

cAMP-dependent phosphorylation of PTB1 promotes the expression of insulin secretory granule proteins in β cells

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Summary

Glucose stimulates the exocytosis of insulin secretory granules of pancreatic β cells. Granule stores are quickly refilled by activation of posttranscriptional mechanisms that enhance the biosynthesis of granule components. Rapid replacement of granules is important to sustain insulin secretion, since new granules appear to be preferentially released. Posttranscriptional regulation of granule biogenesis includes the glucose-induced nucleocytoplasmic translocation of polypyrimidine tract binding protein 1 (PTB1), which binds mRNAs encoding granule proteins, and thus promotes their stabilization and translation. Glucagon-like peptide 1 (GLP-1) potentiates glucose-stimulated insulin gene expression and secretion by increasing cAMP levels in β cells. Here, we show that elevation of cAMP levels causes the protein kinase A-dependent phosphorylation and nucleocytoplasmic translocation of PTB1, thereby preventing the rapid degradation of insulin mRNA and enhancing the expression of various granule proteins. Taken together, these findings identify PTB1 as a common downstream target of glucose and GLP-1 for the posttranscriptional upregulation of granule biogenesis.

Introduction

Pancreatic β cells produce insulin, the most important hormone for the control of glucose homeostasis in chordates (Steiner, 1977). In β cells, insulin is stored within organelles termed secretory granules (SGs). The elevation of glucose levels triggers the fusion of β cell SGs with the plasma membrane and the release of insulin. Insulin, in turn, promotes the removal and consumption of glucose by peripheral tissues, thereby reducing glycemia. Absolute and relative deficits of insulin production and secretion lead to the development of diabetes mellitus, one of the most common metabolic disorders in human.

In addition to stimulating SG exocytosis, glucose induces the concerted expression of insulin and most other components necessary for the assembly of new SGs in β cells (Goodge and Hutton, 2000; Guest et al., 1989). Increasing evidence suggests that newly-synthesized neurosecretory granules, including insulin SGs, preferentially undergo exocytosis versus older granules (Gold et al., 2000; Halban, 1982; Duncan et al., 2003; Solimena and Gerdes, 2003). Thus, rapid induction of granule biogenesis is likely to be important to sustaining insulin secretion. This induction relies on both transcriptional and posttranscriptional mechanisms (Giddings et al., 1983; Permutt and Kipnis, 1972; Wicksteed et al., 2001). Posttranscriptional mechanisms, in particular, can entirely account for the increased insulin biosynthesis in the 2 hr following glucose stimulation (Itoh and Okamoto, 1980). The rapidly increased translation of insulin mRNA depends on its 5' and 3' untranslated regions (UTRs) (Wicksteed et al., 2001). More recently, we have found that glucose promotes the rapid nucleocytoplasmic translocation of polypyrimidine tract binding protein 1 (PTB1) in rat pancreatic islets and insulinoma INS-1 cells (Knoch et al., 2004). Cytosolic

PTB1, in turn, binds and stabilizes mRNA encoding proteins of SGs, thus enhancing their translation. Conversely, knockdown of PTB1 in INS-1 cells by RNA interference led to the depletion of SGs. Taken together, these data point to PTB1 as a key factor which posttranscriptionally regulates the expression of insulin SG components.

Glucagon-like peptide-1 (GLP-1) is an enteric hormone that is secreted in response to ingestion of nutrients and strongly enhances the insulinotropic action of glucose through the activation of its adenylate cyclase-coupled receptor, thus stimulating cAMP production and protein kinase A (PKA)-dependent phosphorylation (Drucker, 2003; Hinke et al., 2004). Pharmacological activation of PKA in rat pheochromocytoma PC12 cells was recently shown to induce the phosphorylation of PTB1 on serine 16 (Xie et al., 2003). This serine is part of the nuclear export signal embedded within the N-terminal nuclear localization signal of the protein. Its phosphorylation both in heterokaryon assays and in *Xenopus* oocytes led to the accumulation of PTB1 in the cytosol (Xie et al., 2003). The aim of the present studies was to test whether PKA phosphorylates PTB1 and modulates its nucleocytoplasmic transport also in β cells, thereby accounting, at least in part, for the ability of cAMP-elevating agents such as GLP-1 to promote the biogenesis of insulin SGs.

Results

To investigate whether cAMP promotes the phosphorylation and nucleocytoplasmic translocation of PTB1, INS-1 cells were incubated with 1 mM IBMX, which inhibits cAMP-degrading phosphodiesterases. IBMX treatment led to a clear phospholabeling of PTB1 compared to untreated cells (Figure 1A, top panel). A similar pattern was detected when the same cell

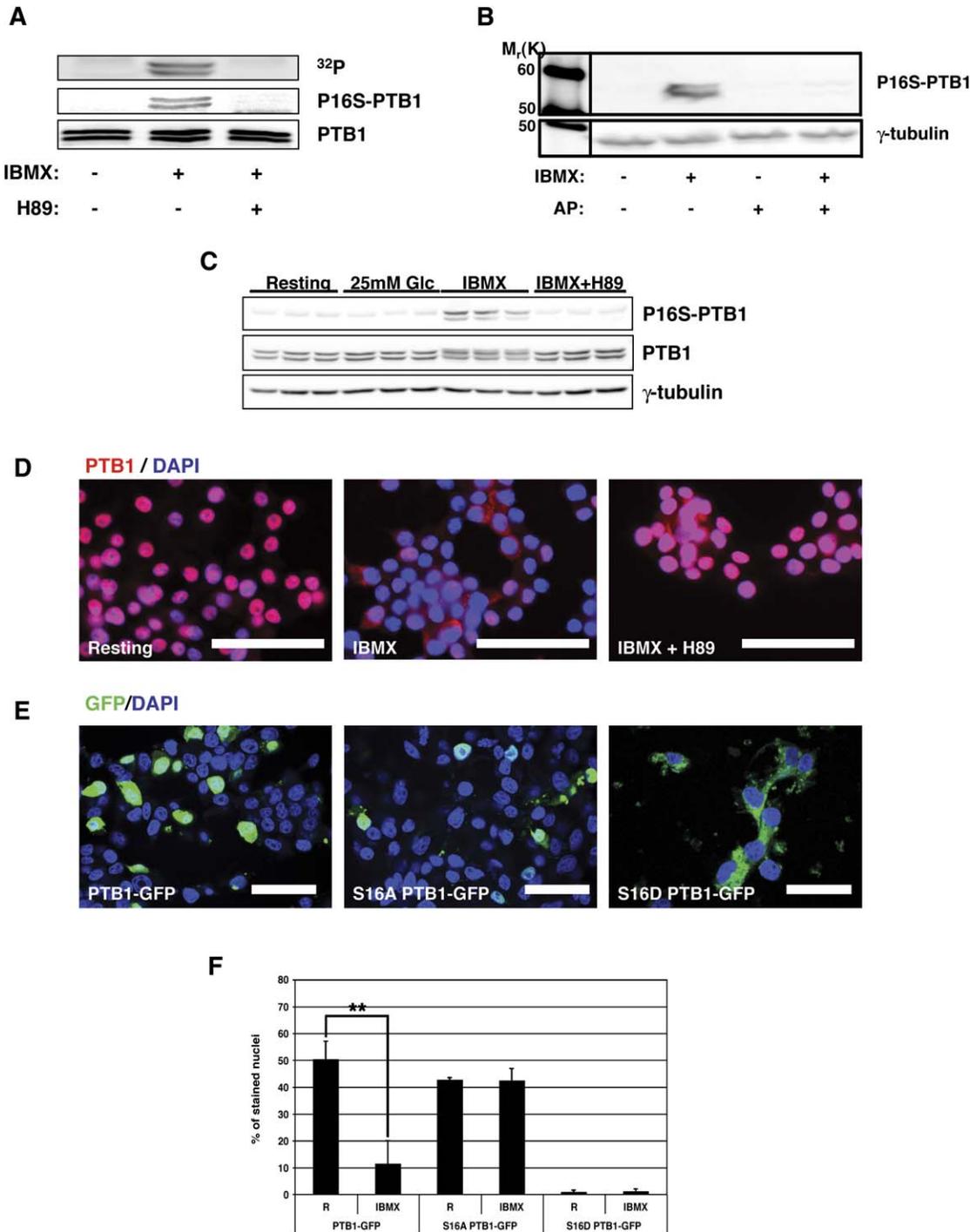


Figure 1. cAMP promotes the phosphorylation and nucleocytoplasmic translocation of PTB1 in INS-1 cells

A) Autoradiography (top panel) for PTB1 and immunoblots for phospho-PTB1 (P16S-PTB1, middle panel) and PTB1 (bottom panel) immunoprecipitated from extracts of INS-1 cells labeled with ^{32}P -orthophosphoric acid. Cells were kept for 1 hr in 0 mM glucose (resting) with or without 10 μ M H89 before being treated with 1 mM IBMX for 2 hr.

