not altered by hyperglycemia (Figure 1B). Moreover, the level of other Rab3a and Rab27a effectors such as MyRIP and RIM2 and of two proteins regulating the activation state of Rab3a, Rab3-GAP and Rab3-GEF (Oishi *et al*, 1998) was not significantly different from cells incubated at low glucose. Several other proteins known to be involved in the regulation of insulin exocytosis, including the SNAREs Syntaxin-1, SNAP-25 and VAMP-2 and the SNARE-interacting proteins Complexin-1, Munc18.1 and Tomosyn, were also not affected by the incubation at 20 mM glucose. The expression of Synaptotagmin IX that functions as a Ca^{2+} sensor in insulin exocytosis (Iezzi *et al*, 2004) was slightly increased after culture at 20 mM glucose (Figure 1B). To verify if glucose affects the expression of the components of the secretory machinery also in primary β -cells, we incubated rat pancreatic islets for 96 h at 33 mM glucose, a condition that is known to result in secretory defects analogous to those observed in INS-1E cells (Zhou *et al*, 2003). Real-time PCR analysis revealed that prolonged incubation of rat pancreatic islets at elevated glucose concentration caused a significant reduction in the expression of Granuphilin, Noc2, Rab3a and Rab27a (Figure 2). This effect was not the result of a generalized decrease in gene expression because the level of VAMP-2 and Rab3-GAP (data not shown) was not altered and the expression of other genes was augmented (see below).

In INS-1E cells, a decrease in the expression of Granuphilin, Noc2 and Rab3a was already detectable in the presence of 10 mM glucose and was maximal at 15 mM (Figure 3A). The effect of 20 mM glucose was apparent after 24 h incubation and was maximal after 2 days (Figure 3B). The reduction in Granuphilin, Noc2 and Rab3a expression was not the result of irreversible cell damage. In fact, 36 h incubation at 2 mM glucose after 2 days exposure to 20 mM glucose was sufficient to recover normal levels of the three proteins (Figure 4A) and to restore exocytosis (data not shown).

Glucose is the most important physiological stimulus of insulin secretion. To test whether the inhibition of Granuphilin, Noc2 and Rab3a synthesis is linked to chronic stimulation of insulin release, INS-1E were cultured in the presence of Tolbutamide, a pharmacological agent that triggers insulin secretion by closing ATP-sensitive K^+ -channels and depolarizing the cell membrane (Doyle and Egan, 2003). At the concentrations used in this study (100 μ M), the amount of insulin released in the presence Tolbutamide was comparable to that secreted in response to 20 mM glucose (not shown). However, Tolbutamide did not affect expression of Granuphilin and Noc2 (Figure 4B). Moreover, 200 μ M

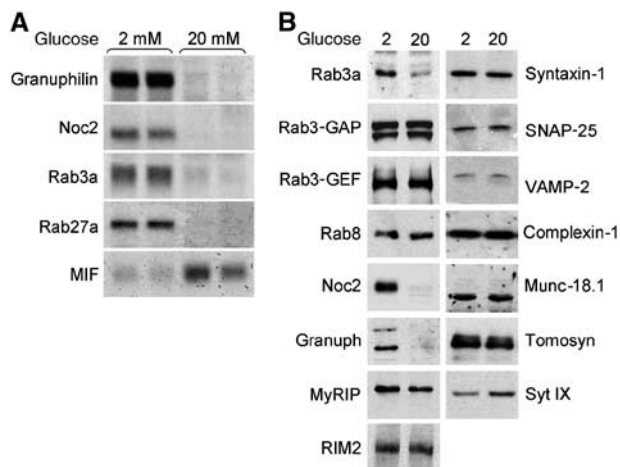


Figure 1 Effect of prolonged exposure to elevated glucose concentrations on mRNA and protein levels of Granuphilin, Noc2, Rab3a and Rab27a. (A) INS-1E cells were cultured in the presence of 2 or 20 mM glucose. After 2 days, total RNA was extracted and the expression of the indicated genes analyzed by Northern blotting. (B) INS-1E cells were cultured for 2 days at 2 or 20 mM glucose. Expression of the indicated proteins involved in the regulation of insulin exocytosis was determined by Western blotting. The figure shows representative results obtained in at least three independent experiments.

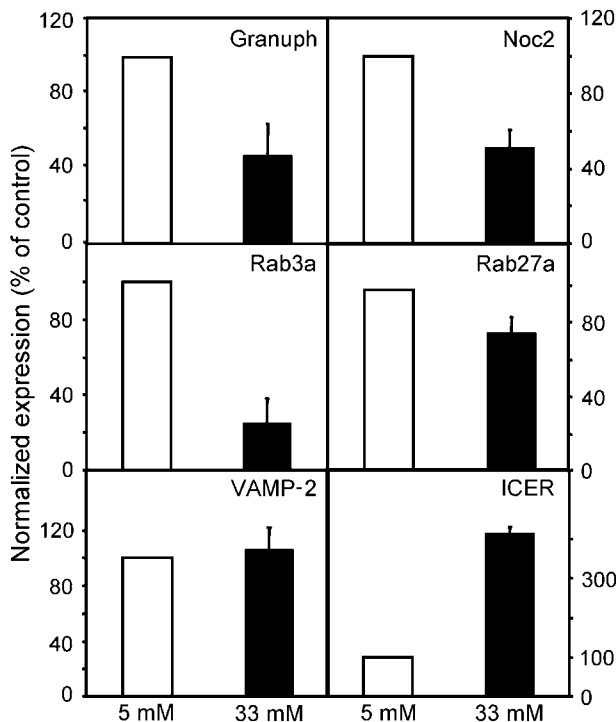


Figure 2 Effect of high glucose on the expression of key regulators of insulin exocytosis in rat pancreatic islets. Freshly isolated rat pancreatic islets were cultured for 96 h at 5 (open bars) or 33 mM glucose (filled bars). Expression of the indicated genes was assessed by real-time PCR analysis. The results were normalized with the values obtained for tubulin in the same sample. Normalization using Rab3GAP values gave similar results. The data obtained in control islets were set to 100%. The results are the mean \pm s.e.m. of four to five independent experiments performed in triplicates. The mRNA levels of Granuphilin, Noc2, Rab3a Rab27a and ICER at 33 mM glucose are significantly different ($P < 0.001$, $n = 4-5$, Student's *t*-test) than in control cells. The expression of VAMP-2 at 5 and 33 mM glucose was not significantly altered.

