Complexin I regulates glucose-induced secretion in pancreatic β-cells

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Summary

The neuronal-specific protein complexin I (CPX I) plays an important role in controlling the Ca^{2+}-dependent neurotransmitter release. Since insulin exocytosis and neurotransmitter release rely on similar molecular mechanisms and that pancreatic β-cells and neuronal cells share the expression of many restricted genes, we investigated the potential role of CPX I in insulin-secreting cells. We found that pancreatic islets and several insulin-secreting cell lines express high levels of CPX I. The β-cell expression of CPX I is mediated by the presence of a neuron restrictive silencer element located within the regulatory region of the gene. This element bound the transcriptional repressor REST, which is found in most cell types with the exception of mature neuronal cells and β-cells. Overexpression of CPX I or silencing of the CPX I gene (*Cplx1*) by RNA interference led to strong impairment in β-cell secretion in response to nutrients such as glucose, leucine and KCl. This effect was detected both in the early and the sustained secretory phases but was much more pronounced in the early phase. We conclude that CPX I plays a critical role in β-cells in the control of the stimulated-exocytosis of insulin.

Key words: Complexin I, Small interfering RNA, Secretion, Pancreatic β-cells, Transcriptional repressor REST

Introduction

Insulin, the major anabolic hormone controlling glucose homeostasis, is released from pancreatic β-cells in response to a glucose stimulus and other secretagogues (Malaisse et al., 1979; Hedeskov, 1980; Prentki et al., 1997). Insulin secretion occurs through a complex metabolic network in which the increased flux of glucose elevates the intracellular ATP/ADP ratio (Gembal et al., 1992; Ashcroft et al., 1994; Maechler et al., 1998; Detimary et al., 1998; Lang, 1999). This results in the closure of the ATP-sensitive potassium channel (KATP channels) and subsequent cellular depolarization (Inagaki et al., 1995; Sakura et al., 1995). In turn, depolarization causes the influx of extracellular Ca^{2+} by the activation of the L-type voltage-dependent Ca^{2+} channels triggering the initial fusion of insulin-containing dense core vesicles with the plasma membrane (Heinemann et al., 1994). The vesicle fusion requires the assembly of a ternary core complex formed by soluble-*N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). The core complex includes the vesicle-associated SNARE VAMP and two plasma membrane SNAREs, syntaxin-I and SNAP25 (Wheeler et al., 1996; Regazzi et al., 1996b; Gerber and Sudhof, 2002). The assembly of the core complex is a tightly regulated step and is modulated by several proteins that interact directly with SNAREs (Regazzi et al., 1996a; Coppola et al., 2002). However, the precise mechanism by which stimuli such as glucose modulate SNARE complex function remains unknown.

Ca^{2+}-dependent exocytosis in neuronal cells requires the assembly of the same ternary complex formed by VAMP, syntaxin-I and SNAP25 (Sudhof, 1995; Sutton et al., 1998). By selectively interacting with syntaxin-I within the ternary SNARE core complex, complexins (CPXs), also named synaphins, play critical roles in regulating the Ca^{2+}-dependent neurotransmitter release (McMahon et al., 1995; Pabst et al., 2000; Hu et al., 2002; Tokumaru et al., 2001; Chen et al., 2002). CPXs form a family of two small related proteins (CPX I and II) (Takahashi et al., 1995; McMahon et al., 1995). CPX I is found mainly in neuronal cells and selective disruption of the gene in mice leads to a strong ataxia and reduces transmitter release caused by decreased Ca^{2+} sensitivity of the synaptic secretion process (McMahon et al., 1995; Reim et al., 2001). Overexpression of CPX I also markedly suppresses exocytosis of small synaptic vesicles (Itakura et al., 1999; Archer et al., 2002). Similar to CPX I, there are many other genes whose expression is limited to neuronal cells. In other cell types, the expression of these genes is blocked by the presence of a neuron-restrictive silencer element (NRSE) within their regulatory region (Mori et al., 1990; Howland et al., 1991; Li et al., 1993; Bessis et al., 1995). This element binds the RE-1 silencer transcription factor (REST), a transcriptional repressor expressed in most cell types with the exception of mature neuronal cells (Schoenherr and Anderson, 1995; Chong et al., 1995; Schoenherr et al., 1996; Jones and Meech, 1999). Different studies have now shown that REST is also absent in β-cells, therefore, allowing expression of some of its target genes (Atouf et al., 1997; Abderrahmani et al., 2001; Kemp et al., 2003).

The restricted pattern of CPX I to neuronal cells suggests

that it could be a potential REST-target gene and could also play an important role in stimulus-induced secretion in β-cells. We show that there is a high expression of CPX I in pancreatic islets and in several insulin-secreting cell lines, and we demonstrate that its selective presence in neuronal and β-cells is determined by REST through a NRSE present within its regulatory region. Moreover, we found that overexpression or selective inhibition of CPX I expression markedly decreases the secretory response of β-cells elicited by nutrients, indicating that CPX-I is an essential component of the molecular machinery controlling insulin exocytosis.

Materials and Methods

Cell culture and transfection

The insulin-secreting cell lines INS1 and βTC3 were cultured as previously described (Abderrahmani et al., 2001). Transient transfection experiments were performed using the Effectene transfection Kit (Qiagen) with a DNA/Effectene ratio of 1/25, according to manufacturer's protocol.

Western and northern blotting analyses

For western blots, cells were washed once in cold PBS and lysed in passive lysis buffer (Promega). A total of 20 μg of proteins was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane by electroblotting. Membranes were blocked by incubation for 60 minutes in a buffer containing 0.1% Tween 20 and 5% milk and incubated overnight at 4°C with the primary antibodies against CPX I/II (Synaptic System). The immunoreactive bands were visualized using a chemiluminescent substrate (Amersham) after incubation of the nitrocellulose filters for 60 minutes with secondary antibodies conjugated to horseradish peroxidase. RNA isolation and northern blot analysis were performed as described previously (Abderrahmani et al., 2001).

