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Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis

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Pancreatic β-cells store insulin in secretory granules that undergo exocytosis upon glucose stimulation. Sustained stimulation depletes β-cells of their granule pool, which must be quickly restored. However, the factors promoting rapid granule biogenesis are unknown. Here we show that β-cell stimulation induces the nucleocytoplasmic translocation of polypyrimidine tract-binding protein (PTB). Activated cytosolic PTB binds and stabilizes mRNAs encoding proteins of secretory granules, thus increasing their translation, whereas knockdown of PTB expression by RNA interference (RNAi) results in the depletion of secretory granules. These findings may provide insight for the understanding and treatment of diabetes, in which insulin secretion is typically impaired.

Secretory granules store peptide hormones in peptide-secreting endocrine cells. Various stimuli induce secretory granules to fuse with the plasma membrane and release their content into the extracellular space. Similarly to most secretory proteins, peptide hormones are co-translationally translocated into the lumen of the rough endoplasmic reticulum, transported to the Golgi complex and then sorted into nascent secretory granules. Along this route peptide hormones can undergo multiple post-translational modifications, including proteolytic cleavage and glycosylation. Thus, the generation of secretory granules is a slow process, requiring in excess of 30 min. As sustained stimulation progressively depletes cells of secretory granules, transcriptional and post-transcriptional mechanisms should be quickly activated to renew secretory granule stores. The existence of one or more factors that coordinate the synthesis of secretory

Table 1 Morphometry of INS-1 cells treated with siRNA oligonucleotides

	n	Minimum number of SGs per cell section	Maximum number of SGs per cell section	%	Mean number of SGs per section ± sem	Mean (μm^2) surface area ± sem
Untreated cells	71	7	59	100	18.8 ± 1.1	90.0 ± 5.6
Cells treated with siRNA oligos 1+ 2	140	0	47	100	6.6 ± 0.8 ($P < 0.000005$)	84.1 ± 2.7 ($P < 0.30$)
Cells treated with siRNA oligos 1+ 2 with ≤ 2 SGs	85	0	2	60.7	0.3 ± 0.1 ($P < 0.000005$) ($P < 0.000005$)*	84.1 ± 3.6 ($P < 0.33$) ($P = 1$)*
Cells treated with siRNA oligos 1+ 2 with > 2 SGs	55	7	47	39.3	16.6 ± 1.1 ($P < 0.1$)	84.1 ± 4.1 ($P < 0.35$)
Cells treated with scrambled siRNA oligo	25	6	54	100	18.7 ± 2.5 ($P = 0.37$)	nd
Cells treated with siRNA oligo for F-Luc	25	6	56	100	18.1 ± 2.5 ($P = 0.28$)	nd

The total number of cells per group (*n*) was from three independent experiments. As no differences between these experiments were found by analysis of variance (ANOVA), all data were pooled. Statistical analysis was performed using a t-test or, in case variances were not equal, a Welch test. Cells treated with siRNA oligonucleotides were compared for secretory granule content and size (*P* values in parenthesis) with cells in the untreated group. Each of the two distinct groups of cells treated with siRNA oligonucleotides 1 and 2 for PTB was also independently compared with the untreated group (*P* values in parenthesis) and with each other (P values with an asterisk). SG, secretory granule; nd, not determined.

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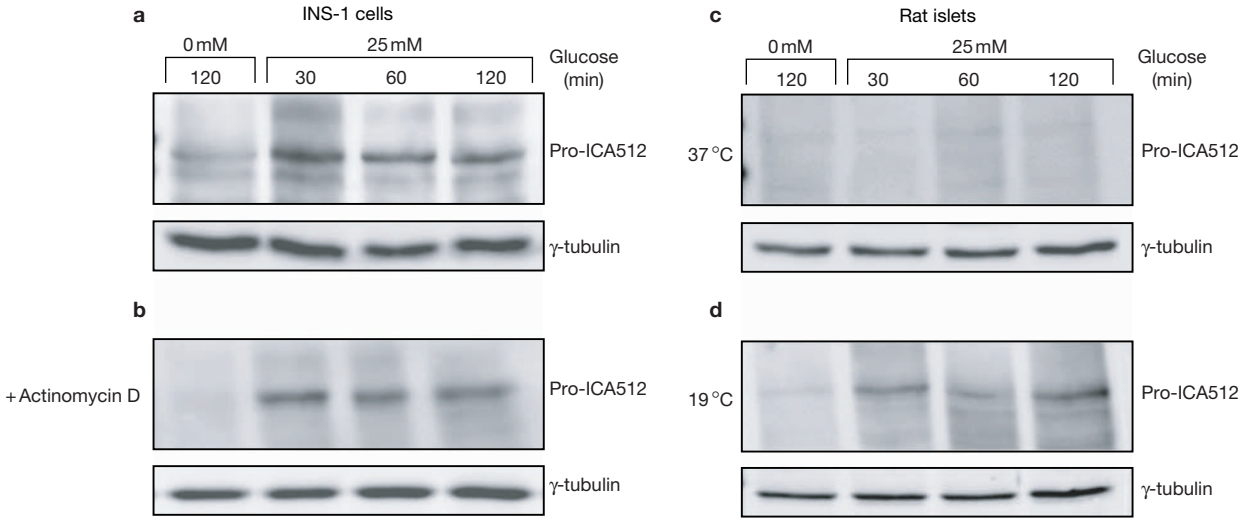

Figure 1 Glucose stimulation of pro-ICA512 expression in insulinoma and rat islet cells. (**a**–**d**) Western blots for pro-ICA512 and γ-tubulin from Triton X-100 extracts of INS-1 cells (**a**, **b**) or purified pancreatic islets (**c**, **d**). Cells were kept at rest (0 mM glucose) or stimulated (25 mM glucose) for the indicated times at 37 °C (**a**–**c**) or 19 °C (**d**), with (**b**) or without (**a**, **c**, **d**) 5 μg ml^{-1} actinomycin D.

granule proteins, and therefore secretory granule biogenesis, has been postulated[1]. The identity of such factors, however, remains unclear.