Diazoxide, a drug that inhibits insulin release by impeding the closure of ATP-sensitive K^+ -channels (Doyle and Egan, 2003) did not prevent the drop in the expression of the two Rab GTPase effectors (Figure 4B).

We then tested whether the effect of glucose was mimicked by incubation with cAMP-raising agents. Indeed, chronic exposure to Forskolin and IBMX resulted in a strong reduction in Granuphilin, Noc2 and Rab3a expression in INS-1E cells (Figure 4C). A similar decrease in the expression of these genes was also observed in MIN6 cells (data not shown) and isolated rat pancreatic islets by real-time PCR analysis (Figure 4D). The effect of glucose and cAMP-raising agents on Granuphilin expression was reproduced using a luciferase reporter gene under the control of the human *Granuphilin* promoter. In fact, 2 days culture in the presence of glucose, Forskolin or IBMX resulted in a sizable decrease in luciferase activity (Figure 5A). Incubation of the cells in the presence of the protein kinase A (PKA) inhibitor H89 prevented the effect of glucose and Forskolin/IBMX on the activity of the Granuphilin promoter (Figure 5B) and restored the expression of Granuphilin and Noc2 (Figure 5C), confirming that the effect of glucose involves a PKA-mediated event.

Close inspection of the promoter region of *Granuphilin*, *Noc2*, *Rab3a* and *Rab27a* revealed the presence of putative cAMP-responsive elements (CRE) potentially capable of bind-

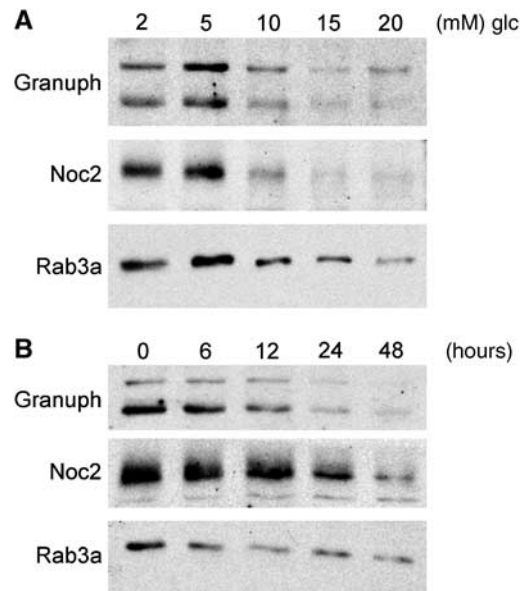


Figure 3 Dose and time dependency of the effect of glucose on Granuphilin, Noc2 and Rab3a expression. (A) INS-1E cells were cultured for 2 days in the presence of the indicated glucose (glc) concentrations. The level of Granuphilin, Noc2 and Rab3a was assessed by Western blotting. (B) INS-1E cells were incubated for the indicated periods in the presence of 20 mM glucose. The amount of Granuphilin, Noc2 and Rab3a remaining in the cells was estimated by Western blotting.

ing the transcriptional repressor ICER (Table I). Interestingly, ICER expression has been shown to be increased in pancreatic islets exposed to hyperglycemic conditions (Zhou *et al*, 2003). Indeed, we found that in pancreatic islets cultured at elevated glucose concentration, the decrease in the expression of Granuphilin, Noc2, Rab3a and Rab27a is associated with a four-fold increase in the expression of ICER (Figure 2). Similar data were obtained after 72 h at high glucose. In contrast, 48 h culture of pancreatic islets at 33 mM glucose changed neither the expression of ICER nor that of the components of the secretory machinery (data not shown), suggesting a direct relationship between these events. Western blot analysis performed after culture at high glucose concentration or in the presence of IBMX/Forskolin confirmed that the reduction in the expression Granuphilin, Noc2, Rab3a and Rab27a is paralleled by the induction of ICER also in INS-1E cells (Figure 6A). As expected, the increase in ICER levels triggered by glucose and IBMX/Forskolin was inhibited by H89 (data not shown). The induction of ICER and its ability to bind to the elements identified in the genes involved in insulin exocytosis was further investigated by EMSA (Figures 6B and 7). Incubation of nuclear extracts of INS-1E with a radiolabeled probe corresponding to the consensus sequence recognized by ICER resulted in the same binding pattern observed in other cell systems (Lamas *et al*, 1997). This complex binding pattern is likely to be due to the interaction of the probe with different CREB and cAMP response element modulator (CREM) isoforms. Treatment of the cells with Forskolin/IBMX or with 20 mM glucose resulted in a strong induction of two fast migrating complexes (Figure 6B). The major band was identified as ICER because it comigrates with exogenously expressed ICER- γ , and the formation of this complex is

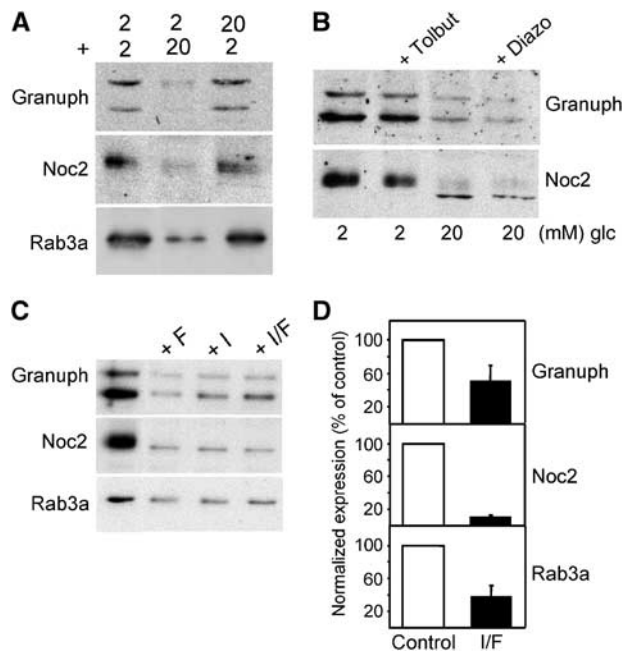


Figure 4 The effect of glucose is reversible and can be mimicked by cAMP-raising agents but not with pharmacological agents that affect insulin secretion. (A) The cells were incubated either at 2 mM (2) or 20 mM (20) glucose for 36 h. The medium was then replaced and the cells incubated for a second period of 36 h at 2 or 20 mM glucose. At the end of the experiment, the cells were homogenized and the extracts analyzed by Western blotting with the indicated antibodies. (B) The cells were cultured for 2 days at 2 mM glucose in the presence or absence of 100 μM Tolbutamide (Tolbut) or at 20 mM glucose with or without 200 μM Diazoxide (Diazo). The expression of Granuphilin and Noc2 was analyzed by Western blotting. (C) INS-1E cells were cultured for two days at 2 mM glucose with or without Forskolin (10 μM) (F) and IBMX (100 μM) (I). The level of Granuphilin, Noc2 and Rab3a was assessed by Western blotting. (D) Freshly isolated rat pancreatic islets were incubated with (filled bars) or without (open bars) Forskolin (10 μM) (F) and IBMX (100 μM) (I) for 72 h. Expression of Granuphilin, Noc2 and Rab3a was assessed by real-time PCR analysis. The values obtained in control islets were normalized to 100%. The results are the mean ± s.e.m. of three independent experiments performed in triplicates.