Immunohistochemistry

Islets were isolated from C57BL/6 wild-type mice (aged 2-3 months) by intraductal digestion of the pancreas with the Liberase RI Purified Enzyme Blend (Roche Diagnostics) according to the manufacturer's protocol. Islets were hand-picked to purity and cultured for 3 days in βTC3 culture medium supplemented with 1% Hepes 1 M pH 7.4 and 0.1% β-mercaptoethanol 50 mM (Life Technologies). Islets were fixed for 15 minutes in 1% paraformaldehyde and then blocked for 60 minutes in a solution containing 1% bovine serum albumin and 0.3% Triton X-100 (Sigma). Incubation with the primary antisera [guinea pig anti-porcine insulin (Linco Research), dilution 1:500; rabbit anti-complexin (Synaptic Systems) dilution 1:500] was performed at 4°C for 24 hours. After several washes in PBS, islets were incubated for 45 minutes with secondary Alexa 488-conjugated anti-rabbit and Alexa 568-conjugated anti-guinea pig IgG (Molecular Probes) at a dilution of 1:500. Images were obtained using a confocal microscope (Leica Microsystems) and then subsequently assembled using Photoshop (Adobe Systems).

Plasmids construction and post transcriptional gene silencing by small interfering RNA (siRNA)

The plasmid encoding CPX I (CMV-CPX I) was constructed by inserting the PCR-amplified CPX I cDNA. Primers were designed to amplify the full-length open reading frame of rat CPX I and were: sense 5′-CGCGGATCCATGGAGTTCGTGATGAAA-3′ and antisense 5′-GCTCTAGATTACTTCTTGAACATGTC-3′. The PCR fragment was cloned into the *Bam*HI and *Xba*I sites of the pCDNA3 vector. To construct the tagged CPX I cDNA with green fluorescent protein (GFP-CPX I), the CPX I cDNA was subcloned in frame into the *Bam*HI and *Xba*I cloning sites of the GFP-pCDNA3 vector. Sequence analysis of inserts was performed by Microsynth (Balgach, Switzerland). To design target-specific siRNA duplex against CPX I, we selected sequences of the type $AA(N_{21})$ (N, any nucleotide) from the coding sequence of the mouse CPX I mRNA. Positions +481 to +499 relative to the transcription start site were suitable for the design of a specific siRNA directed against both mouse and rat CPX I. The selected siRNA sequence was also submitted to BLAST search to ensure that it was specific to the target mRNA. Oligonucleotides contained both the 19-nt sense and 19-nt antisense strands separated with a short spacer from the reverse complement of the same sequence and five thymidines as termination signal. The primers used were: sense 5′-GATCCCC*GATGAGAGCATCCTGGACA***TT**CAAGAGA-*TGTCCAGGATGCTCTCATC*TTTTTGGAAA-3′ and antisense 5′-AGCTTTTCCAAAAA*GATGAGAGCATCCTGGACA*TCTCTTG**AA**-*TGTCCAGGATGCTCTCATC*GGG-3′. The complementary target sequences of CPX I and thymidines are underlined and in bold, respectively. These primers are hybridized and ligated downstream of the H1-RNA promoter by *Hin*dIII/*Bgl*II sites of the pSUPER vector (Brummelkamp et al., 2002) (kindly provided by Dr R. Agami, Nederland Cancer Institute, Amsterdam).

Secretion experiments

For exocytosis measurement, cells (5×10^5) were plated in 24-wells dishes and were transiently transfected with 1 μg of a plasmid encoding human growth hormone (hGH). Two days after transfection, cells were cultured in 2 mM glucose for 24 hours. Transfected cells are then washed and preincubated for 60 minutes in Krebs-Ringer/bicarbonate-Hepes buffer (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM Hepes, 0.1% bovine serum albumin) containing 2 mM glucose (basal conditions). The medium was then removed, and the cells are incubated for 30 minutes in the same buffer (basal conditions) or in a buffer (stimulatory conditions) containing 20 mM glucose (including 10 μM forskolin and 100 μM IBMX) or 20 mM leucine (plus 2 mM glutamine) or 24 mM KCl. Exocytosis from transfected cells was assessed by ELISA (enzyme-linked immunoabsorbent assay; Roche) of the amount of hGH released into the medium during the incubation period, as previously described (Iezzi et al., 1999).

Nuclear protein extract preparation and electromobility shift assays (EMSA)

Cells were grown in medium in 10 cm dishes. Thereafter, nuclear extracts were prepared and EMSAs were performed as previously reported (Abderrahmani et al., 2001). Oligonucleotides used as labeled probes were as follow: NRSE CPX I, forward 5′-GAG-CAGCAGTACGGACACCAAC-3′ and reverse 5′-GGTTGGTGTC-CGTACTGCTGCT-3; NRSE IB1 (forward: 5′-GGCTTCAGCACC-GCGGAGAGCGCCATCTCC-3′ and reverse: 5′-CCGGAGATGGC-GCTCTCCGCGGTGCTGAAG-3′). Complementary forward and reverse oligonucleotides were hybridized and then filled in by the Klenow fragment of DNA polymerase I (Roche Diagnostics) in the presence of [γ-^{32}P]deoxycytosine triphosphate (Amersham). Free nucleotides were separated by centrifugation through a G-50 column. For binding, 5 μg of nuclear proteins were preincubated on ice, in the presence or absence of an excess of unlabeled competitor DNA, for 10 minutes in 20 μl of a solution containing 20 mM Hepes (pH 7.6), 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 250 mM KCl and 2 μg of poly(dI-dC) · poly(dI-dC). Approximately 100 fmol of double-strand labeled oligonucleotide were mixed with nuclear proteins and the mixture was incubated for 20 minutes on ice. For supershift assays, 5-10 μg of nuclear proteins were preincubated on ice with or without REST antibody (a generous

gift from G. Mandel, Howard Hughes Medical Institute, New York) for 30 minutes before addition of the labeled probe. Samples were loaded onto a 6% nondenaturing polyacrylamide gel with 0.25× Tris-borate-EDTA (TBE) buffer. The gels were fixed in a solution of 10% acetic acid and 30% methanol, dried, and exposed to Hyperfilm-MP (Amersham).