β-cells of pancreatic islets are the endocrine cells that produce insulin, the most important hormone for the control of glucose homeostasis in vertebrates. Glucose stimulates calcium-dependent exocytosis of insulin secretory granules, as well as the rapid biosynthesis of insulin and other secretory granule components[2], including chromogranin A[2] and the prohormone convertases 1/3 (PC1/3)[3,4] and 2 (PC2)[4]. Glucose enhances transcription[5] and translation[6] of the insulin gene. Increased translation accounts entirely for the up-regulation of insulin biosynthesis within the first 2 h after stimulation[7], whereas activation of transcription is a slower process. Increased translation results from the stimulation of initiation, elongation and signal-recognition-particle-mediated translocation of the nascent pre-pro-insulin polypeptide into the lumen of the endoplasmic reticulum[8], as well as from a reduced degradation of *insulin* mRNA[9]. Recently it has been shown that glucose stimulation increases the stability of *insulin* mRNA by inducing the binding of PTB to its 3′-untranslated region (UTR)[10,11]. PTB[12] (also known as heterogeneous nuclear ribonucleoprotein I) is a pre-mRNA splicing repressor[13] that has also been implicated in cap-independent translation[14], cytoplasmic RNA transport[15], poly (A) site cleavage[16] and mRNA stability[10,17]. There are several alternatively spliced variants of PTB[18], the largest of which is a 59K protein[19] with four RNA recognition motif domains. Here we show that this PTB isoform is required for the biogenesis of insulin secretory granules.

RESULTS

Post-transcriptional induction of ICA512 by glucose

Our findings originated from the study of ICA512/IA-2, a receptor-tyrosine-phosphatase-like protein associated with insulin secretory granules and neurosecretory granules in general[20]. Cleavage of pro-ICA512 by a furin-like convertase generates a 65K transmembrane fragment (ICA512-TMF) that is enriched in secretory granules[20]. Glucose stimulation of rat insulinoma INS-1 cells induces biosynthesis of pro-ICA512 (ref. 21). This induction was already apparent in cells stimulated for 30 min and persisted after stimulation for 120 min (Fig. 1a). Rapid induction of pro-ICA512 biosynthesis was not detected in islets stimulated at 37 °C (Fig. 1c), but was evident after stimulation at 19 °C (Fig. 1d), which prevents exit of secretory proteins from the *trans*-Golgi network. This was not unexpected, as pro-ICA512 is converted very rapidly into ICA512-TMF[22], and secretory proteins mature more efficiently in β-cells than in insulinoma cells. As glucose stimulated pro-ICA512 biosynthesis even in the presence of actinomycin D (Fig. 1b), which blocks transcription, such a rapid response must depend on post-transcriptional mechanisms.

PTB stabilizes mRNAs for secretory granule proteins

Quantitative real-time PCR showed that *ICA512* mRNA increased 1.46, 1.33 and 2.31 fold in INS-1 cells stimulated with 25 mM glucose for 30, 60 and 120 min, respectively. This increase exceeded that of *insulin* mRNA, which was enhanced 1.43 fold after stimulation for 120 min (Fig. 2a). To establish whether glucose stimulation promoted the stability of *ICA512* mRNA, the labelled 3′-UTR of rat *ICA512* mRNA was incubated with cytosolic extracts from INS-1 cells that had been kept in resting (0 mM glucose) or stimulating (25 mM glucose) buffer for 60 or 120 min. Equivalent amounts of intact *ICA512* mRNA 3′-UTR were recovered after its incubation with extracts from cells kept at rest or stimulated for 60 min (50% decay after 105 min; Fig. 2b and c). Extracts from cells stimulated for 120 min, however, stabilized *ICA512* mRNA 3′-UTR (50% decay after 150 min). An even greater and more rapid stabilization was observed after incubation of *ICA512* mRNA 3′-UTR with extracts from glucose-stimulated pancreatic islets (resting islets: 50% decay after 84 min; islets stimulated for 60 min: 50% decay after 192 min; Fig. 2b, c). Taken together, these results indicate that stabilization of *ICA512* mRNA can partially account for the rapid glucose-dependent stimulation of pro-ICA512 biosynthesis.

The *ICA512* mRNA 3′-UTR contains two evolutionary conserved consensus binding sites for PTB (Fig. 2d). To determine whether glucose promotes *ICA512* mRNA stability through the binding of PTB to its 3′-UTR, two biotinylated oligonucleotides, each including one of the two putative PTB-binding sites in rat *ICA512* mRNA, were independently incubated with cytosolic extracts from resting or glucose-stimulated INS-1 cells. The extracts of cells stimulated for 120 min displayed a binding activity that caused a similar retardation of both