B) Immunoblots with affinity-purified antibody P16S-PTB1 on extracts from INS-1 cells incubated with 1 mM IBMX. Cell lysates were treated or untreated with alkaline phosphatase (AP) as described (Ort et al., 2001).

C) Immunoblots for P16S-PTB1, PTB1 and γ -tubulin on cell extracts from INS-1 kept in resting medium or stimulated with 25 mM glucose, 1 mM IBMX, or 1 mM IBMX + 10 nM H89.

D) Immunofluorescence for PTB1 (red) in INS-1 cells treated with IBMX or IBMX + H89 as in (A). Nuclei were counterstained with DAPI (blue). Scale bar represents 50 μ m.

E) Confocal microscopy on INS-1 cells transfected with the indicated PTB1-GFP constructs. Nuclei were blue stained by DAPI. Scale bar represents 31 μ m.

F) Number of PTB1-GFP positive nuclei from 8 fields as in (D) for each experimental condition. P values were calculated with the Student's t test and are indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.001. Error bars show standard deviations from at least three independent experiments.

extracts were immunoblotted with the antibody P16S, which was raised against a peptide mimicking PTB1 phosphorylated on serine 16 (Figure 1A, middle panel). Both signals, however, were reduced by coincubation with 10 μ M H89 (Figure 1A, bottom panel), which inhibits PKA. Recognition of the PTB1 doublet in IBMX-treated cells by P16S was alkaline phosphatase-sensitive, hence confirming the selective reactivity of this antibody with phosphorylated PTB1 (Figure 1B). However, serine 16 was not phosphorylated following glucose stimulation (Figure 1C). In resting INS-1 cells PTB1 was mostly found in the nucleus, while in IBMX-stimulated cells this nuclear signal was reduced concomitantly with an increased cytosolic labeling (Figure 1D). The IBMX-induced redistribution of PTB1 was also inhibited by cotreatment with H89.

The relationship between phosphorylation of PTB1 on serine 16 and its nucleocytoplasmic translocation in INS-1 cells was directly tested by transient transfection of PTB1-GFP fusion constructs in which serine 16 was replaced either by an alanine (S16A PTB1-GFP) or an aspartate (S16D PTB1-GFP). PTB1-GFP and S16A PTB1-GFP were both enriched in the nuclei of transfected INS-1 cells, whereas S16D PTB1-GFP was exclusively cytosolic (Figure 1E). IBMX treatment significantly reduced the number of nuclei positive for PTB1-GFP, but had virtually no effect on the nuclear signal for S16A PTB1-GFP and S16D PTB1-GFP (Figure 1F). These results demonstrate that cAMP-dependent phosphorylation on serine 16 induces the nucleocytoplasmic translocation of PTB1 in INS-1 cells.

In β cells, cAMP activates PKA and the guanine-nucleotide exchange factor EPAC/cAMP-GEFII (Kashima et al., 2001). Like in PC12 cells, the phosphorylation of PTB1 in INS-1 cells was mostly likely mediated by PKA, because serine 16 is found within a consensus motif for phosphorylation by PKA, and because this phosphorylation was inhibited by H89. To directly assess the role of PKA in PTB1 phosphorylation, the expression of the α -catalytic subunit of PKA (PKA-C α) was knocked down using siRNA oligos. Reduction of PKA-C α expression following RNAi treatment was confirmed by real-time PCR (Figure 2A) and immunoblot (Figure 2C), while the activity of PKA in extracts of siRNA-treated cells was assessed by *in vitro* phosphorylation of a specific substrate (Figure 2B). Real-time PCR data were normalized by parallel amplification of β -actin mRNA, whose levels were the least affected among those of six conventional housekeeping genes in the different experimental conditions we used (Supplemental Experimental Procedures and Figure S1A in the Supplemental Data available with this article online). Knockdown of PKA activity by \sim 50% was accompanied by the reduced phosphorylation of PTB1 (focus on the P16S-PTB1 lines in Figures 2C and 2D) and nucleocytoplasmic translocation of PTB1 in response to IBMX (Figure 2E). Knockdown of the regulatory subunit II of PKA (PKA-RII) led to a similar reduction of PTB1 S16 phosphorylation (Figures S1C–S1E), despite the PKA activity, as expected, was increased (Figure S1F). This result can be explained by taking into account the role of PKA-RII together with A-kinase anchoring proteins in targeting PKA-C to its substrates *in vivo* (Wong and Scott, 2004).

Since PKA can activate the MAP kinases MEK1/2-ERK1/2 (Vossler et al., 1997), cells were stimulated for various lengths of time with IBMX with or without 10 μ M PD98059 or 10 μ M U0126, two selective inhibitors of MEK1/2 (Figure 2F). While the phosphorylation of ERK1/2 induced by IBMX preceded the phosphorylation of PTB1, its inhibition did not affect the latter,

hence pointing to PKA as the kinase directly responsible for PTB1 phosphorylation and translocation.

Next, we monitored the effect of cAMP on the expression of SG components. The levels of β -actin mRNA in INS-1 cells grown in normal medium were comparable with those measured in freshly isolated rat islets (Figure S1B), whereas those of insulin 1, ICA512, PC1/3, and PC2 were 13%, 54%, 59%, and 52%, respectively (Figure 3A). However, the half-lives of insulin 1 mRNA in INS-1 cells ($t_{1/2}$: 19.3 \pm 4.8 hr) and islets ($t_{1/2}$: 16.1 \pm 3.6 hr) kept in medium with 11 mM glucose were comparable (Figure 3B). In INS-1 cells incubated in resting medium, which contained neither glucose nor IBMX, the half-life of insulin 1 mRNA was reduced to 2.16 \pm 0.46 hr (Figure 3C), whereas in IBMX-treated cells, also without glucose, insulin 1 mRNA levels did not decrease. Using real-time PCR we found that in INS-1 cells stimulated with IBMX for 2 hr following 1 hr in resting medium the levels of insulin, ICA512, PC1/3, and PC2 mRNAs, all of which contain PTB1 binding sites in their 3'UTR, and also in the 5'UTR in the case of PC2, were 197%, 250%, 233%, and 165%, respectively, compared to those in cells kept in resting medium for 3 hr (Figure 3D). Such increments were inhibited by H89 and were not apparent in the case of CPE, a SG protein whose expression is not upregulated by glucose (Guest et al., 1989), and whose mRNA, which lacks a consensus for PTB1 binding, is not stabilized by PTB1 upon glucose stimulation (Knoch et al., 2004). The levels of insulin 1 and ICA512 mRNAs in IBMX-treated cells were also comparable to those measured in cells stimulated with 25 mM glucose (Figure 3E). These results indicate that IBMX stimulation was similarly effective as maximal glucose stimulation in restoring the levels of insulin and ICA512 mRNAs levels following their decrease upon cell incubation in resting medium. IBMX stimulation of INS-1 cells pretreated with actinomycin D, which inhibits transcription, still increased insulin 1, ICA512, PC1/3, and PC2 mRNA levels by 172%, 244%, 254%, and 157%, respectively (Figure 3F). Actinomycin D also did not prevent the glucose-stimulated increase in the levels of these mRNAs. Conversely, IBMX treatment could not enhance insulin, ICA512, and PC1/3 mRNA levels following the knockdown of PKA-C α with siRNA oligos (Figure 3G). Taken together, these results indicate that PKA stabilizes mRNAs encoding various SG proteins.