Results

CPX I is highly expressed in pancreatic β-cells

Total RNA prepared from several insulin-secreting cell lines were analyzed by northern blotting for expression of CPX I. Since the mouse and rat CPX I and II mRNAs share high sequence homology by more than 80% (Takahashi et al., 1995), the use of CPX I cDNA as probe allows detection of both CPX I and CPX II transcripts. As expected, CPX I (2.3 kb) and CPX II (5 kb) were detected in the brain, which has been shown previously to express both CPX I and CPX II (Takahashi et al., 1995) (Fig. 1a). High levels of CPX I transcripts were also detected in all examined insulin-secreting cell lines (INS1, MIN6 and βTC3). CPX II mRNA levels were detected in unrelated human carcinoma HeLa and the mouse macrophage-like RAW cells but they were not found in any of studied β-cell lines with the exception of βTC3 cells. However, in this β-cell line CPX II mRNA levels were lower compared to CPX I. Western blotting analysis confirmed the presence of CPX proteins in β-cell lines and in pancreatic islets (Fig. 1b).

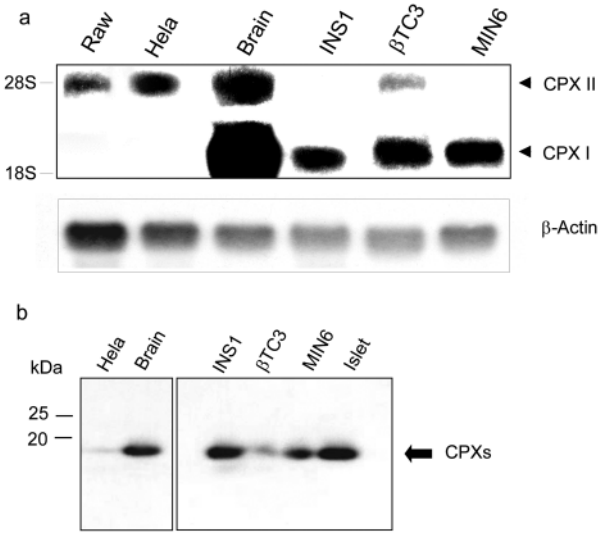


Fig. 1. Complexin 1 (CPX I) is highly expressed in insulin-secreting cells. (a) Total RNA (15 μg) from different tissues were prepared and were subjected to northern blot analysis as described previously (Abderrahmani et al., 2001). RNAs were hybridized with a full-length rat cDNA CPX I probe that shares more than 80% sequence homology with CPX II (Takahashi et al., 1995). A transcript of 2.3 kb corresponding to CPX I is abundantly detected in the brain (used as a positive control for CPXs expression) and in three insulin-secreting cell lines (INS1, βTC3 and MIN6 cells). A band of 5 kb corresponding to CPX II mRNA, is observed in the brain, in HeLa and RAW and at low levels in βTC3 cells (Takahashi et al., 1995; McMahon et al., 1995). CPX II was not detected in INS1 and MIN6 cells. (b) Western blot analysis performed with total protein extracts from insulin-secreting cell lines and pancreatic islets confirmed expression of CPXs protein.

The cellular localization of CPXs was examined by immunofluorescence followed by confocal microscopy. As shown in Fig. 2, CPXs immunoreactivity was mainly localised in the cytosol. The double staining with an anti-insulin antibody confirmed the presence of CPXs in islet cells positive for insulin. Taken together, these data show a high expression of CPX I in insulin-secreting cells.

Expression of CPX I is controlled by the transcriptional repressor REST

Many genes preferentially expressed in neuronal cells and β-cells are regulated by REST. We therefore evaluated whether this was also the case for CPX I. We first searched for the presence of NRSE-like motifs within the gene by computer analysis. A putative NRSE motif located in the 5′ regulatory region was found to be homologous with the consensus NRSE (Table 1). This sequence was conserved in studied species, including human, mouse and rat and the nucleotides known to be required for its functional integrity are preserved. EMSA