selectively prevented by the addition of an anti-CREM antibody (Delmas *et al*, 1993) (Figure 6B). The other minor complex induced by Forskolin/IBMX and glucose was also disrupted by the anti-CREM antibody, and is likely to correspond to a different CREM isoform. Antibodies directed against two unrelated transcription factors, Beta2 and REST did not affect the formation of the complexes with the radiolabeled probe. We then performed competition experiments to assess whether ICER binds to the putative CRE identified in the *Granuphilin* promoter. Indeed, oligonucleotides sequences derived from rat and human promoters efficiently displaced the labeled probe known to be recognized by ICER (Figure 7A). Replacement of two nucleotides in the putative CRE of human Granuphilin (H mut) did not compete for the binding of ICER with the wild-type labeled sequence, confirming the specificity of the interaction with the transcriptional repressor. The binding activity of the putative CRE found in the promoters of Rab3a, Rab27a and Noc2 was tested using competition experiments. As shown in Figure 7B, oligonucleotides containing the putative CRE identified in the three promoters were able to compete for

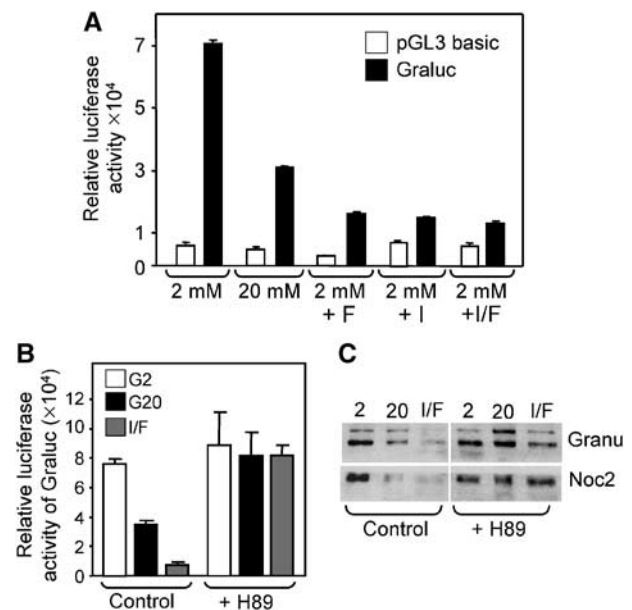


Figure 5 The effect of glucose on the activity of the *Granuphilin* promoter is mimicked by cAMP-raising agents and is prevented by the PKA inhibitor H89. (A) INS-1E cells were transiently transfected either with a plasmid encoding a luciferase reporter gene controlled by a minimal promoter (pGL3 basic, open bars) or driven by the human *Granuphilin* promoter (Graluc, filled bars). After transfection the cells were cultured for 2 days either at 2 mM glucose with or without Forskolin/IBMX or at 20 mM glucose. (B) INS-1E cells transiently transfected with a plasmid encoding a luciferase reporter gene controlled by the human *Granuphilin* promoter were cultured for 2 days either at 2 mM glucose (G2) with or without Forskolin/IBMX (I/F) or at 20 mM glucose (G20). Half of the cells were treated with the PKA inhibitor H89 (10 μM) while control cells were incubated with vehicle. All results from transfection experiments are the mean ± s.e.m. of three independent experiments performed in triplicates. (C) INS-1E cells were cultured for 2 days at 2 mM glucose (2), 20 mM glucose (20) or at 2 mM glucose in the presence of Forskolin/IBMX (I/F). Where indicated, the PKA inhibitor H89 (10 μM) was added to the culture medium. The expression of Granuphilin (Granu) and Noc2 was assessed by Western blotting. The results are representative of three independent experiments.

Table I Promoters of *Granuphilin*, *Rab3a*, *Rab27a* and *Noc2* contain consensus elements for the binding of the transcriptional repressor ICER

Gene	Species	Sequence
<i>G6pase</i> CRE-1		TTGCATCA
<i>G6pase</i> CRE-2		TTACGTAA
Consensus		TGACGTCA
<i>Granuphilin</i>	R	GCAG AGACGTAC GGGCAA
	H	ATTT TTATGTC ACTACGT
	M	CTG CTGACGTGA AGCAAA
<i>Rab3a</i>	R	ATGG TGACGTCA TGGCGC
	H	CTCC TGACGTCA AGGTCA
	M	CTGA TGACGTGA GTTC
<i>Rab27a</i>	R	GC CTGACGAG AGTGAGG
	H	AA CTTGACATA ATCTAG
	M	TAG CTGACTTC AGAT GTA
<i>Noc2</i>	R	CAGAT TGACATCA ATCTAA
	H	GCT CTGACGTCT GCATCG
	M	AAGAT TGACCTT AGAGATC

The consensus for the binding of ICER was obtained by combining the sequences of the cAMP-response element 1 (CRE-1) and 2 (CRE-2) of glucose 6 phosphatase (*G6pase*). The putative binding elements identified in the promoters are shown in bold. The nucleotides matching the consensus are underlined. R, rat; H, human; M, mouse