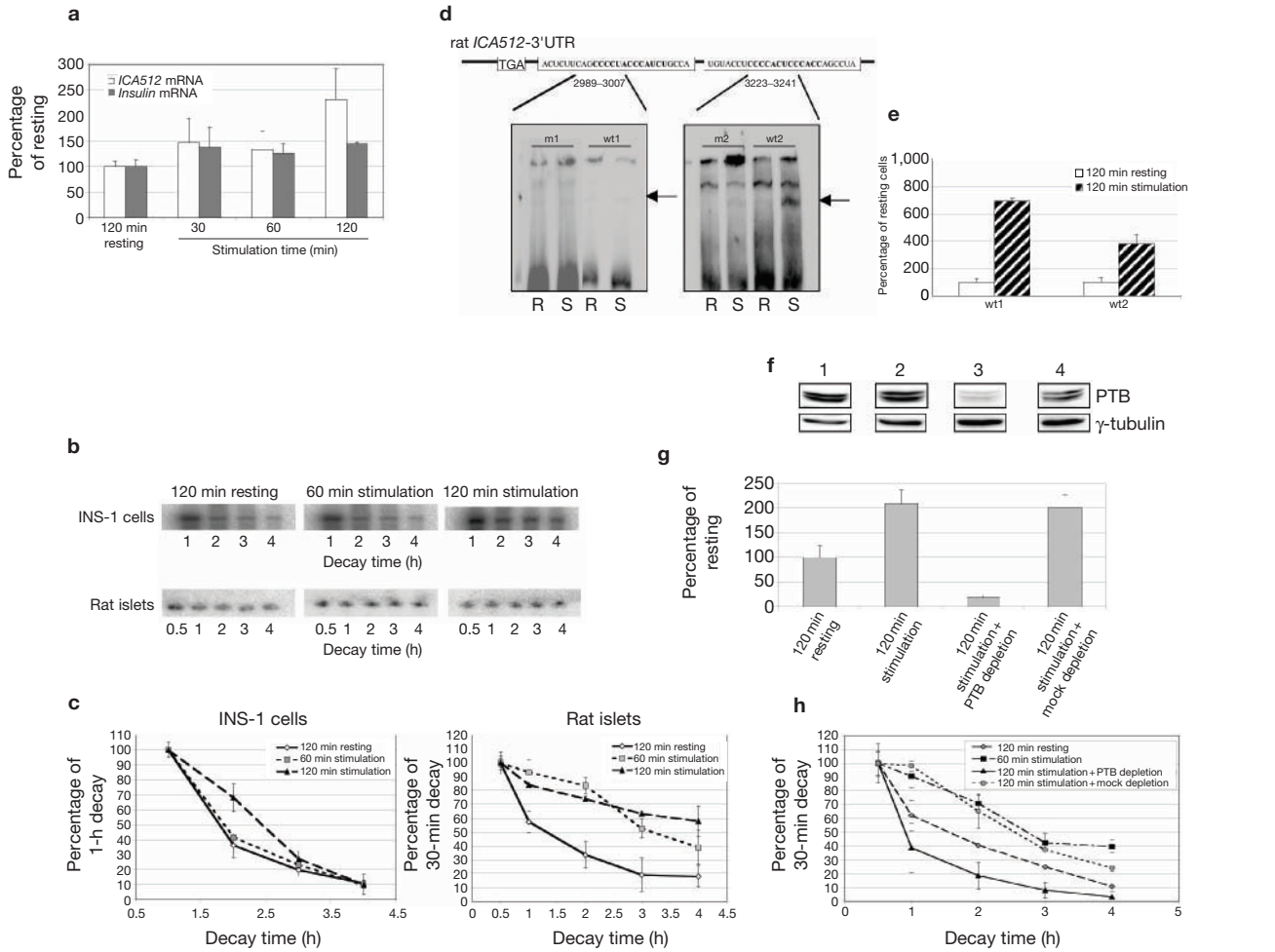

Figure 2 Stabilization of rat *ICA512* mRNA by PTB. (**a**) Quantitative RT-PCR for *ICA512* and *insulin* mRNAs using 1 μg total RNA from INS-1 cells kept at rest or stimulated with glucose for the indicated times. Amounts were normalized against *β-actin* mRNA. (**b**, **c**) Autoradiographs (**b**) and quantification by phosphoimaging (**c**) of ^{32}P-labelled *ICA512* mRNA 3′-UTR incubated for the indicated times with cytosolic extracts from INS-1 cells or islets that were either kept at rest or stimulated with glucose for 60 or 120 min. (**d**) EMSAs of biotinylated RNA oligonucleotides, including either the wild type (wt1, wt2) or mutated (m1, m2) first or second consensus sites for PTB binding in the 3′-UTR of *ICA512* mRNA. Before EMSA, oligonucleotides were incubated with cytosolic extracts from INS-1 cells kept at rest (R) or stimulated (S) with glucose for 120 min. (**e**) Binding of wt1 or wt2 oligonucleotides to PTB, as measured by ELISA. PTB was immunoprecipitated from cytosolic extracts of 120-min resting or glucose-stimulated INS-1 cells using a specific antibody. (**f**) Immunoblots for PTB and γ-tubulin in INS-1 cell cytosolic extracts: lane 1, 120 min resting cells; lane 2: 120 min stimulated cells; lane 3: 120 min stimulated cells plus PTB immunodepletion; lane 4: 120 min stimulated cells plus mock immunodepletion. (**g**) Binding of ^{32}P-labelled full-length *ICA512* mRNA 3′-UTR to cytosolic extracts 1–4 from **f**. (**h**) Decay assays, performed as in **c**, upon incubation of *ICA512* mRNA 3′-UTR with cytosolic extracts 1–4 from **f**.

oligonucleotides in electrophoretic mobility shift assays (EMSAs; Fig. 2d, arrows). However, no retardation was detected after incubation of oligonucleotides with nuclear extracts from cells stimulated for up to 120 min (see Supplementary Information, Fig. S1), or when cytosolic extracts were incubated with oligonucleotides lacking the PTB consensus sequence (Fig. 2d). To unequivocally identify PTB as the binding factor, we developed a solid-phase ELISA in which cytosolic PTB was captured with a specific antibody. Stimulation of INS-1 cells for 120 min enhanced the binding of PTB to the first and second biotinylated oligonucleotides by 6.9- and 3.8-fold, respectively, compared with resting cells (Fig. 2e). Stimulation also increased the binding of PTB to the full *ICA512* mRNA 3′-UTR by 2.1-fold (Fig. 2g). However, immunodepletion of PTB from cytosolic extracts of stimulated cells (Fig. 2f) markedly reduced the recovery of *ICA512* mRNA 3′-UTR (Fig. 2g) and its stability (Fig. 2h), whereas mock immunodepletion with mouse IgGs had no effect. These results demonstrate that upon glucose stimulation, cytosolic PTB binds and stabilizes the 3′-UTR of *ICA512* mRNA.