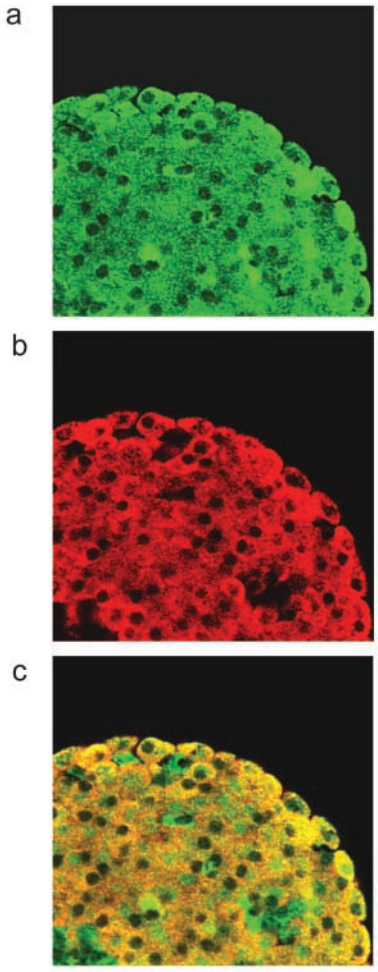
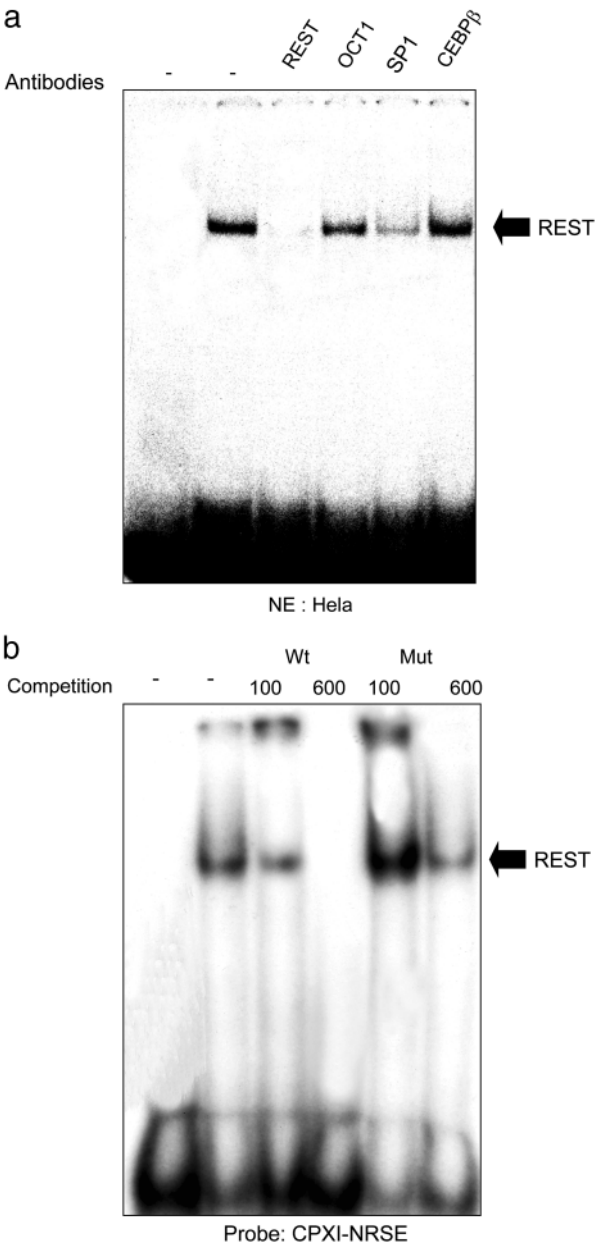


Fig. 2. Immunoreactivity of CPXs is detected in insulin-positive cells from mouse islets**.** Immunohistochemistry for CPXs (a) (1/500 dilution) and for insulin (b) (1/500 dilution) was performed on isolated mouse islets. The overlay of the two images (c) shows that CPXs immunoreactivity is found in insulin-positive islet cells. The images were obtained by confocal microscopy.

Table 1. Sequence comparison of NRSE from mouse *complexin I* gene with consensus NRSE

Gene	Species	Sequence	Location
Consensus		NNCAGCACCNNGGACAGNNNC	
MAPK8IP1	Human	----------G------	5′ regulatory
Complexin I	Human	----------GGC----	5′ regulatory
	Mouse	-------GT-------C----	5′ regulatory
	Rat	-------GT--A---TC---G	5′ regulatory

analysis demonstrated the binding of REST to the putative NRSE. Nuclear protein extracts prepared from HeLa cells, which highly express REST, was incubated with the 21 bp NRSE sequence of the mouse complexin I gene (*Cplx1*) as the labeled probe. The complex formed was slow-migrating compared to the free probe and was disrupted when nuclear extracts was incubated with REST antibodies but not with unrelated antibodies directed against OCT1, SP1 and CEBPβ transcription factors (Fig. 3a). The specificity of the binding activity was assessed by adding 100- and 600-fold molar excess of unlabeled wild-type or mutated NRSE sequence from the MAPK-8-interacting protein gene (*MAPK8IP1)* that has been previously described to specifically bind REST (Abderrahmani et al., 2001). The presence of wild-type sequence competed in the formation of the protein-DNA interaction whereas the mutated oligonucleotides did not decrease the binding activity (Fig. 3b). These experiments show that the neuronal- and β-cell expression of the complexin I gene is determined by REST.



Selective silencing of CPX I decreases stimulus-induced secretion in β-cells

To address the role of CPX I in β-cell exocytosis, we selectively inhibited endogenous levels of CPX I in β-cells. This was achieved by taking advantage of the possibilities offered by the RNA interference (RNAi) process. RNAi is based on short double stranded RNA molecules called small interfering RNAs (siRNAs) that trigger specific silencing of gene expression in a sequence-specific manner (Hammond et al., 2001; Elbashir et al., 2001). siRNAs can be generated in mammalian cells using plasmids that direct the transcription of short hairpin RNAs (Brummelkamp et al., 2002). We have recently used this strategy to initiate RNAi in insulin-secreting cells (Waselle et al., 2003). Computer analyses ensured that oligonucleotides selected for RNAi were specifically designed against mouse and rat CPX I mRNA and that they were divergent from the CPX II sequence. We first tested the effect of the plasmid directing the synthesis of CPX I siRNAs (siRNA CPX I) on the expression of GFP-tagged CPX I (GFP-CPX I). INS1 cells were co-transfected with GFP-CPX I in the presence of an empty plasmid (pSUPER) or of siRNA CPX I. Western blotting analysis showed that the GFP-tagged protein was readily detectable in INS1 cells transiently transfected with the GFP-CPX I construct (Fig. 4). The expression of GFP-CPX I was not affected by the co-transfection of the pSUPER vector but was drastically decreased in the presence of siRNA CPX I. This indicates that expression of CPX I can be