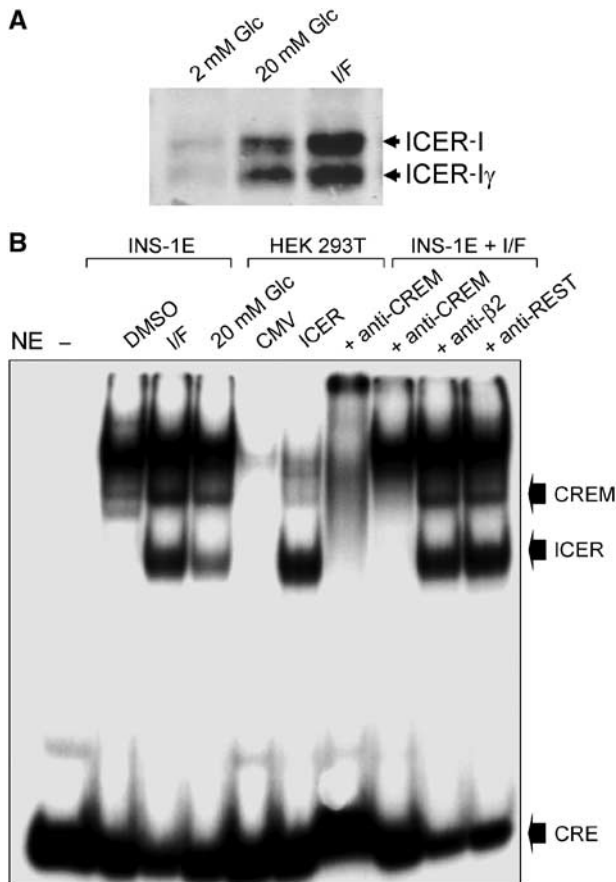


Figure 6 Induction of ICER protein and binding activity in INS-1E cells exposed to cAMP-raising agents or to elevated glucose concentrations. **(A)** INS-1E cells were cultured for 2 days at 2 mM glucose (2 mM Glc), 20 mM glucose (20 mM Glc) or at 2 mM Glc in the presence of IBMX/Forskolin (I/F). The expression of the different ICER isoforms was assessed by Western blotting. **(B)** Nuclear extracts (NE) obtained from INS-1E cells incubated with vehicle (DMSO), treated for 48 h with IBMX and Forskolin (I/F) or 20 mM glucose were incubated with a radioactively labeled oligonucleotide containing the consensus sequence for the binding of ICER. For comparison, nuclear extracts of HEK 293T cells transfected with an empty vector (CMV) or with a plasmid producing ICER-1 γ (ICER) were incubated in parallel with the probe. The radioactive complexes formed during the incubation were analyzed by electrophoresis and visualized by autoradiography. In the sample loaded in the first lane, the nuclear extract was omitted (-). In the last four lanes, before the addition of the labeled oligonucleotide, the nuclear extracts were preincubated with antibodies recognizing all CREM isoforms (anti-CREM), the transcription factor Beta2 (anti-Beta2) or the transcriptional repressor REST (anti-REST). The positions of ICER, the other CREM isoform increased by glucose and of the unbound CRE probe are indicated. The results are representative of three independent experiments.

the binding of ICER, suggesting that Rab3a, Rab27a and Noc2 are also targets of the transcriptional repressor.

The binding of ICER to the CRE element identified in the promoter of *Granuphilin* was further investigated by co-transfecting INS-1E cells with the plasmid containing a luciferase reporter gene driven by the human *Granuphilin* promoter and with a plasmid encoding ICER-1 γ . As shown in Figure 8A, overexpression of ICER-1 γ led to strong reduction in the activity of the promoter. Introduction of two point mutations within the human *Granuphilin* CRE sequence (H mut) reduced the efficacy of the promoter, indicating that this

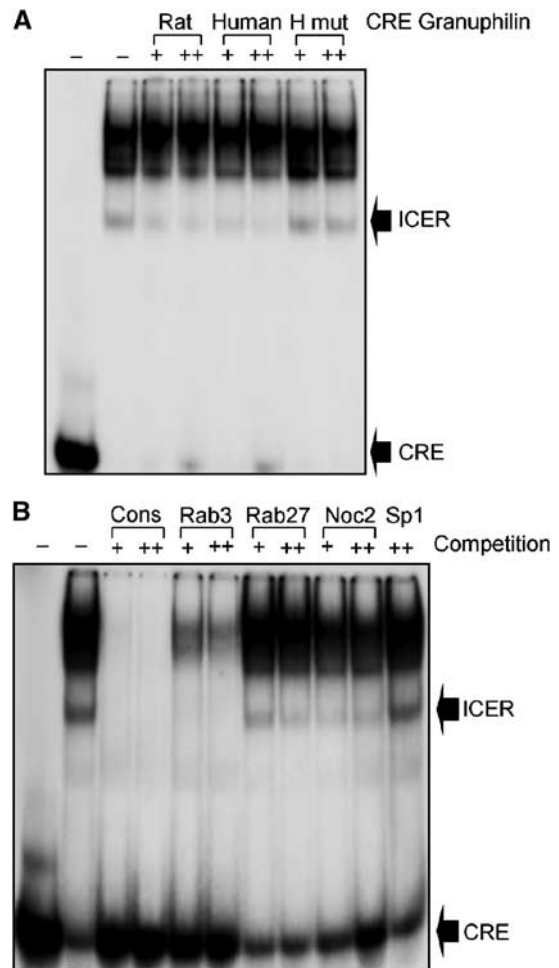


Figure 7 ICER binds to the putative CRE identified in the promoters of *Granuphilin*, *Rab3a*, *Rab27a* and *Noc2*. **(A)** Nuclear extracts (NE) of INS-1E cells treated with IBMX and Forskolin were incubated with a radioactive probe containing the consensus sequence for the binding of ICER. In the first lane, the nuclear extracts were omitted. Where indicated, the incubation was performed in the presence of 100- (+) or 400-fold (++) molar excess of unlabeled oligonucleotides corresponding to the putative CRE of rat or human *Granuphilin* promoters. In the last two lanes, competition was performed with a mutated CRE sequence of human *Granuphilin* promoter (H mut). The radioactive complexes were analyzed by electrophoresis and visualized by autoradiography. The positions of the ICER complex and of the unbound probe are indicated. **(B)** Nuclear extracts of INS-1E cells treated with IBMX and Forskolin were incubated with a radioactive probe containing the consensus sequence for the binding of ICER. Where indicated the incubation was performed in the presence of 100- (+) or 400-fold (++) molar excess of unlabeled oligonucleotides corresponding to the consensus sequence (cons), to rat Rab3a CRE, rat Rab27a CRE, rat Noc2 CRE or to an unrelated sequence (Sp1). In the first lane the nuclear extracts were omitted. The positions of the ICER complex and of the free probe are indicated. The results are representative of three independent experiments.