In addition to insulin and ICA512, numerous other secretory granule components contain conserved putative PTB-binding sites in their mRNA 3′-UTR (see Supplementary Information, Table 1). As tests, we analysed the stability of the 3′-UTRs of mRNAs encoding PC1/3 and PC2, which are upregulated by glucose[4]. RNA decay assays showed that glucose stabilizes rat *PC1/3* mRNA 3′-UTRs (resting cells: 50% decay after 126 min; cells stimulated for 60 min: 50% decay after 234 min) and *PC2* mRNA 3′-UTRs (resting cells: 50% decay after 114 min; cells stimulated for 60 min: 50% decay after 210 min) even more effectively than *ICA512* mRNA 3′-UTR (Fig. 3). Glucose, however, had no effect

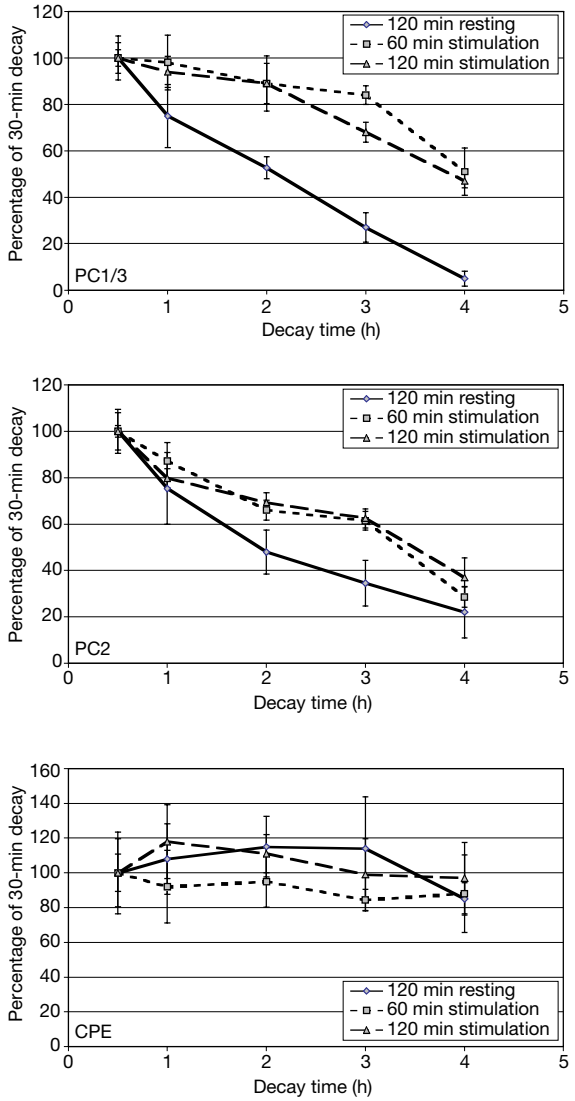

Figure 3 Stability of *PC1/3*, *PC2* and *CPE* mRNAs. Quantification by phosphoimaging of ^{32}P-labelled *PC1/3*, PC2 and *CPE* mRNA 3′-UTRs incubated for the indicated times with cytosolic extracts from INS-1 cells either kept at rest for 120 min or stimulated with glucose for 60 or 120 min.

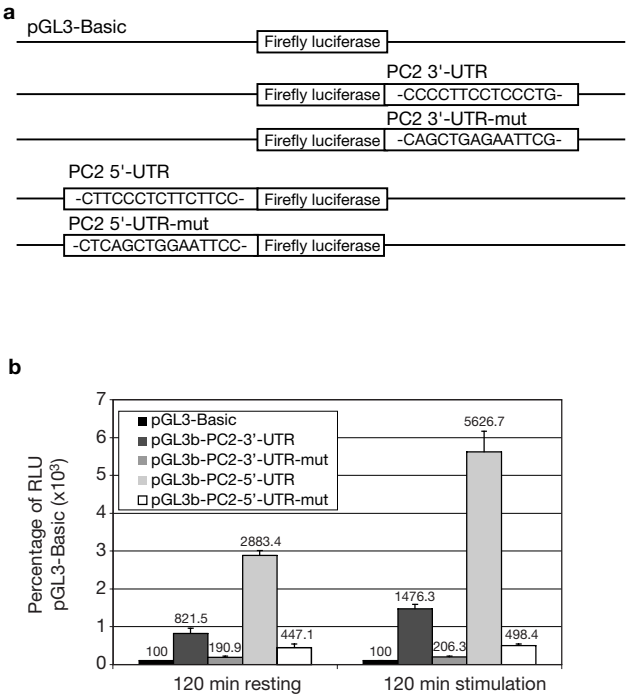

Figure 4 Glucose-regulated luciferase expression in INS-1 cells upon inclusion of PTB-binding sites. (**a**) Schematic representation of firefly luciferase cDNA constructs in pGL3-Basic. pGL3 PC2 3′-UTR and pGL3 PC2 5′-UTR contain the corresponding UTRs from rat PC2. Two additional pGL3 constructs were generated with mutations in the PTB-binding sites of PC2 (pGL3 PC2 3′-UTR-mut and pGL3 PC2 5′-UTR-mut). (**b**) Relative luciferase units (RLUs) in resting INS-1 cells, or INS-1 cells transfected with the pGL3 constucts shown in **a** and stimulated for 120 min. RLU in resting or stimulated INS-1 cells transfected with pGL3-Basic was set as 100%.

on the stability of carboxypeptidase E (*CPE*) mRNA 3′-UTR. CPE is partially responsible for insulin processing within secretory granules, but its expression is not regulated by glucose[2]. Notably, *CPE* mRNA 3′-UTR lacks a consensus for PTB binding and was significantly more stable than *ICA512*, *PC1/3* and *PC2* mRNA 3′-UTRs.

Increased translation of mRNAs binding PTB

To verify whether PTB functions as a glucose-dependent regulator of mRNA stability and translation *in vivo*, INS-1 cells were transiently transfected with firefly luciferase constructs carrying either the original 5′- and 3′-UTRs, or also the corresponding regions of rat *PC2* mRNA (Fig. 4a). The choice of rat *PC2* UTRs was convenient because its 3′-UTR and 5′-UTR both contain a single putative PTB-binding site. Inclusion of PC2 3′-UTR increased luciferase activity by 8.2- and 28.8-fold under resting and stimulated conditions, respectively (Fig. 4b). Luciferase activity was further enhanced by including PC2 5′-UTR (an increase of 14.7-fold at rest and 56.2-fold upon stimulation). However, mutagenesis of the PTB-binding sites in the 3′- or 5′-UTR of PC2 was sufficient to reduce luciferase activity at rest (PC2 3′-UTR-mut, 4.29-fold decrease; PC2 5′-UTR-mut, 7.16-fold decrease), and by even more after stimulation (PC2 3′-UTR-mut, 6.44-fold decrease; PC2 5′-UTR-mut, 11.29-fold decrease). These data demonstrated that PTB promotes the expression of mRNAs even in resting conditions and that its activity is glucose-regulated. Furthermore, they suggest that PTB may not only increase mRNA stability but also mRNA translation by binding to 5′-UTRs, as already shown in the case of its interaction with viral internal ribosome entry sites[14,23].