We then verified that cAMP/PKA also promote the expression of SG proteins encoded by mRNAs including PTB1 binding sites. As previously shown (Knoch et al., 2004), glucose rapidly increased the expression of pro-ICA512 (Figure 4A, top panel). IBMX also enhanced the expression of pro-ICA512 (Figure 4A, middle panel), while no additional increment was observed upon treatment with 1 mM IBMX and 25 mM glucose (Figure 4A, bottom panel), conceivably because at these high concentrations each agent already caused a maximal stimulation. Similar to glucose, IBMX-induced pro-ICA512 expression was already apparent after 30 min. stimulation, i.e. before PKA-dependent PTB1 was detectable (Figure 2F). Moreover, the levels of pro-ICA512 were higher in 1 hr rather than in 2 hr IBMX-stimulated cells, when PTB1 phosphorylation is maximal. These data indicate that glucose- and IBMX-induced expression of SG components relies on multiple posttranslational mechanisms, some of which become active prior to PTB1 phosphorylation and translocation (see more in the discussion). The induction of pro-ICA512 by IBMX was prevented by knockdown of PKA-C α (Figure 4B). Other SG proteins upregulated by IBMX included PC2

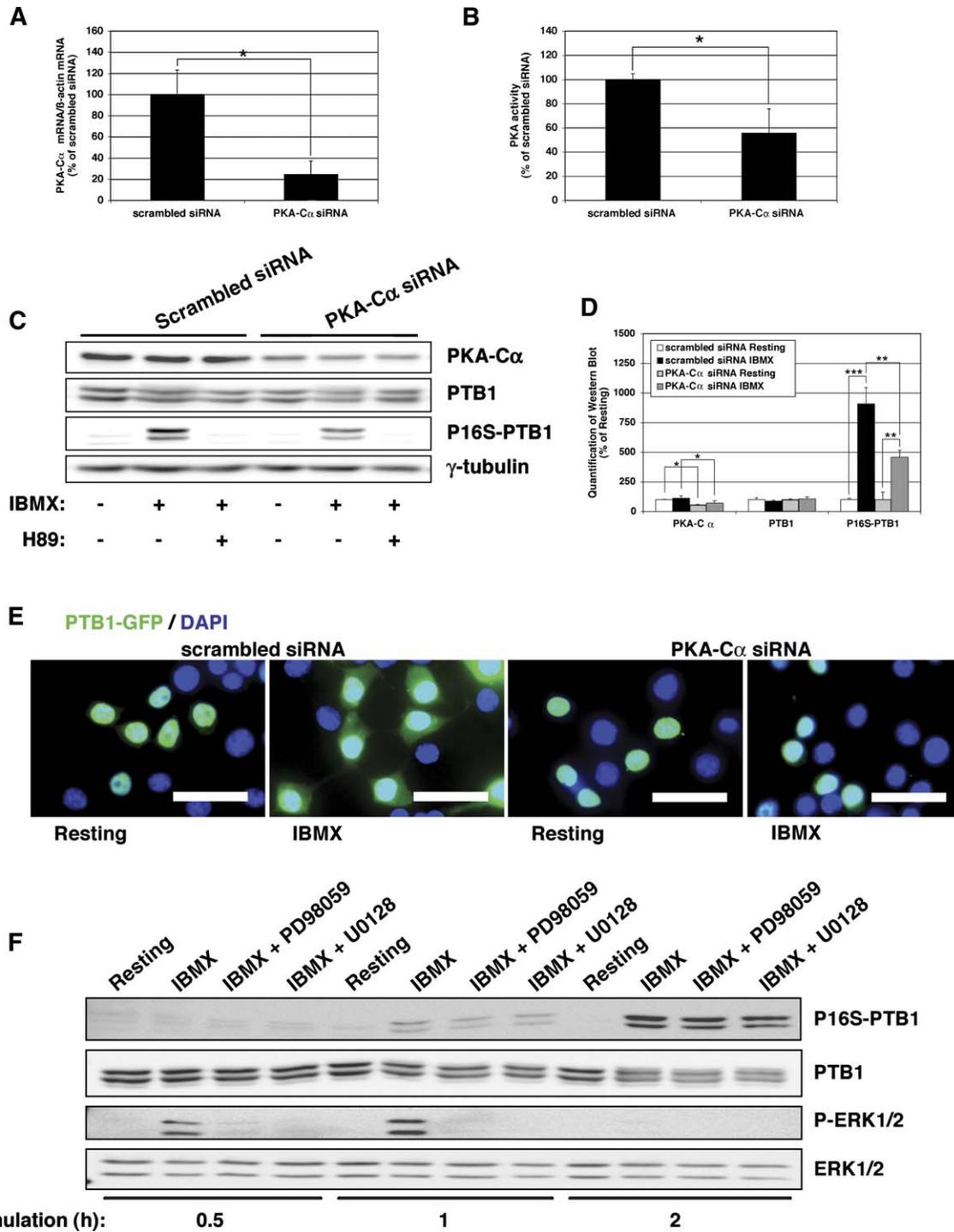


Figure 2. PTB1 is phosphorylated by PKA

A) PKA-C α mRNA levels in INS-1 cells as quantified by real-time PCR 4 days after transfection with short-interfering RNA (siRNA) oligos for PKA-C α or control scrambled siRNA oligos. Values were normalized for β -actin mRNA.

B) PKA activity in extracts from INS-1 cells treated as in (A).

C) Immunoblots for PKA-C α , PTB1, P16S-PTB1, and γ -tubulin in extracts from INS-1 cells treated as in (A) and incubated with or without 1 mM IBMX or 1 mM IBMX + 10 μ M H89.

D) Quantification of the immunoblots showed in (C).

E) Fluorescence microscopy of INS-1 cells transfected with PTB1-GFP, treated with control or PKA-C α siRNA oligos as in (A), and then incubated with or without 1 mM IBMX. Scale bar represents 25 μ m.

F) Immunoblots for PTB1, P16S-PTB1, ERK1/2, or phospho-ERK1/2 on INS-1 cell extracts. Cells were kept in resting medium with or without 5 μ M PD98059 or 10 μ M U0128 for 1 hr before treatment with 1 mM IBMX for the indicated times.

P values were calculated with the Student's t test and are indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.001. Error bars show standard deviations from at least three independent experiments.

and chromogranin A (Figures 4C and 4D), whereas various proteins unrelated to SGs, including γ -tubulin, calnexin, mannosidase II, and PDI, were unaffected (Figure 4C). The role of PTB1 in IBMX-induced expression of SG components was directly tested by reducing PTB1 levels by RNA interference. Analyses by real-time PCR and western blotting confirmed that upon depletion of PTB1 IBMX could not any longer elicit increments in the levels of insulin, ICA512, PC1/3, and PC2 mRNAs (Figure 4E) and pro-ICA512 (Figure 4F). Specific knockdown of PTB1 was validated by confirming the reduction of PTB1 mRNA and of a renilla luciferase construct including at the 3'UTR rat PTB1 target sequence (Figures S1F and S1G).

To test whether PTB1 binding sites in the 3'UTR of mRNAs encoding SG proteins are critical for regulation of gene expression by cAMP/PKA, we independently cloned the 3'UTR of ICA512 and PC2 downstream of a firefly luciferase reporter gene (pGL3-Basic) (Figure 5A). Stimulation with IBMX did not significantly affect luciferase activity in INS-1 cells transfected with pGL3-Basic, but induced an \sim 2-fold increase in cells transfected with Luc-ICA512-3'UTR and Luc-PC2-3'UTR compared to resting cells (Figure 5B). This increase was not observed in cells cotreated with H89 or transfected with a Luc-PC2-3'UTR construct (Luc-PC2-3'UTR-mut) in which the single consensus site for PTB1 binding was mutated. Further evidence for the involvement of PKA-induced phosphorylation in the upregulation of gene expression was obtained by measuring luciferase activity in cells cotransfected with Luc-ICA512-3'UTR and various PTB-V5 alleles (Figure 5C). Luciferase activity was increased approximately 2- and 4-fold in cells expressing PTB-V5 and S16D PTB-V5, respectively, compared to nontransfected cells or cells expressing S16A PTB-V5.