element contributes to the control of the expression of the Rab effector. As expected from the binding experiments in Figure 7A, the expression of the luciferase construct containing the point mutations in human *Granuphilin* CRE was not repressed by co-transfection of ICER-1 γ (Figure 8A) or by incubation of the cells with Forskolin and IBMX (data not shown). The involvement of the transcriptional repressor was further demonstrated by assessing the expression of

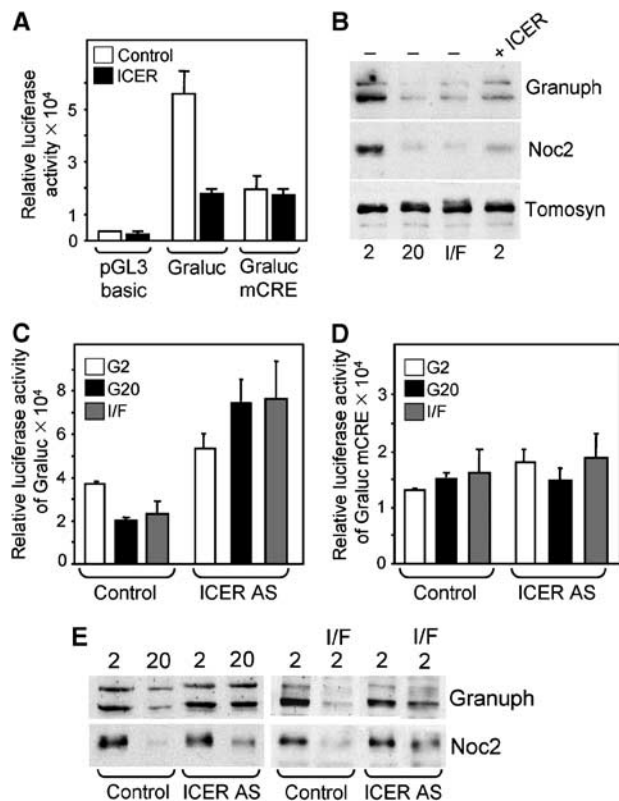


Figure 8 The transcriptional activity of the *Granuphilin* promoter and the cellular content of Granuphilin and Noc2 are linked to the expression level of ICER. (A) INS-1E cells were transiently transfected with plasmids encoding a luciferase reporter gene driven by a minimal promoter (pGL3 basic), by wild-type human *Granuphilin* promoter (Graluc) or by the human *Granuphilin* promoter in which the putative CRE was mutated (Graluc mCRE). These plasmids were co-transfected either with an empty vector (empty bars) or with a plasmid leading to the overexpression of ICER γ (filled bars). Luciferase activity was measured 2 days later. (B) INS-1E cells transiently transfected with the plasmid encoding the ICER- γ expression plasmid (ICER) were incubated for 2 days at 2 mM glucose, 20 mM glucose or at 2 mM glucose in the presence IBMX/Forskolin (I/F). The expression of Granuphilin, Noc2 and Tomosyn was assessed by Western blotting. (C) INS-1E cells were transiently transfected with the plasmid encoding a luciferase reporter gene under the control of the *Granuphilin* promoter (Graluc) and with an empty vector or a vector encoding ICER antisense (ICER AS). The cells were culture at 2 mM glucose (G2), 20 mM glucose (G20) or at 2 mM in the presence of Forskolin and IBMX (I/F). The luciferase activity was measured 2 days later. (D) Same conditions as in (C) except that the luciferase reporter gene is driven by a *Granuphilin* promoter in which the CRE element is mutated (mCRE). (E) INS-1E cells transfected with an empty vector or with ICER antisense (ICER AS) were incubated at 2 mM glucose, 20 mM glucose or at 2 mM glucose in the presence of IBMX and Forskolin (I/F). The expression of Granuphilin and Noc2 was assessed by Western blotting. The results are representative of three independent experiments.

Granuphilin and Noc2 in INS-1E cells transiently transfected with the ICER- γ -overexpressing plasmid. Under our experimental conditions, the transfection efficiency is estimated to be about 30–50%. Despite this relatively low transfection rate, we were able to detect a significant decrease in Granuphilin and Noc2 but not of Tomosyn in cells transfected with ICER- γ (Figure 8B). To visualize the amount of Granuphilin, Noc2, Rab3a and Rab27a remaining selectively in the cells overexpressing the transcriptional repressor, INS-

1E cells were transiently co-transfected with the ICER- γ construct and with a plasmid encoding the green fluorescent protein (GFP). After 2 days, the fluorescent cells coexpressing GFP and ICER- γ were analyzed by immunofluorescence with antibodies against Granuphilin, Noc2, Rab3a and Rab27a. In agreement with the data in Figure 8B, in the cells overexpressing ICER- γ , the level of these four proteins was strongly reduced (Supplementary Figure 2).

If the decrease in the expression of the genes controlling insulin exocytosis is mediated by ICER than silencing of the transcriptional repressor should prevent the effect of glucose and Forskolin/IBMX. Indeed, co-transfection of an antisense plasmid that specifically reduces ICER level (Lamas *et al*, 1997; Pfeffer *et al*, 1998; Maronde *et al*, 1999; Hussain *et al*, 2000) abolished the inhibition of the activity of the Granuphilin promoter caused by glucose and Forskolin/IBMX (Figure 8C). In cells expressing the antisense plasmid, the luciferase activity was significantly higher than in control cells suggesting relieve of a constitutive inhibition of the Granuphilin promoter. Transfection of the antisense plasmid had no effect on the luciferase activity driven by a Granuphilin promoter containing a mutated CRE element, confirming the specificity of the effect (Figure 8D). In agreement with these data, Western blot analysis demonstrated that transient transfection of INS-1E cells with the ICER antisense construct prevents the reduction in Granuphilin and Noc2 expression caused by glucose and Forskolin/IBMX (Figure 8E). Very similar results were obtained by transfecting the cells with a small interfering RNA directed against a sequence that is unique to ICER (Supplementary Figure 3).