Depletion of secretory granules after knockdown of PTB

To establish whether PTB has an effect on insulin secretory granule biogenesis, its expression in INS-1 cells was downregulated by RNAi[24] using small-interfering RNA (siRNA) oligonucleotides for PTB. The transfection efficiency of INS-1 cells with Cy3-conjugated siRNA oligonucleotides was estimated to be 50–60% by fluorescence microscopy (data not shown). Four days after transfection with a mixture of siRNA oligonucleotides 1 and 2, the overall expression of PTB was reduced by 44% (Fig. 5a; also see Supplementary Information, Fig. S2c) and by 40% in the cytosol (Supplementary Information, Fig. S2a, b), compared with control cells. Also decreased were secretory granule components with consensus sites for PTB binding in their mRNA 3′-UTR (see Supplementary Information, Table 1), including ICA512, PC1/3, PC2,

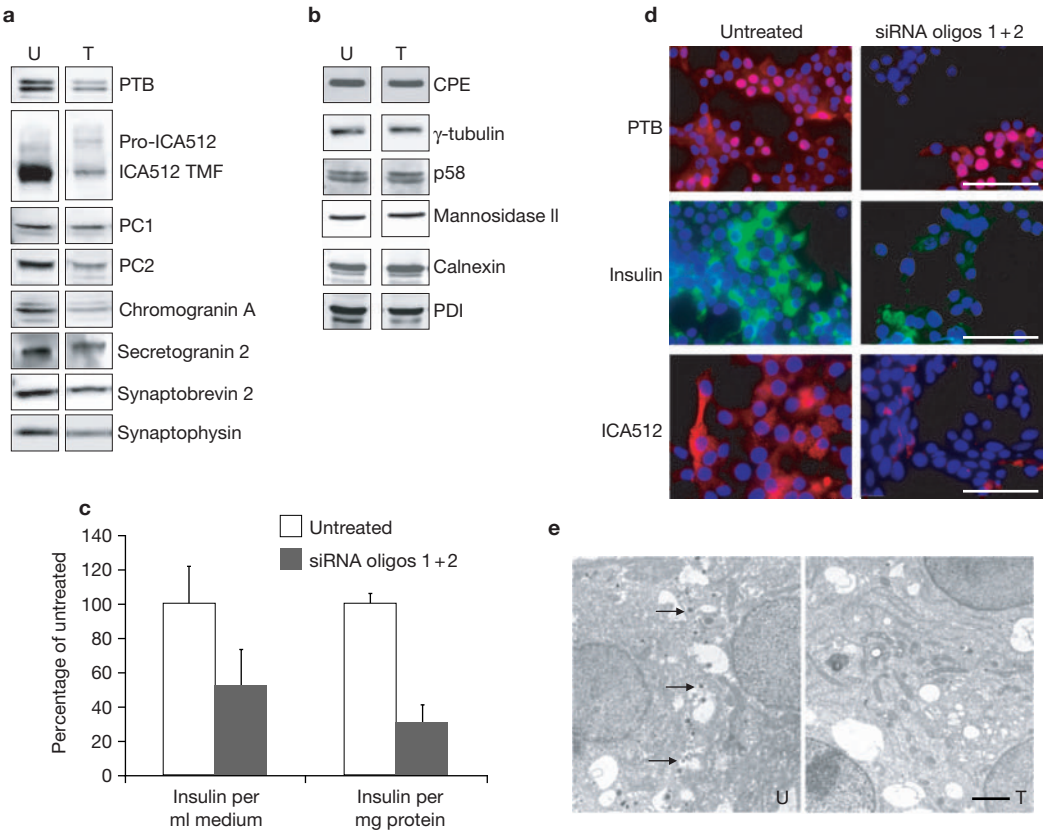

Figure 5 Depletion of secretory granules in INS-1 cells upon knockdown of PTB. (**a**, **b**) Western blots of PTB, various secretory granule (**a**) and housekeeping proteins (**b**) in INS-1 cells either untreated (U) or treated (T) with siRNA oligonucleotides. (**c**) Insulin in the medium and in total protein extracts of INS-1 cells either untreated or treated with siRNA oligonucleotides, as measured by radioimmunoassay. (**d**) Immunofluorescence microscopy images of PTB (red), insulin (green) and ICA512 (red) in INS-1 cells either untreated or treated with siRNA oligonucleotides. Nuclei were counterstained with DAPI (blue). Scale bars represent 72 μm in top panel, and 55 μm in bottom panels. (**e**) Electron micrographs of INS-1 cells either untreated (U) or treated (T) with siRNA oligonucleotides. In untreated cells, secretory granules (arrows) were mostly aligned along the plasma membrane. Scale bar represents 1.78 μm.