Fig. 3. The NRSE-like motif of the mouse complexin I gene binds REST. (a) EMSA with ^{32}P-labeled CPXI-NRSE using nuclear extracts from HeLa cells. A slow migrating complex (arrow) is observed. This complex was disrupted by REST antibodies but not by unrelated antibodies against transcription factors OCT1, SP1 and CEBPβ. (b) This DNA-binding activity was competed by adding a 100- or 600-fold molar excess of unlabeled wild-type NRSE of the *MAPK8IP1* gene (WT) previously described to specifically bind REST (Abderrahmani et al., 2001). This binding activity was not disrupted by adding unlabeled mutated NRSE of the *MAPK8IP1* gene (MUT). NE, nuclear extracts.

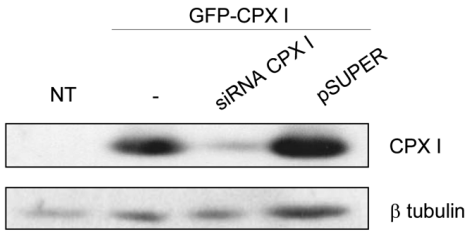


Fig. 4. CPX I expression is efficiently silenced by plasmids directing the synthesis of small interfering RNAs (siRNA). Oligonucleotides were synthesized to specifically target CPX I and were cloned into pSUPER vector (see Material and Methods). The efficiency of siRNA was assessed by cotransfection of GFP-CPX I in the presence or in the absence of siRNA CPX I in INS1 cells. CPX I expression was detected using anti-GFP antibody. The western blot shows ectopic expression of CPX I, in the absence of siRNA CPX I (–) and in the presence of empty vector pSUPER (control) compared to untransfected cells (NT). However, CPX I expression is suppressed by adding siRNA CPX I.

efficiently reduced by the generation of its specific siRNA. The rat INS1 and mouse βTC3 insulin-secreting cells were then assessed for their ability to secrete in response to glucose, the main secretagogue of insulin secretion, and to leucine, an activator of mitochondrial metabolism, in the presence or absence of siRNA CPX I. Exogenously expressed human growth hormone (hGH) has been previously shown to be targeted to secretory granules of and to be co-released with insulin after triggering of exocytosis (Iezzi et al., 1999). hGH secretion was therefore used to monitor exocytosis from transiently transfected cells. In the absence of siRNA CPX I, the secretagogues elicited a 5- and 3-fold increase in hGH release in INS1 and βTC3, respectively (Fig. 5a,b). In the presence of siRNA CPX I, basal hGH secretion was unaffected but the ability of the cells to secrete in response to 20 mM glucose or 20 mM leucine was decreased by 50% for both INS1 and βTC3 cells (Fig. 5a and b, respectively). In neuronal cells, CPX I has been proposed to act at the Ca^{2+}-triggering step during the process of neurotransmitter release (Hu et al., 2002).