Finally, we determined whether overexpression of ICER- γ affects the secretory process of insulin-secreting cells. For this purpose, INS-1E cells were transiently cotransfected with the ICER- γ -overexpressing plasmid and with a plasmid encoding the human growth hormone (hGH). We have previously shown that hGH is targeted to secretory granules and is co-released with insulin during exocytosis (Iezzi *et al*, 1999). This approach allows a direct assessment of the secretory activity of transiently transfected cells independently of their capacity to produce insulin. At 3 days after transfection, the cells were incubated under resting conditions or under conditions that stimulate insulin release. We found that overexpression of ICER- γ results in a significant decrease in unstimulated secretion and an even more pronounced reduction in hormone release in the presence of secretagogues (Figure 9). These findings demonstrate that inappropriate levels of ICER have deleterious effects on the secretory capacity of pancreatic β -cells.

Discussion

Type 2 diabetes is characterized by insulin resistance combined with a progressive loss of pancreatic β -cell function. Prolonged hyperglycemia and hyperlipidemia have detrimental effects on insulin-secreting cells and accelerate β -cell failure. Long-term exposure of pancreatic islets to physiologically elevated glucose concentrations lead to modification in the expression of many genes, including that of insulin (Weir *et al*, 2001). These important alterations in β -cell function are further exacerbated by the appearance of major defects in the secretory process of β -cells and, in

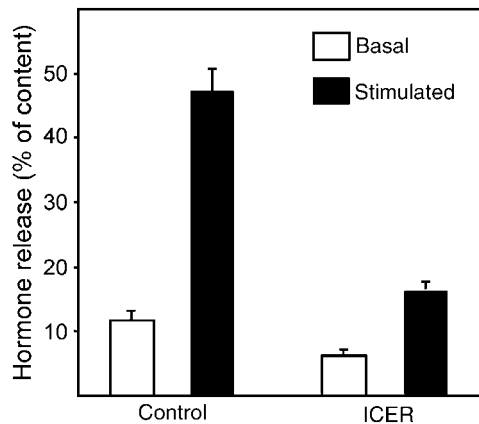


Figure 9 Effect of ICER overexpression on β -cell exocytosis. INS-1E cells were transiently co-transfected with an hGH encoding plasmid and with an empty plasmid (control) or a plasmid leading to the overexpression of ICER-1 γ (ICER). After 3 days culture in normal RPMI medium, the cells were incubated during 45 min at 2 mM glucose (basal condition, open bars) or in the presence of 20 mM glucose, 10 μ M Forskolin and 100 μ M IBMX (stimulatory condition, filled bars). The total amount of hGH present in the cells and the fraction released in the medium during the 45 min incubation period were measured by ELISA. The results are the means \pm s.e.m. of three independent experiments performed in triplicates.

particular, a decreased capability to release insulin in response to secretagogues (Eizirik *et al*, 1992; Zhou *et al*, 2003). Excessively elevated glucose and fatty acids down-regulate the expression of important genes controlling insulin synthesis and β -cell metabolism, partially explaining the loss in the secretory response (Deeney *et al*, 2000). However, the release of insufficient amounts of insulin could also result from defects in the exocytotic process. Despite recent progress in the identification of the major components controlling insulin exocytosis (Lang, 1999; Easom, 2000; Rorsman and Renström, 2003), the capacity of the secretory machinery to adapt to physiological and pathological conditions has been poorly investigated. In Goto-Kakizaki (GK) rats, a spontaneous model of type 2 diabetes, the number of secretory granules docked at the plasma membrane is reduced and insulin exocytosis is defective (Nagamatsu *et al*, 1999). This finding was attributed to dysregulation in the expression of SNARE proteins (Nagamatsu *et al*, 1999; Gaisano *et al*, 2002). However, only the expression of few constituents of the secretory machinery was analyzed. The Rab GTPases Rab3a and Rab27a and their effectors Noc2 and Granuphilin are associated with secretory vesicles of endocrine and neuroendocrine cells and are major players in the regulation of hormone and neurotransmitter release (Izumi *et al*, 2003; Cheviet *et al*, 2004b; Südhof, 2004). Evidence for a central role for these proteins in pancreatic β -cell exocytosis has been provided both *in vitro* (Iezzi *et al*, 1999; Ozaki *et al*, 2000; Coppola *et al*, 2002; Yi *et al*, 2002; Waselle *et al*, 2003; Cheviet *et al*, 2004a) and *in vivo* (Yaekura *et al*, 2003; Matsumoto *et al*, 2004; Gomi *et al*, 2005; Kasai *et al*, 2005). Indeed, mice lacking Rab3a, Rab27a or Noc2 display impaired glucose-induced insulin release and elevated plasma glucose levels. In this study, we demonstrate that the production of these proteins is dynamically regulated by glucose. Chronic exposure of INS-1E cells to elevated glucose concentrations leads to a dramatic decrease in the expression of the genes encod-

ing for these proteins. The effect of glucose is not the result of irreversible cell damage because, after returning the cells to resting conditions, normal levels of Granuphilin, Noc2 and Rab3a and normal secretory rates can be recovered within few hours. Although the effect was quantitatively less pronounced, prolonged exposure of pancreatic islets to elevated glucose concentrations led to similar results. Several factors could account for the quantitative difference between the two systems. It should be noted that Rab3a, Rab27a and Noc2 are not expressed exclusively in insulin-secreting cells. In the other cells of the pancreatic islet, the expression of these genes may not be modulated by glucose. It is also possible that INS-1E cells display a higher sensitivity to glucose compared to primary β -cells.

The effect of glucose on the expression of Rab GTPases and of their effectors was mimicked by Forskolin and IBMX and was prevented by a pharmacological PKA inhibitor, suggesting the involvement of the cAMP pathway. Glucose is known to raise cAMP both in insulin-secreting cell lines and in primary β -cells (Hellqvist *et al*, 1984; Briaud *et al*, 2003; Costes *et al*, 2004; Allagnat *et al*, 2005). The increase in cAMP elicited by glucose is relatively small compared to pharmacological stimuli such as Forskolin (Allagnat *et al*, 2005). However, even minor elevations in cAMP levels that persist for several hours may be sufficient to trigger long-term effects on gene expression such as the one described in this study. In addition, glucose elicits a variety of other signaling cascades that could potentially synergize with cAMP and contribute to the activation of PKA.