chromogranin A, secretogranin II, synaptobrevin 2 and synaptophysin (Fig. 5a; also see Supplementary Information, Fig. S2a–c). Cell insulin content was reduced by 69% (0.683 ± 0.040 μg mg-protein^{-1} in control cells versus 0.209 ± 0.017 μg mg-protein^{-1} in treated cells), whereas secreted insulin was reduced by 48% (2.97 ± 0.65 ng ml^{-1} in control cells versus 1.56 ± 0.32 ng ml^{-1} in treated cells; Fig. 5c and Supplementary Information, Fig. S2b, c). Conversely, treatments with control siRNA oligonucleotides did not significantly alter the expression of these secretory granule components (see Supplementary Information, Fig. S2c). Knockdown of PTB, however, had no effect on CPE levels (Fig. 5b; also see Supplementary Information, Fig. S2b, c). There were also no significant changes in the expression of housekeeping proteins, such as markers of the endoplasmic reticulum (calnexin and protein disulfide isomerase (PDI)), the Golgi complex (p58 and mannosidase II) or the cytosol (tubulin; Fig. 5b). Intriguingly, both rat *calnexin* and *PDI* mRNA 3′-UTRs contain a consensus for PTB binding, but their expression was not stimulated by glucose (see Supplementary Information, Fig. S2d), suggesting the existence of additional regulatory factors. The reduction of insulin and ICA512 concomitantly with PTB knockdown was confirmed by light immunomicroscopy (Fig. 5d and Supplementary Information, Fig. S2e). Electron microscopy showed the virtual absence of secretory granules in 60% of the cells treated with siRNA oligonucleotides 1 and 2 for PTB (Fig. 5e and Table 1). The number of secretory granules per cell per section was reduced from 18.8 ± 1.1 in control cells to 6.6 ± 1.1 in cells treated with siRNA oligonucleotides for PTB. The latter cells could be clearly distinguished in two categories. One group of cells (60%) contained ≤ 2 secretory granules, with an average of 0.3 ± 0.1 secretory granules. The other group (40%) contained ≥ seven secretory granules per cell per section, with an average (16.6 ± 1.1) that was similar to control cells. As these numbers correlate well with the transfection efficiency measured by fluorescence microscopy, cells with ≤ two secretory granules are likely to represent cells in which PTB was downregulated by RNAi. Conversely, treatments with siRNA oligonucleotides that were either scrambled or targeted the mRNA of transfected firefly luciferase had no impact on secretory granule content (Table 1). Despite the loss of secretory granules, cells in which PTB was knocked down were otherwise normal, with neither changes in size (Table 1) and shape, nor in the general appearance of organelles such as the nucleus, the endoplasmic reticulum, the Golgi complex or mitochondria. These data demonstrate that PTB is a key factor for the biogenesis of secretory granules.

Glucose-stimulated translocation of PTB

To gain insight into the mechanisms that control activation of PTB, we investigated whether glucose regulated its subcellular localization. As in

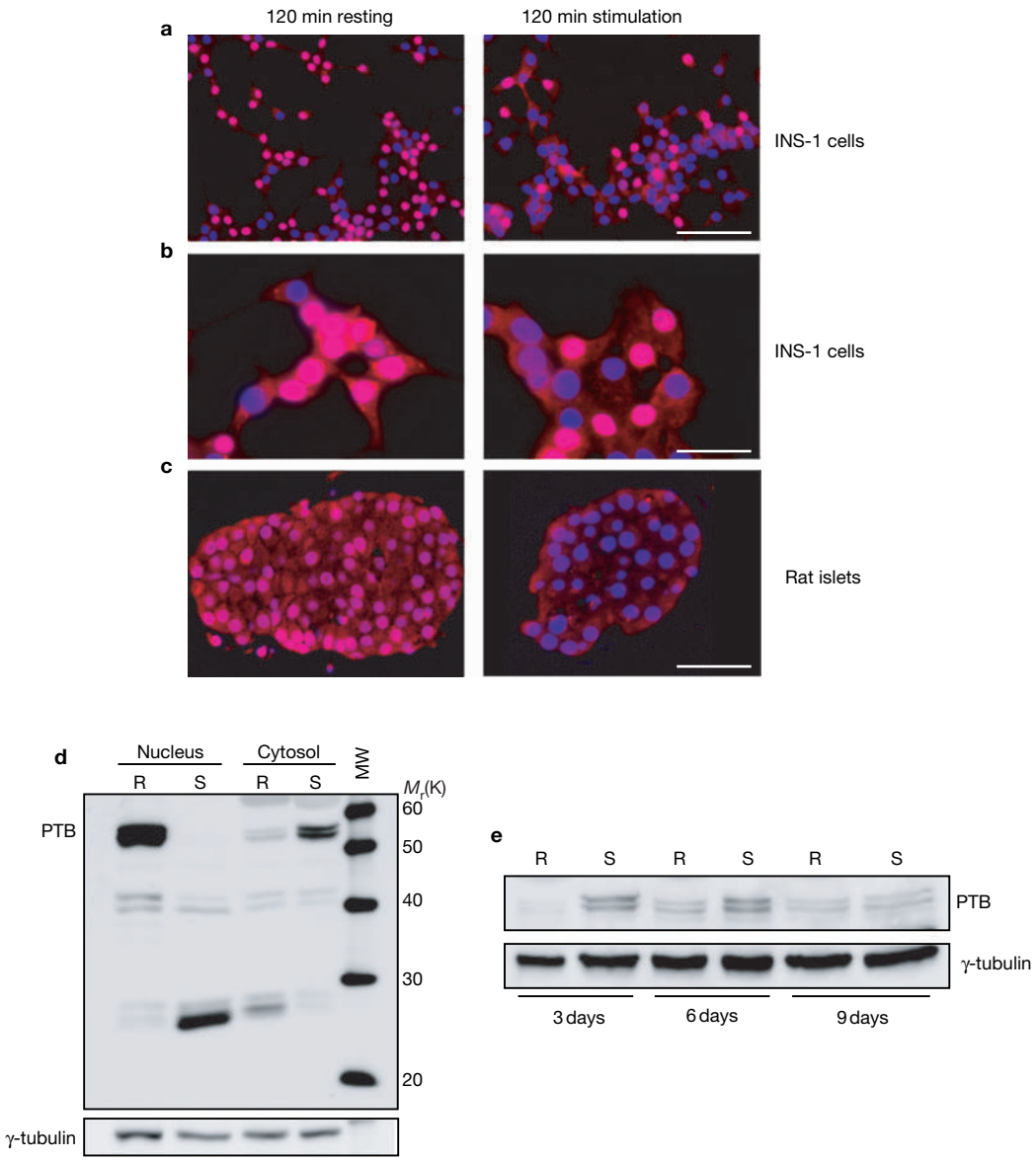

Figure 6 Nucleocytoplasmic translocation of PTB upon glucose stimulation of β-cells. (**a**–**c**) Low- (**a**, **c**) and high-power (**b**) immunofluorescence microscopy images of PTB (red) in INS-1 cells (**a**, **b**) and islets (**c**) kept at rest or stimulated with glucose for 120 min. Nuclei were counterstained with DAPI (blue). Scale bars represent 85 μm in **a**, 30 μm in **b**, and 74 μm in **c**. (**d**, **e**) Western blot of PTB and γ-tubulin from both nuclear and cytosolic fractions (**d**) or cytosolic fractions only (**e**) from islets incubated for 120 min under resting conditions (R) or in glucose stimulation buffer (S). Islets were cultured *in vitro* for 1 day (**d**) or 3, 6 and 9 days (**e**). MW, standard molecular weights.