Next, we tested whether GLP-1, which physiologically enhances glucose-stimulation of β cells by activating PKA, could increment the expression of SG components through PTB1 similarly to IBMX. Treatment of INS-1 cells with GLP-1 increased the levels of both ICA512 and PC1/3 mRNAs, albeit less than IBMX, while no significant increments were measured in the case of insulin 1 and CPE mRNAs (Figure 6A). We also analyzed whether 3'UTRs of SG protein mRNAs are involved in the upregulation of gene expression by GLP-1. Treatment with GLP-1 significantly increased the luciferase activity in INS-1 cells transfected with Luc-ICA512-3'UTR or Luc-PC2-3'UTR, although less than IBMX, while it did not affect the luciferase activity of cell transfected with pGL3-Basic or Luc-PC2-3'UTR-mut (Figure 6B). Overall, the reduced effects of GLP-1 compared to IBMX correlated with the lower levels of cAMP induced by the former in INS-1 cells and purified rat islets (Figure 6C). These data strongly suggest that PTB1 is part of the signaling pathway through which GLP-1 rapidly enhances the translation of mRNAs encoding SG proteins. Accordingly, GLP-1 treatment led to the increased phosphorylation of PTB1 in both INS-1 cells and islets (Figure 6D). The use of whole islets rather than isolated β cells for these analyses was justified, as immunomicroscopy on rat pancreatic tissue sections showed a comparable expression of PTB1 in islet β and α cells (Figure 6E).

Discussion

In this study, we have shown that the induction of PKA activity by IBMX, which increases cAMP levels in β cells, causes the phosphorylation and nucleocytoplasmic transport of PTB1. Cytosolic

PTB1, in turn, quickly upregulates the expression of insulin and other SG components encoded by mRNAs containing PTB1 binding sites in their 3'UTR. Specifically, binding of cytosolic PTB1 to these mRNAs, as we have previously shown to occur following glucose stimulation of β cells, leads to their stabilization and increased translation, hence prompting the biogenesis of insulin SGs. These findings are of physiological relevance, as GLP-1, which activates PKA, also promotes the phosphorylation of PTB1, and conceivably, its nucleocytoplasmic translocation. Accordingly, we show that GLP-1 rapidly enhances the levels of ICA512 and PC1/3 mRNAs and that this increment, at least in the case of PC2, depends on the presence of a PTB1 binding site in the 3'UTR. Treatment with GLP-1, however, did not significantly increase the levels of insulin mRNA in INS-1 cells and freshly isolated islets, conceivably because cAMP levels, and thus PTB1 phosphorylation, were lower than following IBMX treatment. Evidence that pro-ICA512 expression was already increased in cells stimulated with IBMX for 30 min, while phosphorylated PTB1 was not detected before stimulation with IBMX for 1 hr, points to the existence of multiple posttranscriptional mechanisms that are sequentially activated in order to enhance granule biogenesis. Once activated, these mechanisms are likely to remain operative until stimulation gradually subsides. Strict dependency of some of these processes on glucose presence could explain why the levels of pro-ICA512 were lower in cells stimulated for 2 hr with IBMX, but without glucose, compared to cells equally stimulated for 1 hr only, notwithstanding that maximal PTB1 phosphorylation was detected after IBMX treatment for 2 hr. In vivo secretion of GLP-1 precedes the increase of hyperglycemia. Hence, PKA-dependent PTB1 phosphorylation and translocation should already be ongoing by the time glucose levels reach the threshold required for β cell stimulation, and thus they should be able to operate in concert with glucose in enhancing SG biogenesis.

INS-1 cells are a widely used model system to study β cells function (Asfari et al., 1992). While being less responsive than primary β cells, these cells respond to stimulation with high glucose for 2 hr with a 2.5- to 3-fold enhanced insulin secretion (Ort et al., 2001) and 2-fold increased levels of total insulin (data not shown). As in primary β cells, such increment of insulin expression can only result from enhanced mRNA translation, since insulin transcription is not yet induced within this short period of time after exposure to high glucose. Our data also indicate that the half-life of insulin 1 mRNA in INS-1 cells and rat islets grown in normal medium with 11 mM glucose is comparable. Thus, our measurements in INS-1 cells are likely to be physiologically significant. The half-life of insulin 1 mRNA, however, was significantly shorter than previously reported by Welsh et al. for the combined rat insulin 1 and 2 mRNAs (Welsh et al., 1985). These authors found indeed that the half-life of insulin mRNA in rat islets incubated with 3.3 mM glucose is 29.0 ± 6.1 hr, and that it increases by 2.6-fold ($t_{1/2}$: 76.8 ± 20.9 hr) in 17 mM glucose. In the same study, it was shown that in rat insulinoma RIN-5F cells the half-life of insulin mRNA was 26.0 ± 5.1 hr in either low and high glucose, but it still increased by 2.2-fold ($t_{1/2}$: 57.6 ± 8.0 hr) upon treatment with cholera toxin, which enhances cAMP levels. The reason for the discrepancy between our results and those by Welsh and coworkers is not clear and may be attributed, at least in part, to methodological differences between these studies. Our mRNA decay assays, however, show that in INS-1 cells incubated without glucose