Using a combination of approaches, we provide strong evidence that the effect of glucose is mediated by the inducible transcription factor ICER, a member of the CREM family of basic leucine zipper transcription factors that is thought to serve as a dominant-negative repressor of cAMP-dependent gene expression. First, prolonged exposure to high glucose increases ICER transcriptional activity and ICER mRNA level confirming recent data obtained by others (Zhou *et al*, 2003). Second, we have identified functional elements for the binding of ICER in the promoters of *Granuphilin*, *Noc2*, *Rab3a* and *Rab27a*. Third, we demonstrate that overexpression of ICER-1 γ suppresses the expression of the two Rab GTPases and of their effectors, and that the decrease in the level of these proteins elicited by glucose can be prevented by transfection of the cells with an ICER antisense construct. Finally, we show that exocytosis is impaired in INS-1E cells transfected with ICER-1 γ , a result in good agreement with a recent study reporting a decrease in stimulus-induced secretion in rat pancreatic islets overexpressing a CREM isoform closely related to ICER-1 γ (Zhou *et al*, 2003). Taken together these data identify ICER as a central regulator of the secretory function of pancreatic β -cells. Alterations in the level of this transcriptional repressor are expected to result in defects in insulin exocytosis and could predispose to diabetes. In line with this assumption, ICER-1 γ expression was found to be increased in pancreatic islets of type 2 diabetic rats (Inada *et al*, 1998) and transgenic mice with β -cell-directed overexpression of ICER-1 γ suffer from severe diabetes (Inada *et al*, 2004). Plasma insulin concentrations in these transgenic animals are extremely low due to abnormal islet morphology, reduced β -cells mass and low insulin production. Expression of proteins regulating insulin exocytosis was not investigated in this mice model but glucose-induced insulin release from

ICER-1 γ overexpressing β -cells was abolished (Inada *et al*, 2004), pointing to an additional defect in the secretory machinery. Therefore, the presence of inappropriate levels of one or more members of the ICER family have deleterious impacts on several important pancreatic β -cell functions, including proliferation, insulin production and insulin secretion. Because of the combination of adverse effects of ICER on β -cells even relatively low but persistent increases in the amount of these transcriptional repressors are likely to have major consequences on glucose homeostasis and could, added to the other detrimental effects resulting from chronic hyperglycemia, contribute to the development of type 2 diabetes. Induction of ICER in response to physiological agonists such as glucagon is normally transient (Hussain *et al*, 2000). However, prolonged exposure of β -cells to glucose leads to an elevation of the transcriptional repressor both in cell lines and in primary β -cells that persists for several days (Zhou *et al*, 2003; present study). The causes of the alteration in the control of ICER expression after prolonged exposure to supraphysiological glucose levels are still poorly understood. ICER expression has been well described to be coupled to the cAMP/PKA pathway in several cell types (Mukherjee *et al*, 1998; Nervina *et al*, 2003). In agreement with the data obtained in other cell systems, we found that inhibition of the PKA-dependent pathway prevents both the induction of ICER and the decline in the expression of the components of the exocytotic machinery caused by glucose and cAMP-raising agents. Our observations indicate that the effect of glucose is at least dependent on the activation of PKA. The determination of the precise signaling cascade underlying the long-term effects of glucose is a central issue and will be one of the main challenges for future studies investigating the contribution of ICER to β -cell failure.

The results presented in this study have relevant implications not only for elucidating the mechanisms controlling insulin release under normal and disease states but also for understanding the regulation of exocytosis in other cell systems. In fact, the secretory process of most endocrine and exocrine glands, neurons and cells of hematopoietic origin are regulated by one or more ICER targets identified in this study (Izumi *et al*, 2003; Cheviet *et al*, 2004b; Südhof, 2004; Tolmachova *et al*, 2004). In view of our findings, induction of ICER expression is anticipated to have profound impacts on exocytosis in all these secretory systems. Indeed, ectopic expression of ICER in a pituitary corticotroph cell line has already been shown to cause a complete block of hormone release (Lamas *et al*, 1997). A variety of physiological and pathological stimuli are known to induce the expression of ICER in endocrine glands and in different brain regions (Mioduszevska *et al*, 2003). We propose that a rise in ICER expression is part of adaptive processes in which the activity of the exocytotic machinery needs to be modified. These processes could include certain forms of synaptic plasticity and circadian fluctuations in hormone release.

In conclusion, we highlighted a role for the transcriptional repressor ICER in the control of the expression of four key components of the exocytotic machinery regulating hormone and neurotransmitter release and demonstrated its potential involvement in physiopathological conditions affecting β -cell function. Future studies will have to determine the precise signaling cascade leading to persistent induction of ICER in

the presence of elevated glucose concentrations and to pinpoint the role of ICER in the modulation of exocytosis in other secretory systems.

Materials and methods

Materials

The antibodies against Noc2, MyRIP/Slac2-c, Tomosyn, SNAP-25 and CREM were provided by Dr RD Burgoyne (University of Liverpool, UK), Dr A El-Amraoui (Institut Pasteur, Paris, France), Dr R Jahn (University of Göttingen, Germany) Dr H Hirling (EPFL, Lausanne, Switzerland) and Dr E Lalli (IPMC Nice University), respectively. The antibodies directed against Rab3-GAP and Rab3-GEP were generous gifts of Dr U Blank (Inserm U 699, Paris, France). The following antibodies were obtained from commercial sources: Rab3a, RIM2, VAMP-2, Complexin-1 were from Synaptic System (Göttingen, Germany); Rab27a, Rab8, Munc-18.1 and Synaptotagmin IX were from BD Biosciences (San Jose, USA); Syntaxin-1a was from Sigma Aldrich (St-Louis, MI). Generation of the antibody against Granuphilin has been described previously (Coppola *et al*, 2002). The pSV-ICER expression plasmid (Molina *et al*, 1993) was provided by Dr Sassone-Corsi, Strasbourg, France.