other cell types[24], PTB was mainly found in the nucleus of resting INS-1 cells; immunoreactivity in the cytosol was low and mostly concentrated in particles whose identity remains unclear (Fig. 6a, b). Stimulation for 120 min reduced PTB-positive nuclei by 72%, whereas cytosolic immunoreactivity was enhanced, although cytosolic PTB-positive particles were decreased (Fig. 6a, b). In stimulated cells, cytosolic PTB appeared more diffuse and reticulated, suggesting that it was targeted to the endoplasmic reticulum. Glucose-induced nucleocytoplasmic translocation of PTB was even more pronounced in isolated pancreatic islets, as shown by immunocytochemistry (Fig. 6c) and western blotting (Fig. 6d). Together with the translocation of the 59K PTB isoform, glucose enhanced the levels of a 27K PTB species primarily in the nucleus. Most probably, this represents a carboxy-terminal proteolytic fragment of the 59K form[25], as no other spliced variants were detected by RT-PCR in INS-1 and islet cells (see Supplementary Information, Fig. S3). Interestingly, glucose-stimulated translocation of PTB in purified pancreatic islets in culture decreased markedly with time (Fig. 6e).

DISCUSSION

We have identified PTB as a factor required for the biogenesis of insulin secretory granules. Many secretory granule components and other proteins associated with the neuroendocrine phenotype contains one or more consensus sites for PTB binding in their 3′-UTRs, and at least in the case of PC2 also in the 5′-UTR. Glucose stimulation of β-cells promotes the nucleocytoplasmic translocation of PTB, as well as the stabilization

of mRNAs encoding secretory granule components by the cytoplasmic pool of PTB. Thus, it is conceivable that mRNA stabilization is a mechanism by which PTB up-regulates the expression of β-cell secretory granule proteins in response to glucose. As most of these proteins are found in secretory granules of other peptide-secreting endocrine cells and neurons, it is possible that PTB also supports secretory granule biosynthesis in these cells in response to different stimuli. Stabilization of mRNAs for secretory proteins by cytosolic PTB in response to treatments that increase intracellular calcium levels, such as phorbol esters and ionomycin, has been reported in T-lymphocytes[17]. Calcium alone, however, may not be sufficient to activate PTB in β-cells, as stimulation with high potassium or sulphonylureas increases intracellular calcium levels and insulin secretion, but does not induce ICA512 (ref. 21) and insulin[26] biosynthesis. PTB activation may therefore depend on other second messengers, such as cAMP, which has been shown to promote insulin expression[27,28]. Indeed, while this manuscript was in revision it was shown that cAMP-dependent phosphorylation of PTB by protein kinase A induces its nucleocytoplasmic transport in neuroendocrine PC12 cells[29]. In addition to mRNA stabilization, cytosolic PTB could conceivably enhance other post-transcriptional mechanisms, such as mRNA translation, which participate in the rapid stimulus-dependent up-regulation of insulin expression[8,30]. Secretory granule biogenesis may also be promoted at the post-translational level by chromogranin A[31]. This hypothesis, however, remains controversial[32,33], and a mechanistic explanation for how chromogranin A would exert this control is lacking.

As newly synthesized secretory granules are preferentially recruited for exocytosis[34,35], alterations in the ability to promote rapid secretory granule biosynthesis may affect the pattern of insulin secretion. This may be relevant in type-2 diabetes, in which insulin secretion is typically impaired because of the inadequate release of newly synthesized secretory granules. Pharmacological activation of PTB, however, may sustain the production and exocytosis of insulin secretory granules under persistent hyperglycemia. □

METHODS

Islet isolation and cell culture. Pancreatic islets were isolated from female Wistar rats by collagenase digestion, purified by density gradient centrifugation, and cultured (400 per 60-mm culture dish) as described previously[36]. INS-1 cells were grown as described[37].

Cell extracts and immunoblotting. Stimulation of INS-1 cells (7×10^5 per 35-mm well) and pancreatic islets (400–1,000) with 25 mM glucose for 0–120 min was performed as described[21]. After stimulation, cells were washed with cold PBS and then extracted in lysis buffer (20 mM Tris-HCl at pH 8.0, 140 mM sodium chloride, 1 mM EDTA, 1% Triton X-100 and 1% protease inhibitor cocktail (Sigma, St Louis, MO)). Cytosolic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) in accordance with the manufacturer's instructions. For immunodepletion experiments, cytosolic extracts of INS-1 cells were incubated overnight at 4 °C with 5 μg monoclonal antibody 1 directed against the C-terminal region of PTB (Zymed Lab, San Francisco, CA) or mouse IgGs (BioRad, Hercules, CA) before addition of protein G–Sepharose (Pharmacia, Freiburg, Germany) and centrifugation. Protein concentration in the detergent-soluble material was measured using the BCA assay (Pierce). Cell extracts were separated by SDS–PAGE and immunoblotted as described[21] using the following antibodies: mouse monoclonals anti-PTB, anti-ICA512 (ref. 21), anti-calnexin (Transduction Laboratories, San Diego, CA), anti-chromogranin A (Immunon, Pittsburgh, PA), anti-PDI (Stressgen, Victoria, Canada), anti-γ-tubulin and anti-insulin (Sigma), anti-synaptophysin and anti-synaptobrevin 2 (Synaptic Systems, Goettingen, Germany); rabbit polyclonals anti-secretogranin 2 (gift from W. Huttner), anti-mannosidase II (ref. 38), anti-p58 (ref. 39), anti-PC1/3, anti-PC2 and anti-CPE (Chemicon, Temecula, CA). Chemiluminescence was performed using the Supersignal West Pico Substrate (Pierce) as substrate and detected with a LAS 3000 Bioimaging System (Fuji, Tokyo, Japan).