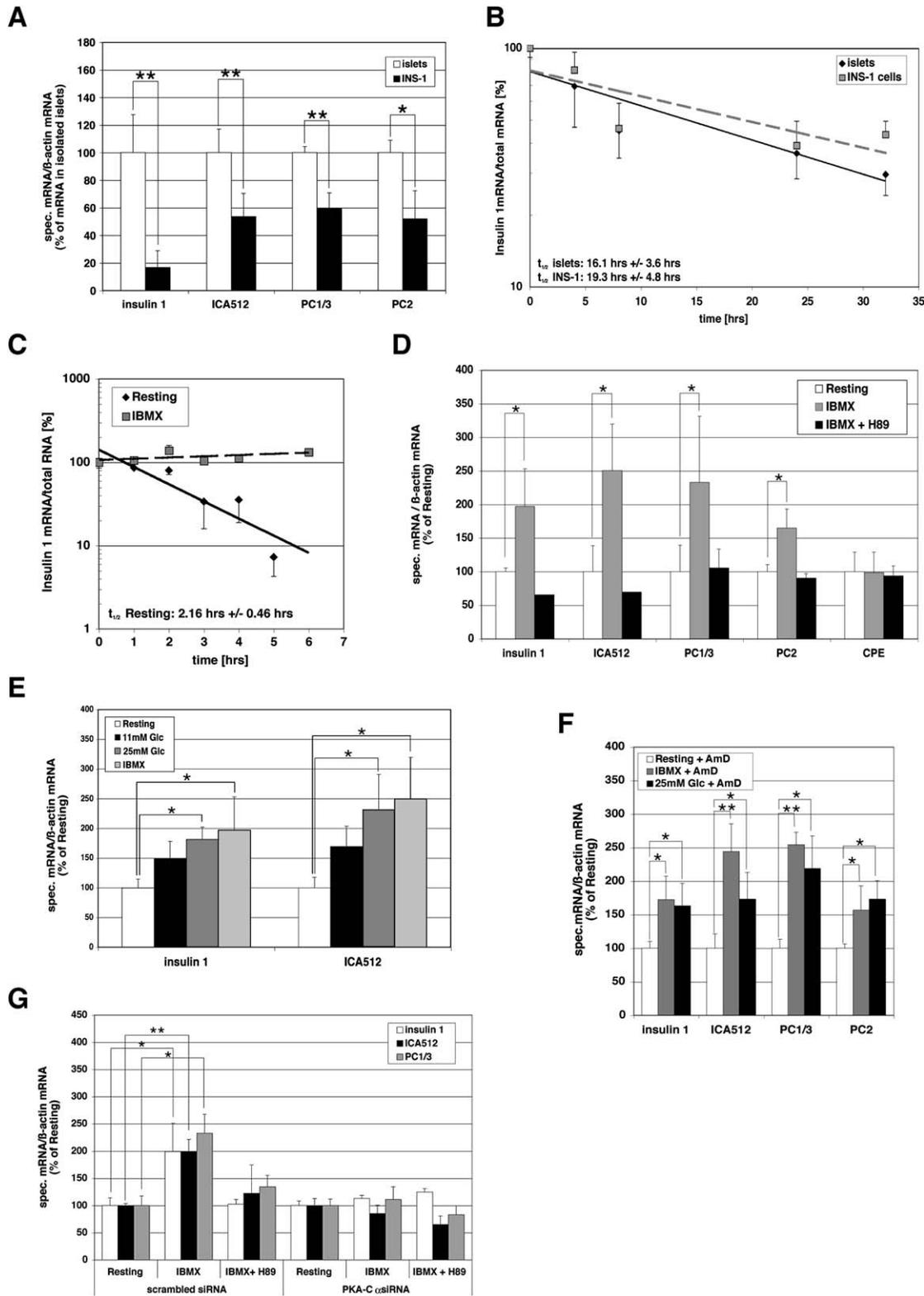


Figure 3. cAMP/PKA-induced stabilization of SG mRNAs

A) Levels of insulin 1, ICA512, PC1/3, and PC2 mRNAs in purified rat pancreatic islets and INS-1 cells grown in normal medium with 11 mM glucose. Quantification was performed by real-time PCR using β -actin mRNA for normalization. For each gene, the levels detected in islets were equaled to 100%.
B) Decay of [3 H]-labeled insulin 1 mRNA in INS-1 cells (broken line) and purified rat islets (continuous line) incubated for up to 40 hr in normal medium with 11 mM glucose.
C) Decay of [3 H]-labeled insulin 1 mRNA in INS-1 cells in 0 mM glucose (resting buffer) with (broken line) or without 1 mM IBMX (continuous line) for up to 6 hr.
D–G) Real-time PCR for the indicated SG markers and β -actin on total RNA from INS-1 cells. In **(D)** cells were incubated for 1 hr in resting buffer, and then for an additional 2 hr in resting buffer without (resting) or with 1 mM IBMX or 1 mM IBMX + 10 μ M H89. **(E)** mRNA levels in cells incubated for 2 hr in resting buffer, 11 mM glucose (normal

the half-life of insulin 1 mRNA was only 2.16 ± 0.46 hr. This very short half-life is consistent with the results independently obtained by real-time PCR, which showed an increment of insulin mRNA by 2-fold after IBMX treatment for 2 hr. Notably, IBMX treatment completely prevented the decay of insulin mRNA and its effect was not adversely affected by blocking transcription with actinomycin D.

In PC12 cells, which resemble β cells in respect to many of their neuroendocrine features, treatment with forskolin induced the phosphorylation of PTB1, but did not significantly change its localization (Xie et al., 2003). In the case of INS-1 cells, treatment with forskolin was surprisingly ineffective in increasing cAMP levels as well as PTB1 phosphorylation and nucleocytoplasmic transport (data not shown). Conceivably, IBMX-mediated inhibition of phosphodiesterases, of which PDE3B is the most relevant isoform in β cells (Ahmad et al., 2000; Harndahl et al., 2002), is more effective than forskolin-mediated activation of β cell adenylate cyclase, which include the isoforms type I, III, VI and VIII (Delmeire et al., 2004). Notably, we did not detect phosphorylation of PTB1 in INS-1 cells stimulated with glucose (this study), although we observed a redistribution of PTB1 from the nucleus to the cytosol (Knoch et al., 2004). Translocation of PTB1 in the cytosol without detectable changes in its phosphorylation has also been observed in HeLa cells infected with poliovirus (Back et al., 2002; Xie et al., 2003), possibly because of its cleavage by the virus-encoded protease3C^{pro} (Back et al., 2002). Glucose stimulation of pancreatic islets is also accompanied by the generation of a PTB1 C-terminal proteolytic fragment (Knoch et al., 2004), which is close in size to the mainly cytosolic 26 kDa C-terminal fragment observed in poliovirus-infected cells (Back et al., 2002). In glucose-stimulated islets, however, such 27 kDa PTB1 fragment was only detected in the nuclear fraction, whereas most cytosolic PTB1 migrated as a 59 kDa polypeptide, i.e., at the molecular weight of full-length PTB1. These differences may account for the fact that in β cells stimulated with glucose translocation of intact PTB1 into the cytosol correlates with increased stability and translation of SG protein mRNAs, whereas in poliovirus-infected cells cytosolic PTB1 fragments may contribute to the molecular switching from translation to replication of polioviral RNA (Back et al., 2002). Taken together, these findings suggest that other mechanisms, in addition to PKA-dependent phosphorylation, can elicit the translocation of PTB1 in the cytosol. The identification of these mechanisms will be an important task for the future. It will also be interesting to establish in which compartment PKA phosphorylates PTB1, and thus whether this modification promotes the exit of PTB1 from the nucleus or rather favors its retention in the cytosol.

The synergy of GLP-1 and glucose on insulin expression and secretion has been proposed to occur at different levels (Hinze et al., 2004). For instance, both glucose and GLP-1 activate calcineurin by increasing the levels of intracellular Ca^{2+} . Calcineurin, on one hand, can promote insulin transcription by dephosphorylation, and thereby activation, of the transcription factor NFAT (nuclear factor of activated T cells). Activation of

calcineurin by somatostatin, on the other hand, has also been shown to inhibit insulin release from mouse β cells (Renstrom et al., 1996), thereby counteracting the stimulatory effect of glucose and GLP-1 on secretion. Glucose and GLP-1 can also be synergic in stimulating type VIII adenylate cyclase, whose activity is increased by Ca^{2+} /calmodulin and $G_{s\alpha}$. The activity of Ca^{2+} /calmodulin-activated calcineurin and cAMP-dependent PKA may further converge in regulating the phosphorylation of molecules involved in SG exocytosis, as both enzymes are recruited to the same site at the plasma membrane by the shared anchoring protein AKAP79/150 (Lester et al., 2001). Here, we have demonstrated that increased nucleocytoplasmic transport and binding of PTB1 to the 3'UTR of mRNAs encoding SG proteins is an additional pathway by which glucose and GLP-1 prompt the rapid biosynthesis of SG components, and thereby the assembly of new insulin SGs. Taken together with previous data (Xie et al., 2003), our findings raise the possibility that cAMP/PKA-dependent phosphorylation of PTB1 is a posttranscriptional mechanism shared by different neuroendocrine cells to promote the biogenesis of SGs.

Experimental Procedures

Antibodies

The rabbit anti-phospho-PTB1 antibody P16S was generated as described (Xie et al., 2003) and purified by affinity chromatography using agarose beads coupled to the immunogenic phosphopeptide. Other primary antibodies were from the following sources: mouse monoclonal antibodies anti-PTB1 (Zymed), anti-ICA512 (Ort et al., 2001), anti-calnexin (Transduction Laboratories), anti-chromogranin A (Immunon), anti-protein disulfide isomerase (PDI, Stressgen), anti- γ -tubulin (Sigma), anti-mannosidase II (Velasco et al., 1993) and anti-V5 (Invitrogen); rabbit polyclonal antibodies anti-ERK1/2, anti-phospho-ERK1/2 and anti-catalytic subunit 1 of rat PKA (PKA-C α) (Cell Signaling), anti-protein convertase 2 (PC2), anti-carboxypeptidase E/H (CPE) and anti-glucagon (Chemicon), and antiregulatory subunit II of rat PKA (PKA-RII) (Upstate); guinea pig polyclonal antibodies anti-insulin (Abcam). Goat anti-mouse, anti-rabbit and anti-guinea pig IgG conjugated with Alexa 488 or Alexa 568 were purchased from Molecular Probes. Goat anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Bio-Rad.