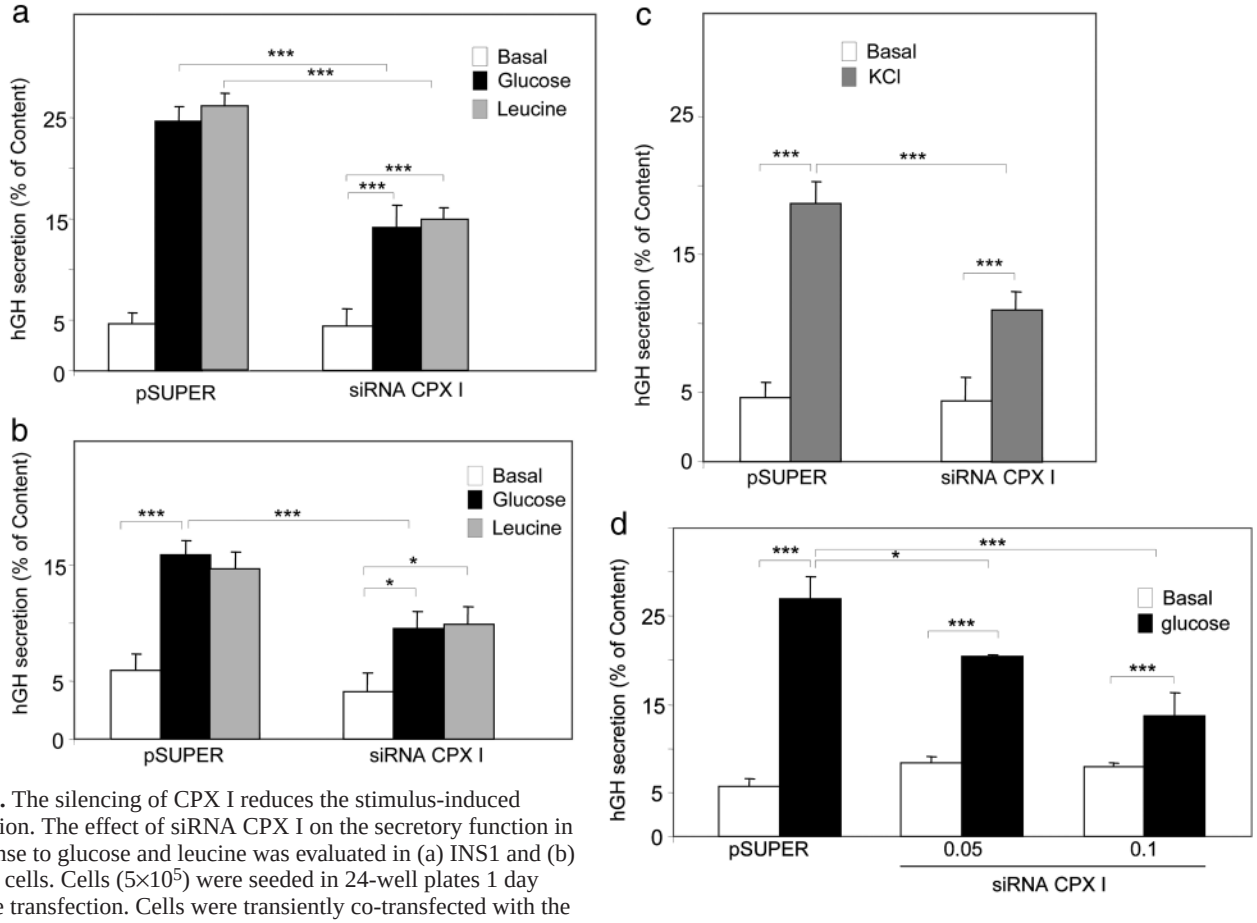


Fig. 5. The silencing of CPX I reduces the stimulus-induced secretion. The effect of siRNA CPX I on the secretory function in response to glucose and leucine was evaluated in (a) INS1 and (b) βTC3 cells. Cells (5×10^5) were seeded in 24-well plates 1 day before transfection. Cells were transiently co-transfected with the plasmid encoding hGH as a reporter gene for secretion and with siRNA CPX I or pSUPER. Two days after transfection, the cells were cultured in 2 mM of glucose for 24 hours. Cells were then preincubated in KRBH for 60 minutes in 2 mM glucose (basal condition) and were subsequently incubated for 45 minutes in KRBH either under basal condition or with 20 mM glucose (plus 10 μM foskolin and 100 μM IBMX) or 20 mM leucine (plus 2 mM of glutamine). The amount of hGH released into the buffer and remaining inside the cells under basal and stimulatory conditions was determined by ELISA. Results were expressed as a percentage of hGH content. In the absence of siRNA CPX I, INS1 and βTC3 cells were able to secrete hGH in response to glucose and leucine by 5-fold and 3-fold, respectively. By adding siRNA CPX I, cells were still able to secrete hGH in response to glucose and leucine compared to basal condition but the secretory response was significantly decreased by about 50% compared to cells transfected with pSUPER. (c) The 50% decrease of hGH secretion was also observed in response to 24 mM KCl in INS1 cells. (d) The decrease of stimulus-induced secretion by the silencing of CPX I occurs in a dose-dependent manner. The figure shows the mean ± s.e.m. of at least three independent experiments measured in triplicate (*P<0.01, ***P<0.001).

Our data strongly suggest that in β-cells, CPX I plays a similar role after Ca^{2+} influx induced by secretagogues. For this reason, we assessed the ability of cells transfected with the CPX I silencer to secrete in response to 24 mM of KCl, a secretagogue that acts downstream of glycolysis by depolarising the β-cell membrane. Depolarisation leads to the entry of Ca^{2+}, independently of mitochondrial metabolism, by activating the L-type voltage-dependent Ca^{2+} channels. In the absence of siRNA CPX I, 24 mM of KCl increased hGH secretion 5-fold in INS1 cells. In the presence of siRNA CPX I, KCl-induced secretion was reduced by 50% compared with control cells (Fig. 5c). Similar results were obtained with βTC3 cells (data not shown). We next evaluated whether the decrease of stimulus-induced exocytosis by the silencing of CPX I occurs in a dose-dependent manner. In the presence of 0.05 μg and 0.1 μg of siRNA CPX I, the hGH secretion was significantly decreased by 34% and 50%, respectively, compared with control cells (Fig. 5d). The maximum decrease of hGH secretion was reached at 50% even introducing high amount of siRNA CPX I (data not shown). These results indicate that endogenous expression levels of CPX I are required for normal stimulus-induced secretion.

Insulin secretion in response to glucose exposure is biphasic, with a first phase peaking after about 5 minutes, a decrease to almost basal levels and a second phase beginning after 10 minutes and lasting for hours (Straub et al., 2002). The early secretory phase is thought to reflect mainly the release of a pool of granules docked or very close to the plasma membrane. The sustained phase necessitates the recruitment of granules from a reserve pool. To determine which of the two secretory phases is affected by the silencing of CPX I we measured the amount of hGH secreted within the first 10 minutes (early phase) and between 10 and 45 minutes (sustained phase). The decrease in CPX I expression caused a reduction in both secretory phases (Fig. 6). However, the effect was much more pronounced in the early phase when secretion was practically abolished.