mRNA decay assay. The 3′-UTRs of rat ICA512, PC1, PC2 and CPE were subcloned into pCRII-TOPO with both T7 and SP6 promoters (Invitrogen, Carlsbad, CA). α-^{32}P-UTP-labelled RNA was synthesized with the T7-MEGA script kit (Ambion, Woodward, Austin, TX). Decay assays were performed as described[40] with minor modifications by incubating labelled (10^4 cpm) *in vitro* transcripts with 10 μg cytosolic extracts from resting or stimulated cells for 0–240 min. Reactions were stopped with 6× gel loading buffer and separated on a 6% polyacrylamide–7 M urea gel. Dried gels were exposed and quantified with a BAS 1800II phosphoimager (Fuji) using Image Gauge v3.45 software.

RNA–EMSA. For EMSAs, the following biotin-conjugated oligonucleotides were used: 5′-ACUCUUCAGCCCCUACCCAUCUGCC (first PTB-binding site in rat ICA512, wt1); 5′- ACUCUUCAGCAAAAAGGGAUCUGCC (mutated first PTB-binding site, m1); 5′-UGUACCUCCCCACUCCCACCAGCCUA (second PTB-binding site in rat ICA512, wt2); 5′-UGUACAUAGGAACUAGGACCAGCCUA (mutated second PTB-binding site, m2). The binding reaction was carried out using the LightShift Chemoluminescent EMSA Kit (Pierce). 10 μg cytosolic or nuclear extracts from resting or stimulated INS-1 cells were incubated with 5 pmol RNA oligonucleotides in binding buffer containing 100 mM potassium chloride and 1.5 mM magnesium chloride. After a 30-min incubation, 5 U RNAse T1 were added to each reaction and incubated for 10 min. After an additional 10-min incubation with 110 μg heparin, the reaction was stopped with 5 μl loading buffer. Electrophoresis, blotting, UV cross-linking and detection were performed according to manufacturer's instructions.

PTB binding assay. 96-well plates were coated with the monoclonal anti-PTB antibody. Binding reactions between INS-1 cell cytosolic extracts and biotinylated RNA oligonucleotides or ^{32}P-labelled full-length ICA512 3′-UTR were performed as above and UV cross-linked before incubation with anti-PTB-coated plates for 2 h. After incubation with horseradish-peroxidase-conjugated streptoavidin for 1 h, bound peroxidase activity was measured at 405 nm with 2,2′azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid (ABTS; Sigma) as substrate. Bound radioactivity was measured by β-counting.

Real-time PCR. Total RNA from 6×10^5 INS-1 cells was isolated with Trizol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. 1 mg of total RNA was used for the reverse transcriptase reaction, with 2 μM specific antisense primers for β-actin, ICA512 and insulin. mRNA expression was analysed by quantitative real-time PCR using the LightCycler system (Roche Diagnostics, Mannheim, Germany) as described[41].

Luciferase assays. Subcloning of rat PC2 3′-UTR and 5′-UTR into pGL3-Basic (Promega, Madison, WI), encoding firefly luciferase and multiple site-directed mutagenesis of PTB-binding sites, were performed using standard protocols. pGL3 constructs and phRL (Promega), encoding renilla luciferase, were co-transfected into INS-1 cells with Lipofectamine (Invitrogen). Firefly luciferase activity was measured 4 days after transfection and normalized with that of renilla luciferase using the dual luciferase system (Promega) according to manufacturer's instructions.

RNA interference. INS-1 cells (6×10^5 per 35-mm well) were grown for 2 days before transfection. 21-mer siRNA oligonucleotides 1 and 2 for rat PTB were synthesized with the Silencer siRNA Construction Kit (Ambion) using the following primers: sense primer 1, 5′-AAGATACCTAGTGATGTCACTCCTGTCTC; antisense primer 1, 5′-AAAGTGACATCACTAGGTATCCCTGTCTC; sense primer 2, 5′-AAGGACCGCAAGATGGCACTGCCTGTCTC; antisense primer 2, 5′-AACAGTGCCATCTTGCGGTCCCCTGTCTC. siRNA oligonucleotides 1 and 2 were mixed in a 1:1 ratio. Cells were transfected with 1 μg of the siRNA oligo mixture per well using Lipofectamine. Four days after transfection, cells were harvested in lysis buffer for immunoblotting or processed for immunocytochemistry.

Immunocytochemistry. INS-1 cells and islets were fixed with 3% paraformaldehyde. After embedding in gelatin, 5-μm islet sections were prepared. INS-1 cells and islet sections were then permeabilized with 0.2% saponin or 0.3% Triton X-100 and incubated with monoclonal antibodies against PTB, insulin or ICA512 for 1 h. After washing and incubation with goat-anti-mouse Alexa488- or Alexa568-conjugated secondary antibodies (Molecular Probes, Eugene, OR),

nuclei were counterstained with DAPI (Sigma). Images were acquired with a CoolSnap-HQ CCD camera (Roper Scientific, Tuscon, AZ) attached to an Olympus BX61 microscope and processed with Metamorph 4.65 software (Universal Imaging, Downington, PA).

Electron microscopy. INS-1 cells grown on glass coverslips were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and processed for standard Epon embedding. Surface areas of cell sections were analysed using a SIS Megaview camera with analysis software installed on a Tecnai12 electron microscope (FEI; Hillsboro, OR).

Insulin RIA. Total insulin content in the cells and in the medium was measured with the Sensitive Rat Insulin RIA Kit (Linco Research, St Charles, MO).

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: see *Nature Cell Biology* website for details.

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