Islet isolation and cell culture

Pancreatic islets were isolated from female Wistar rats by collagenase digestion and density gradient centrifugation as described previously (Gotoh et al., 1985). Rat insulinoma INS-1 cells were cultured as described (Asfari et al., 1992). Cells were kept in resting medium (15 mM HEPES, [pH 7.4], 5 mM KCl, 120 mM NaCl, 24 mM NaHCO_3 , 1 mM MgCl_2 , 2 mM CaCl_2 , 0 mM glucose, 1 mg/ml ovalbumin) for 1 hr before being stimulated for 2 hr by the addition of 25 mM glucose, 1 mM 3-isobutyl-1-methylxanthin (IBMX, Sigma) or 100 nM GLP-1 (Phoenix Pharmaceuticals) to the resting medium unless otherwise indicated. Preliminary experiments showed that the INS-1 cells placed at resting medium with 0 mM or 2.8 mM glucose for 1 hr prior to stimulation with 17 mM and 25 mM glucose performed similarly in term of their insulin secretion stimulation index, levels of RNAs for SG proteins, as well as induction of pro-ICA512, chromogranin A and γ -tubulin protein content (data not shown). In the case of stimulation with 25 mM glucose the concentration of NaCl was reduced to 70 mM in order to maintain an equal osmolarity. 10 μM H89 and 5 μM PD98059 (Calbiochem) or 10 μM U0128 (Cell Signaling) or 5 $\mu\text{g/ml}$ actinomycin D (Sigma) were added where indicated to the resting and stimulating media.

medium), 25 mM glucose, or in resting buffer with 1 mM IBMX. (F) mRNA levels in cells preincubated with 5 $\mu\text{g/ml}$ actinomycin D in resting buffer for 1 hr prior to incubation in the same buffer with 5 $\mu\text{g/ml}$ actinomycin D, with or without 1 mM IBMX, or 25 mM glucose for 2 hr. (G) mRNA levels in cells transfected with scrambled or PKA-C α siRNA oligos and then treated as in (D). The amount of mRNA measured in resting cells was equal to 100% (A and D-G). P values were calculated with the Student's t test and are indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Error bars show standard deviations from at least three independent experiments.

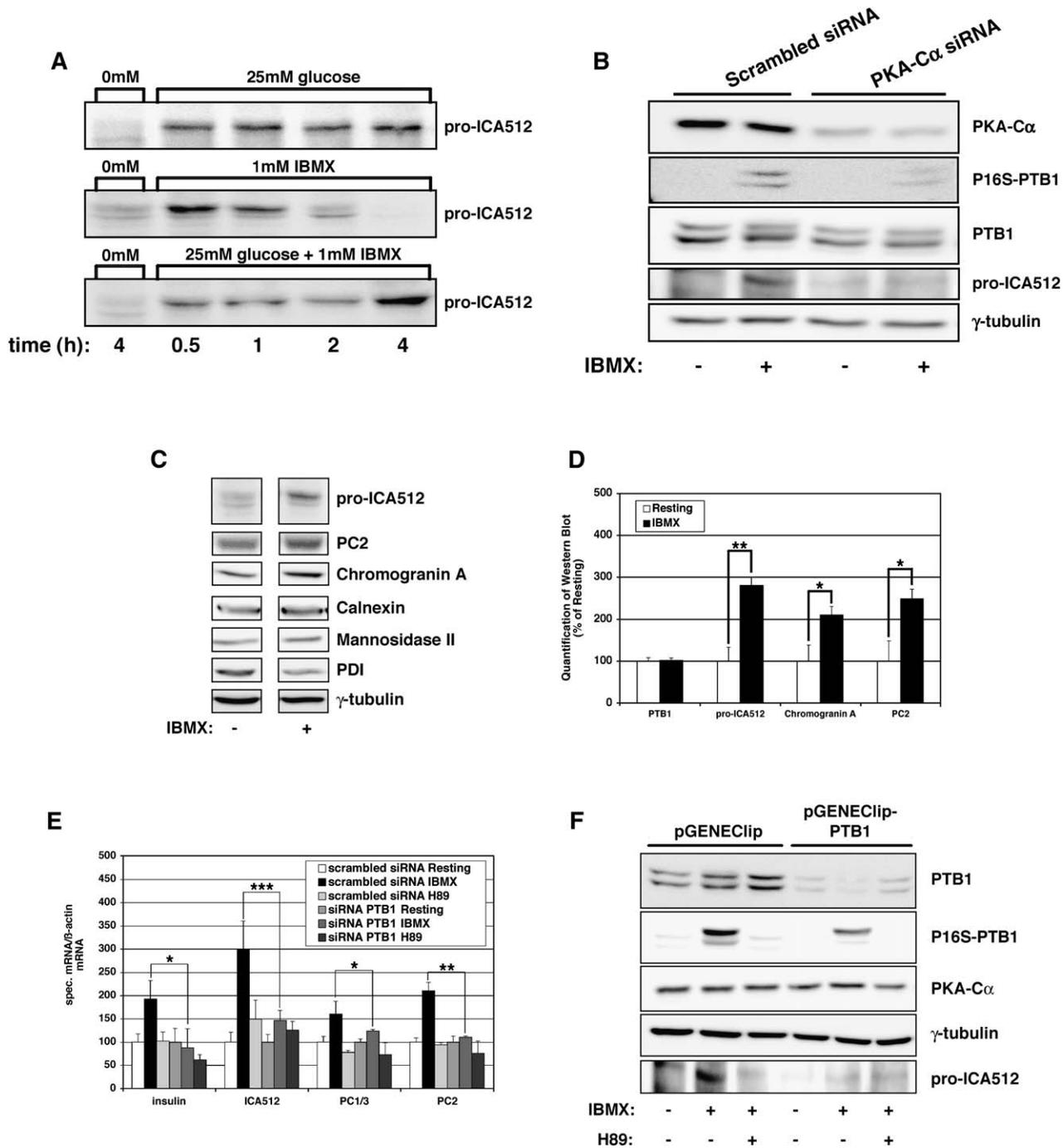


Figure 4. cAMP/PKA-induced expression of SG proteins

A) Immunoblots for pro-ICA512 on extracts from INS-1 cells in 0 mM or 25 mM glucose and 1 mM IBMX, alone or in combination for the indicated times.

B) Immunoblots for the indicated proteins on extracts from INS-1 cells treated with siRNA oligos and stimulated with 1 mM IBMX for 2 hr.

C) Immunoblot for pro-ICA512 and other SG (PC2, chromogranin A) or housekeeping (calnexin, mannosidase II, PDI, and γ -tubulin) proteins on extracts from INS-1 cells untreated or treated with 1 mM IBMX for 2 hr.

D) Quantification of the immunoblots shown in (C).

E and F) Real-time PCR for the indicated SG markers and β -actin on total RNA (E) and immunoblots for the indicated genes (F) on extracts from cells transfected with pGENEClip or pGENEClip-PTB1 and then treated without (resting) or with 1 mM IBMX or 1 mM IBMX + 10 μ M H89.

P values were calculated with the Student's t test and are indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Error bars show standard deviations from at least three independent experiments.