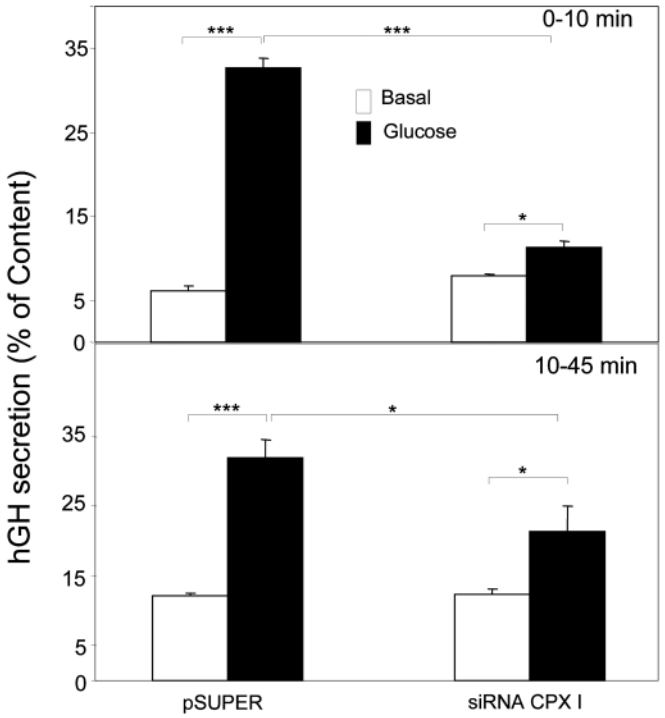


Fig. 6. Differential effect of CXP I silencing on early and late secretory phases. INS-1 cells were transiently cotransfected with a plasmid encoding hGH and with the empty pSUPER vector and siRNA CPX I. After 3 days, the cells were incubated under basal condition (Basal) or in the presence of 20 mM glucose. After 10 minutes, the incubation medium was collected and replaced with fresh buffer for an additional 35-minute period. Cellular hGH content and the fraction of hGH released by the cells during the first 10 minutes of incubation (top panel) and during the successive 35 minutes (lower panel) were measured by ELISA. The figure shows the mean ± s.e.m. of at least three independent experiments measured in triplicate (*P<0.01, ***P<0.001).

Stimulus-induced secretion of β-cells is reduced by CPX I overexpression

Expression levels of CPXs are important for stimulated exocytosis and overexpression of CPXs was shown to reduce the secretion of neurotransmitters in neuronal cells (Archer et al., 2002; Itakura et al., 1999). The effect of CPX I overexpression on stimulus-induced secretion of insulin-secreting cells was evaluated. Overexpression of CPX I was first confirmed by western blotting analysis. The transient transfection of the plasmid encoding CPXI (CMV-CPX I) in INS1 cells significantly increased the CPX I expression level (Fig. 7a). Overexpression of CMV-CPX I did not alter the hGH basal secretion but markedly reduced secretion in response to glucose and to KCl, both in INS1 cells and in βTC3 cells (Fig. 7b and c, respectively). These data confirm that expression levels of CPX I are critical for nutrient-induced secretion in β-cells.

Discussion

Previous studies showed that CPX I is an important regulator for Ca^{2+}-dependent exocytosis in neuronal cells. We show that there is a high expression of CPX I in pancreatic β-cells. The neuronal- and β-cell expression of CPX I was due to the presence of a NRSE within the 5′-regulatory region that interacted with the transcriptional repressor REST. We also demonstrated that overexpression or the selective reduction of CPX I expression levels decreased the stimulus-induced secretion. These data indicate the requirement of adequate CPX I expression levels for normal stimulated exocytosis of insulin in β-cells.

The observation that CPX I is regulated by REST provides new evidence about the role of REST-target genes in normal β-cell function. Several studies have shown that the cell-specific expression of the human *MAPK8IP1* gene encoding islet-brain 1 (IB1)/JNK-interacting protein 1(JIP-1), *pax4* and the mouse *connexin 36* genes is determined by REST through NRSE (Abderrahmani et al., 2001; Kemp et al., 2003; Martin et al., 2003). IB1/JIP-1 and connexin 36 are critical components for the control of stress-induced cell survival and insulin secretion, respectively (Bonny et al., 2000; Haefliger et al., 2003; Le Gurun et al., 2003). Pax4 is a transcriptional factor involved in early determination of the β and δ cell lineage during endocrine pancreas development (Sosa-Pineda et al., 1997). Expression of IB1/JIP-1, connexin 36 and PAX4 was

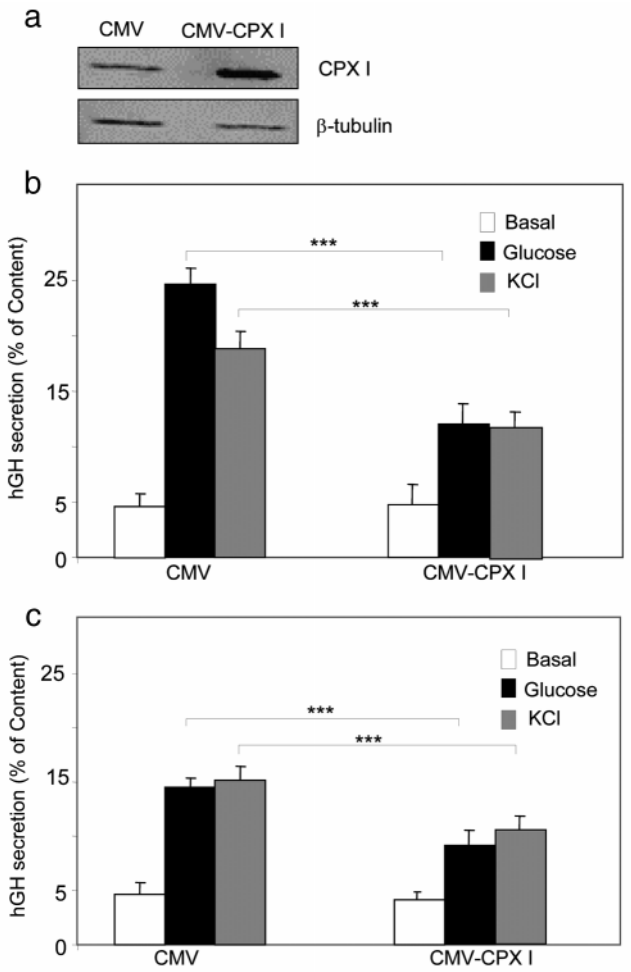


Fig. 7. Overexpression of CPX I reduces nutrient-induced secretory function. (a) Western blot analysis of CPX I overexpression in INS1 cells. The transient transfection of CMV-CPX I increased CPXI expression. The effect of overexpression of CPX I was assessed on the secretory ability of INS1 (b) and βTC3 cells (c) in response to glucose and KCl. Cells were transiently co-transfected with the plasmid encoding CPX I (CMV-CPX I) or the empty vector (CMV) and with the plasmid encoding hGH vector. The release of hGH under basal and stimulatory (20 mM glucose supplemented by forskolin and IBMX or 24 mM of KCl) conditions was measured by ELISA. In both INS1 and βTC3 cells, overexpression of CPX I significantly decreased, by 50%, the amount of secreted hGH in response to glucose and to KCl compared to cells transfected with CMV. The figure shows the mean ± s.e.m. of at least three independent experiments measured in triplicate (***P<0.001).