Plasmid construction

The human Granuphilin promoter was amplified by PCR on human genomic DNA (Sigma, St Louis MI). Complementary single-stranded oligodeoxynucleotides were synthesized to incorporate *KpnI* and *XhoI* sites to the sequences located in the region -4001 to -1, 5' of the transcription start site of the human Granuphilin gene (GenBank Accession No. NT_011651.15) with the following primers sense: 5'-GGGGTACCAGAGTGGTTCAAATTGT-3' (*KpnI* site underlined, Granuphilin gene in bold) and antisense 5'-CGGCTCGAGATTACTCAACTTTTTC-3' (*XhoI* site underlined, Granuphilin gene in bold). The PCR reaction was performed using the Expand PCR system (Roche, Rotkreuz, Switzerland), following supplier conditions. The PCR product was cut with *KpnI* and *XhoI* and cloned into the corresponding sites of the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). Mutation in the CRE sequence of the human Granuphilin promoter (Graluc) was generated by PCR-site-directed mutagenesis using high fidelity *Pfu* DNA polymerase, according to the manufacturer's protocol (QuikChangeTM, Stratagene, La Jolla, CA). *In vitro* mutagenesis was carried out on full-length Graluc construct from double-stranded 8.9 kb plasmid DNA using two oligonucleotides primers each complementary to opposite strands of the vector (sense: 5'-GGCCATATTTTTATTTTAGTACGTGTGTATCTACCAC-3' and antisense: 5'-GGTGGTAGATACACGCTACTAAATAAAAATATGGCC-3'). The CRE sequence is underlined and the mutated nucleotides are in bold. The plasmid encoding ICER antisense (ICER AS) was constructed by inserting PCR-amplified fragment of ICER from pSV-ICER. Primers were: Sense 5'-AGAAAGTCTAGACATGGCTGTAACGAGATGAA-3' and antisense 5'-ACTGTGCAGGATCCCTGGTGAGG CAGC-3'. The PCR fragment was inserted between the *Bam*HI and *Xba*I cloning sites of the pcDNA3 vector. The siRNA duplex against the rat ICER sequence CTGGAGATGAAACTGCTGC was obtained from Eurogentec (Liege, Belgium).

Cell culture and transfection

The insulin-secreting cell line INS-1E (Merglen *et al*, 2004) was cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 UI/ml penicillin, 50 μ g/ml streptomycin, 0.1 mM sodium pyruvate and 0.001% β -mercaptoethanol. Transient transfection experiments were performed using the Lipofectamine 2000 transfection Kit (Invitrogen, Carlsbad, CA).

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 (Sutton *et al*, 1986).

Immunocytochemistry

INS-1E cells were seeded on glass coverslips coated with 20 μ g/ml laminin and 2 mg/ml poly-L-Lysine. The day after, they were incubated for two days in glucose-free RPMI 1640 medium

supplemented with the indicated concentrations of D-Glucose. The cells were fixed in 4% paraformaldehyde and incubated for 2 h with the first antibody diluted in PBS, pH 7.5, 0.1% goat serum, 0.3% Triton-X-100 and 20 mg/ml bovine serum albumin (BSA). The coverslips were rinsed with PBS, incubated for 30 min with the secondary antibody diluted in the same buffer and mounted for confocal microscopy (Leica, model TCS NT, Lasertechnik, Heidelberg, Germany).

Northern and Western blots

Total RNA extraction and Northern blotting were performed as previously described (Abderrahmani *et al*, 2001). For Western blotting, the cells were scraped and lysed by brief sonication. The cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The proteins were detected using specific antibodies and were visualized by chemiluminescence using horseradish peroxidase coupled secondary antibody.

Real-time PCR

Total RNA from pancreatic islets was extracted using the RNA purification kit (Ambion, Austin, Texas), according to the manufacturer's protocol. Reverse transcription reactions were performed as previously described (Plaisance *et al*, 2005). Real-time PCR assays were carried out on a BioRad MyiQ Single-Color Real-Time PCR Detection System using the BioRad iQ SYBR Green Supermix, with 100 nM primers and 1 µl of template (RT reaction) 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. Primers sequences for PCR were:

β-tubulin,
sense 5'-GGAGGATGCTGCCAATAACT-3' and
antisense 5'-GGTGGTGAGGATGGAATGT-3';
Granuphilin,
sense 5'-GAGAATGCCGAGTTCTGGAG-3' and
antisense 5'-GTCTGCTGAAGGAGGGACTG-3';
Noc2,
sense 5'-GGCACACTCTCTGGAGGAAG-3' and
antisense 5'-GGGGAGGGCAATAAATAAA-3';
Rab3a,
sense 5'-GTCAGCACTGTGGGCATAGA-3' and
antisense 5'-TGCAGTGCATTGAAGGACTC-3';
Rab3GAP,
sense 5'-ATGCAGAGTGCCTGTCTCCT-3' and
antisense 5'-ACATGTTGCTGGGGATCTTC-3';
VAMP-2,
sense 5'-TCACTGCCTTGCCAAGTC-3' and
antisense 5'-CTCCAGGACCTTGCCACAT-3';
ICER,
sense 5'-ATGGCTGTAACGGAGATGAAACTG-3' and
antisense 5'-CACCTTGTGGCAAAGCAGTA-3'.

The values obtained were normalized to the amount of tubulin that was measured in parallel for each sample.

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Nuclear protein extract preparation and electromobility shift assays (EMSA)

Nuclear protein extracts EMSA and supershift experiments were performed as previously reported (Abderrahmani *et al*, 2001). Primers used as labelled probes were as follows:

CRE consensus:
sense 5'-GGACGTAGTCTGACGTCAGCGGA-3' and
antisense 5'-CATCAGACTGCAGTCGCCCTCCGA-3';
for competition experiments, primers used were:
rat CRE Rab3a:
sense, 5'-ATGGTGACGTCATG-3' and
antisense 5'-CATGACGTCACCAT-3';
rat CRE Granuphilin:
sense 5'-GCAGAGACGTACGGG-3' and
antisense: 5'-CCCGTACGTCCTCTGC-3';
human CRE Granuphilin:
sense 5'-ATTTTATGTCAGTACCT-3' and
antisense 5'-ACGTACTGACATAAAAAAT-3';
mutated human CRE Granuphilin:
sense 5'-ATTTTATTTAGTACGT-3' and
antisense 5'-ACGTACTAAAAATAAAAAAT-3';
rat CRE Rab27:
sense 5'-AGCCTGACGAGAGT-3' and
antisense 5'-ACTCTCGTCAGGCT-3';
rat CRE Noc2:
sense 5'-CAGATGACATCAAT-3' and
antisense 5'-ATTGATGTCATCTG-3'.

Secretion experiments

INS-1E cells (3×10^5) plated in 24-wells dishes were transiently cotransfected with a construct encoding the hGH (Nicholls, San Juan Capistrano, CA) and with a plasmid permitting constitutive expression of ICER-I γ (Molina *et al*, 1993). After 3 days, 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% BSA) containing 2 mM glucose (basal conditions). The medium was then removed and the cells incubated for 45 min in the same buffer (basal conditions) or in a buffer containing 20 mM glucose, 10 µM Forskolin and 100 µM IBMX (stimulatory conditions). The total amount of hGH produced by transfected cells and the fraction released into the medium during the incubation period were determined by ELISA (Roche Diagnostics, Rotkreuz, CH).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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