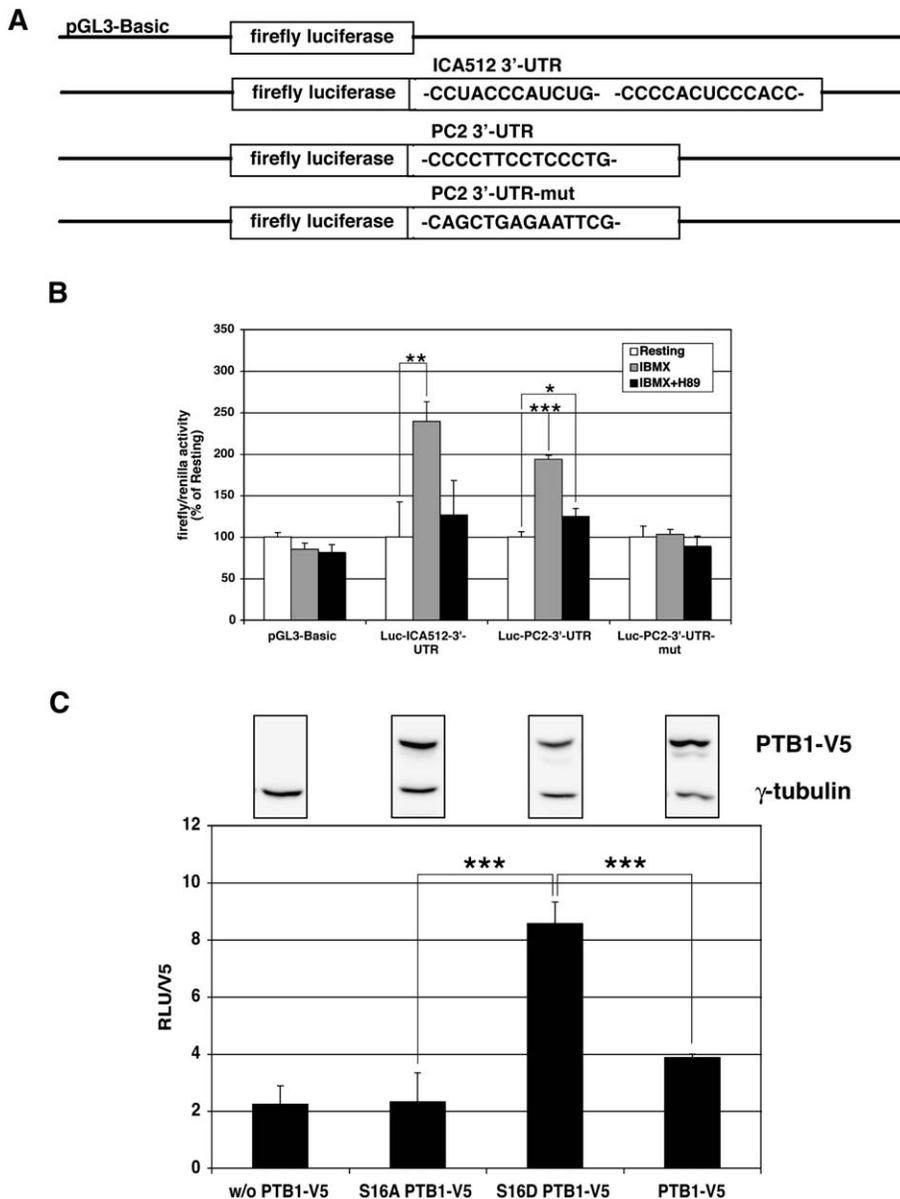


Figure 5. Phospho-PTB1 mediates the cAMP-dependent posttranscriptional upregulation of mRNAs encoding components of insulin SGs

A) Cartoon of pGL3-Basic derived constructs including the 3'UTRs of rat ICA512 and PC2. Motifs for PTB1 binding in these 3'UTRs are shown. In Luc-PC2-3'-UTR-mut, the consensus for PTB1 binding was mutated.

B) Dual luciferase assays in INS-1 cells cotransfected with the indicated firefly luciferase constructs and renilla luciferase. Cells were incubated in 0 mM glucose with or without 1 mM IBMX or 1 mM IBMX +10 μ M H89. The ratio between firefly and renilla luciferase activities in resting cells was set as 100%.

C) Firefly luciferase activity was measured 4 days after cotransfection of INS-1 cells with Luc-ICA512-3'UTR and the indicated PTB-V5 alleles. To reduce levels of endogenous cytosolic PTB1, cells were kept overnight in 2.8 mM glucose. The histograms show the ratio between firefly luciferase activities (relative light units, RLU) and the expression levels of PTB-V5 alleles in the corresponding cells, as determined by immunoblot (top panels).

P values were calculated with the Student's t test and are indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Error bars show standard deviations from at least three independent experiments.

Cloning

The cDNA encoding rat PTB1 was amplified by RT-PCR from INS-1 cell total RNA and cloned into pcDNA3.1 (Invitrogen) or pEGFP-N1 (Clontech) using standard protocols. The 3'UTR of ICA512 (473 bp; NCBI acc. N^o: U40652) and PC2 (209 bp, NCBI acc. N^o: M76706) were cloned into the XbaI site downstream of the firefly luciferase cDNA in pGL3-Basic (Promega). Replacements of serine 16 in PTB1 or mutation of the PTB1 binding site in the 3'UTR of PC2 were carried out with the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

Transfection

INS-1 cells were transiently transfected with cDNA vectors using an Amaxa electroporator, as described (Knoch et al., 2004).

Protein analysis

INS-1 cells and purified islets incubated for various lengths of time with pharmacological agents were extracted in lysis buffer (20 mM TRIS/HCl, [pH 8.0], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail (Calbiochem). Protein concentration in the detergent soluble material was measured by BCA assay

(Pierce). Cell extracts were separated by SDS-PAGE and immunoblotted as described (Knoch et al., 2004). Chemiluminescence was performed using the Supersignal West Pico Substrate (Pierce) and detected with a LAS 3000 Bioimaging System (Fuji). Phospholabeling was performed by metabolic labeling of INS-1 cells with 250 μ Ci 32 P-orthophosphate/35 mm well for 2 hr. Following incubation with IBMX with or without H89, cells were extracted and Triton X-100 soluble material was used for immunoprecipitation with the anti-PTB1 antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography with a BAS 1800 II Phosphoimager (Fuji).

Immunocytochemistry

For immunocytochemistry, INS-1 cells were grown on cover slips and fixed after treatment with 3% paraformaldehyde and permeabilized with 0.2% saponin. Rat pancreatic tissue was fixed by immersion in 4% paraformaldehyde and embedded in paraffin. Microtome sections were microwaved 2 times for 5 min and permeabilized with 0.2% saponin before being sequentially incubated with anti-PTB1 antibodies for 2 hr, washed, and then incubated with anti-insulin or anti-glucagon antibodies for 1 hr. After washing, cell and tissue specimen were incubated with fluorochrome-conjugated secondary antibodies for 1 hr. INS-1 cells nuclei were counterstained with DAPI.