undetectable in cells expressing REST whereas these proteins were abundantly expressed in neuronal cells and β-cells. Similarly, we showed here that CPX I transcripts were undetectable in HeLa and RAW cells. These cells express high levels of REST mRNA compared to insulin-secreting cells that do not contain detectable levels of REST (Abderrahmani et al., 2001). Our observation is then consistent with the fact that preferential presence of CPX I in β-cells is transcriptionally determined by REST. We have shown that in mice, REST is present in progenitor cells and disappears during the β-cell differentiation (A.A., J. Ferrer and G.W., unpublished). The silencing of REST during this early developmental stage is thought to be necessary for the differentiated phenotype of insulin-secreting cells. Since CPX I plays an important role in glucose-induced secretion, the disappearance of REST during β-cell differentiation could be mandatory for the establishment of a full secretory response in mature β-cells.

The transduction mechanism by which an increase in intracellular Ca^{2+} concentration elicits exocytosis of insulin is still poorly understood. Assembly of the SNARE complex formed by syntaxin I, VAMP and SNAP25 is required for insulin exocytosis from dense-core vesicles in β-cells (Regazzi et al., 1996b; Wheeler et al., 1996; Gerber and Sudhof, 2002). Some models of CPX I function have been proposed in neuronal cells. On the one hand, it was shown that CPX I binds syntaxin-I SNARE in the assembled SNAREs complex. This interaction was believed to promote oligomerisation of the complex into higher-order structures that would allow efficient Ca^{2+}-dependent neurotransmitter release (Tokumaru et al., 2001). On the other hand, subsequent work showed that the SNARE complex assembly or its oligomerisation do not need the presence of CPX I (Chen et al., 2002). The model proposed that CPX I stabilizes the syntaxin-I/synaptobrevin interface, thus allowing efficient Ca^{2+}-evoked neurotransmitter release. The CPX I knockout or CPX I overexpression inhibited the stimulated exocytosis in neuronal cells (Itakura et al., 1999; Reim et al., 2001). CPX I was demonstrated to act at or after the Ca^{2+}-triggering step of fast synchronous transmitter release (Reim et al., 2001). Here, we show that overexpression of CPX I also led to an impaired stimulated exocytosis in β-cells. This could be the result of competition for the binding with SNAP25/syntaxin-I and therefore excess CPX I levels may alter the formation of the functional SNARE complex. Thus, overexpression experiments are not appropriate to assess the precise function of CPX I in insulin exocytosis. To overcome this problem we took advantage of the possibilities offered by the RNAi process. Specific silencing of the CPX I gene led to a decrease of stimulated secretion, which was more drastic in the early secretory phase whereas it was less pronounced in the sustained phase. Thus, this result indicates that CPX I expression levels are required for normal stimulus-induced insulin secretion, probably by controlling the Ca^{2+}-triggering step of insulin release in β-cells.

CPX II is expressed at low levels in many different tissues, and in neuronal cells has a function similar to CPX I in the control of SNARE-mediated exocytosis (McMahon et al., 1995). Consistent with this, we found low levels of CPX II transcripts in β-TC3 cells. This observation is in accordance with the fact that CPX I and CPX II are encoded by two different genes which are probably differentially regulated. In rat forebrain and hippocampus, expression levels and cellular localisation of CPX I and CPX II are different (Eastwood et al., 2000). CPX I and CPX II genes are likely to be differentially regulated also in β-cells. Thus, while the CPX I gene is activated, the CPX II gene may be repressed in β-cells. However, CPX II mRNA was not detected in all β-cell lines, indicating that the expression of this gene is regulated in a β-cell line-dependent manner. This could be due to clonal selection occurring during the transformation and generation of cell lines. At present, we cannot exclude a minor role of CPX II in insulin exocytosis.

The data suggest that a loss or a gain of function of CPX I

could be associated with a decrease of glucose-induced insulin secretion. A defect of insulin secretion can lead to diabetes. Dysregulation in CPX I expression could potentially lead to hyperglycemia because of inefficient release of insulin in response to glucose. Blood glucose homeostasis in mice lacking CPX I has not been investigated. In view of our findings, these mice could develop an abnormal elevation of glycemia caused by an impairment of glucose-induced insulin secretion (Reim et al., 2001). As in the case of CPX I, the neuronal and the β-cell-specific expression of IB1/JIP-1 is determined by REST. IB1/JIP-1 also plays a critical role both in neuronal cells and β-cells (Bonny et al., 2000; Whitmarsh et al., 2001; Haefliger et al., 2003; Magara et al., 2003). A rare mutation found in the IB1/JIP-1 gene was associated with a monogenic form of type 2 diabetes (Waeber et al., 2000). Thus, we hypothesised that genes selectively expressed in neuronal cells and β-cells are critical for β-cell function and thereby are potential candidates for type 2 diabetes. We therefore propose that a loss or a gain of function of CPX I could lead to a decrease of glucose-induced insulin secretion observed in diabetic patients.

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