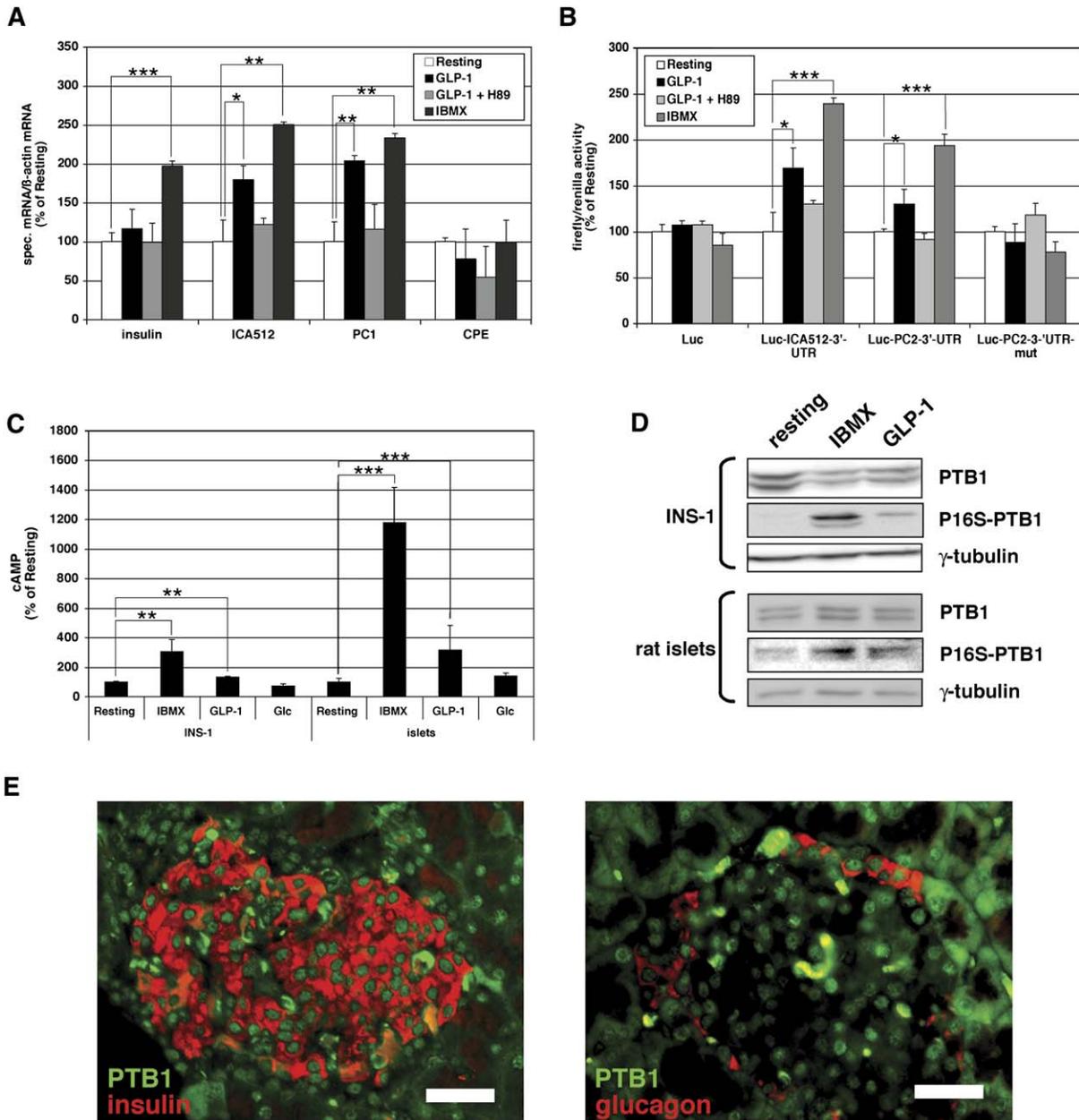


Figure 6. Phospho-PTB1 is involved in GLP-1 stimulation in β cells

A) Real-time PCR for the indicated genes and β -actin on total RNA from INS-1 cells in 0 mM glucose without (resting) or with 100 nM GLP-1, 100 nM GLP-1 + 10 μ M H89, or 1 mM IBMX.

B) Dual luciferase assays in INS-1 cells cotransfected with the indicated firefly luciferase constructs and the control renilla luciferase construct and treated as in (A).

C) cAMP levels in extracts from INS-1 cells and isolated rat islets kept at rest or stimulated with 1 mM IBMX, 100 nM GLP-1, or 25 mM glucose for 2 hr.

D) Western blots for the indicated proteins on extracts from INS-1 cells and isolated rat islets stimulated with 100 nM GLP-1 or 1 mM IBMX.

E) Coimmunostainings of rat pancreatic sections with anti-PTB1 (green) and anti-insulin or anti-glucagon antibodies (red). Scale bar represents 30 μ m.

P values were calculated with the Student's *t* test and are indicated as follows: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Error bars show standard deviations from at least three independent experiments.

Image acquisition and processing were performed as described (Knoch et al., 2004; Lacas-Gervais et al., 2004).

RNA interference

Twenty-one-mer short-interfering double-stranded RNA (siRNA) oligonucleotides for rat PKA-C1 (NCBI accession number: XM341661) were synthesized with the Silencer siRNA Construction Kit (Ambion) using the following primers: sense primer 1 (nt 124-145), 5'-AAGAGTTCCTAGCCAAGCCACC TGTCTC; antisense primer 1, 5'-AATG-GCTTTGGCTAGGAATCCCTGTG

TC; sense primer 2 (nt 1003-1024), 5'-AAGGTGGAAGCTCCCTTCATACCTG TCTC; antisense primer 2, 5'-AATATGAAGGGAGCTCCACCCCTGTCTC; sense primer 3 (nt 1112-1133), 5'-AAGGAGTTTACTGAGTTTACGCTGTG TC; antisense primer 3, 5'-AACTAAACTCAGTAACTCCCTGTCTC. Control scrambled siRNA oligonucleotides were previously described (Knoch et al., 2004). INS-1 cells (5x10⁵/35 mm well) were grown for 2 days before transfection of 0.5 μ g mixed siRNA oligos for PKA-C1 or scrambled oligos/well with Lipofectamin (Invitrogen). Four days after transfection cells were harvested or fixed for immunostaining with 3% paraformaldehyde.

Knockdown of rat PTB1 was performed by cloning the following hairpin (5'-TCTGTCATGGAAGAGTGTAATTTCAAGAGAATTTACACTCTTCCATGACCT) into the GeneClip U1 Hairpin vector (Promega). This hairpin was designed using the siRNA Target Designer software (www.promega.com/siRNA Designer). To verify the specificity of this hairpin-mediated RNAi, the PTB1 target sequence (5'-GTCATGGAAGAGTGTAAT) was cloned into the XhoI site of the siCHECK™-2 vector (Promega) at the 3'-end of the renilla luciferase cDNA. Four micrograms pGENEClip-PTB1 was cotransfected with 0.5 µg siCHECK-PTB1 in INS-1 cells by electroporation (Amaxa) as described (Knoch et al., 2004). Three days after transfection the cells were used for stimulation experiments.

RNA labeling

Four hundred freshly isolated pancreatic islets and 7×10^5 INS-1 cells/35 mm well were cultured for 1 or 4 days, respectively, before being labeled with 10 µCi [³H]-uridine for 4 hr in either 0 mM or 11 mM glucose. Next, cells were incubated for up to 6 or 40 hr in 0 mM glucose with or without 1 mM IBMX or in normal medium with 11 mM glucose. After washing the cells with PBS, total RNA was isolated with the RNeasy Kit (Qiagen) according to the manufacturer's protocol. Samples were cleared from genomic DNA by DNase digestion. Insulin 1 mRNA was isolated using a biotinylated anti-sense oligonucleotide for rat insulin 1 (5'-TTGTGGCACTTGGGGTCCCTC CACTTCACGACGGGACTTGGG) coupled to Dynabeads M-270 Streptavidin (Dyna) according to manufacturer's instructions. Counts (cpm) from total and bead-isolated RNA were measured with a Winspectral Liquid Scintillation Counter 1414 (Wallac). Counts for insulin 1 mRNA were normalized relative to the counts for total RNA measured at each of the corresponding time points. The half-life of insulin mRNA was calculated as described (Welsh et al., 1985).

Real-time PCR

Four hundred islets or 6×10^5 INS-1 cells/35 mm well were cultured for 1 or 4 days, respectively, before the extraction of total RNA. One microgram total RNA was reversely transcribed with SuperScript II reverse transcriptase (Invitrogen) and oligo d(T) primer. mRNA levels were measured by quantitative real-time PCR with the QuantiTect SYBR Green PCR Kit (QIAGEN) and a MX4000 Thermocycler (Stratagene). Normalization of real-time PCR data was performed by parallel amplification of rat β-actin mRNA (see also Supplemental Experimental Procedures and Figure S1A). Primers for β-actin, insulin and ICA512 have been previously described (Steinbrenner et al., 2002). For additional genes we used the following primers: PKA-C1, sense: 5'-GCTCACTGAACCTCCCAATCC, antisense: 5'-GAACCACTATGCCATGAAGATC; PC1/3, sense: 5'-GTACCCAAAACCTCCAGCAG, antisense: 5'-GGCTTGTTGAGCTTTTCCAG; PC2, sense: 5'-TAGGGCCACGCTTCCGC, antisense: 5'-GGTAGGAACGCTATATTTGGG; CPE, sense: 5'-TTGGGAACATGCATGTAATGAG, antisense: 5'-CACGAACCACTCCTCAGCTC.

Luciferase Assays

For dual luciferase assays INS-1 cells were cotransfected with firefly luciferase constructs and phRL (Promega) encoding renilla luciferase. The firefly luciferase activity was measured 4 days after transfection and normalized versus that of renilla luciferase.

PKA Activity

Enzymatic activity of PKA in cell extracts (0.2 µg) was quantified with the non-radioactive PKA Kinase Activity Assay Kit (Stressgen) according to manufacturer's instructions. Absorbance at 450 nm was measured with a microplate reader (Anthos). Activity was defined as the difference between measured absorbance without and in the presence of 6 µM PKA inhibitor peptide (Upstate).

Quantification of cAMP Levels

cAMP amounts in INS-1 cells and isolated islets after incubation in resting buffer or with IBMX, GLP-1 or glucose were measured using the Cyclic AMP EIA kit (Cayman Chemical) according to the manufacturer's instructions.

Statistics

SigmaStat 3.0 (SPSS Inc.) was used for statistical analyses. p values were calculated with the Student's t test and indicated in the figures as follows:

*p < 0.05, **p < 0.01 and ***p < 0.001. Error bars show standard deviations from at least three independent experiments.

Supplemental data

Supplemental Data include Supplemental Experimental Procedures and one figure and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/3/2/123/DC1/>.

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