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Biochemical and Biophysical Research Communications 328 (2005) 38–42
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Increased expression of polypyrimidine tract binding protein results in higher insulin mRNA levels

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Received 23 November 2004
Available online 5 January 2005

Abstract

The aim of this study was to further elucidate the role of the polypyrimidine tract binding protein (PTB) in the control of insulin mRNA stability. We observed that the glucose- or interleukin-1β-induced increase in insulin mRNA was paralleled by an increase in PTB mRNA. To further test the hypothesis that PTB controls insulin gene expression, βTC-6 cells were treated with a PTB-specific siRNA to modify the β-cell content of PTB. Surprisingly, we observed an increase in PTB mRNA and PTB protein levels in response to the siRNA treatment. In addition, the PTB-siRNA treatment also increased insulin mRNA. We conclude that expression of the PTB gene controls insulin production.

Keywords: Insulin mRNA; Polypyrimidine tract binding protein; mRNA stability; siRNA; β-cell

The insulin production from the pancreatic β-cells is regulated by the blood glucose concentration. The short-term (0–3 h) production of insulin is mainly regulated by changes in translation of the pre-existing insulin mRNA, whereas the long-term (>3 h) production is also influenced by alterations in the insulin mRNA level [1]. The insulin mRNA content of the β-cell is regulated not only by transcriptional control, but also by mRNA stability control. Thus, an increased production of insulin in response to a long-term glucose challenge results from an additive effect of stabilization of the insulin mRNA and an up-regulated transcription of the insulin gene [2]. We have recently observed that insulin mRNA is highly abundant in β-cells (approximately 100,000 molecules/cell) and represents up to 30% of the total mRNA pool [3]. In addition, insulin mRNA contents are not significantly affected by a 24 h exposure to actinomycin D [3]. These findings support the notion that the regulation of insulin mRNA levels over a 24 h period is mainly controlled by alterations in messenger stability and to a lesser extent gene transcription.

It has been demonstrated that the 3′-untranslated region (UTR) of insulin mRNA, which contains a pyrimidine-rich sequence, is important for glucose-induced stabilization of insulin mRNA [4]. The pyrimidine-rich sequence is homologous to a previously described mRNA stabilizing site called the hypoxia-inducible protein-binding site (HIPBS) [5], and we have observed that a 55 kDa polypyrimidine tract-binding protein (PTB) binds to this sequence [3,6]. It has been suggested that PTB, by binding to 3′-UTR pyrimidine-rich sequences, not only mediates increased insulin mRNA stability [3], but also augments granule protein mRNA stability in general [7]. It appears that glucose promotes PTB translocation from the nucleus to the cytosol, which results in increased messenger stability and translation [7].

PTB can be found in at least three different isoforms ranging between 55 and 62 kDa, and exists both as oligomer and monomer. The main form is PTB1, which consists of 531 amino acids and has a molecular mass

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doi:10.1016/j.bbrc.2004.12.147

of 57 kDa [8]. PTB contains several RNA binding motifs and is believed to be involved in transcription, splicing, translation, polyadenylation, and localization between the nucleus and the cytosol. There are several other proteins that are known to interact with PTB and it is likely that the action of PTB might involve the assembly of a protein complex [3,9]. There is evidence that the intracellular localization (and function) of PTB is controlled by its phosphorylation. It has been shown that protein kinase A phosphorylates PTB at Ser-16 and that this alters the intracellular localization of PTB to the cytoplasm [10]. In addition, as indicated by a microarray analysis, it appears that the expression of the PTB gene is inducible in insulin producing cells [11].

The overall aim of this study was to clarify whether PTB gene expression participates in the control of insulin mRNA levels. This was carried out by exposing insulin producing βTC-6 cells to glucose and interleukin-1β (IL-1β) to establish whether changes in insulin mRNA are paralleled by changes in PTB mRNA levels. Second, we have treated βTC-6 cells with PTB-specific siRNA to modify the β-cell content of PTB and to observe the effect on insulin mRNA levels.

Materials and methods

Cell cultures. The βTC-6 cell line was purchased from the American Type Culture Collection. The cells were used at passage numbers 15–30 and were kept in Dulbecco's modified Eagle's medium (Gibco) with glutamax-1, penicillin, and 10% FCS (Gibco). The cells were passaged approximately twice a week by trypsination in calcium-free Hanks' balanced salt solution (Sigma) containing 5 mg/ml trypsin and 0.53 mM EDTA.

siRNA transfection. A pre-designed siRNA oligonucliotide directed against murine PTB was purchased from Ambion. The antisense sequence was 5′-CAUGAGAAGGUUGGUAACCtt-3′. To introduce this siRNA into the cells a liposome reagent, Lipofectamine (Invitrogen), was used. Transfection was carried out according to manufacturer's recommendation using 10 μg Lipofectamine and 10, 50, 100 or 150 nM siRNA. The control contained 100 nM of non-targeting siRNA (Dharmacon). The transfection took place in 200 μl OptiMEM (Gibco) for 3 h after which the cells were cultured for 24 h in complete DMEM. All experiments were run in duplicate and after harvest the cells were either used for semi-quantitative real time PCR or Western blot.

Total RNA isolation and cDNA synthesis. Cells were washed once with PBS and 200 μl of Ultra Spec RNA Isolation Solution (Biotecx Laboratories) was added to each sample. Total RNA was extracted according to the instructions of the manufacturer. The cDNA synthesis was performed according to the instructions from FINNZYMES using their M-MulV reverse transcriptase Rnase H^-. The synthesis reaction was terminated by heating at 95 °C for 5 min after which the samples were stored at −20 °C. Before the cDNA samples were analyzed on the real time PCR instrument, they were purified using the Viogene PCR-M Cleanup System. All steps were performed according to manufacturers' recommendations except for the final cDNA elution, which took place in 2× 30 μl water heated to 60 °C.

Semi-quantitative real time PCR. The real time PCR was performed on a Light Cycler instrument (Roche) using the SYBR Green *Taq* Readymix (Sigma). This value is then normalized against the value for β-actin. To ensure the function of β-actin as a housekeeping gene, β-actin levels were compared with those of GAPDH. Both β-actin and GAPDH mRNA levels were unaffected by all treatments in the present study (results not shown), which verifies that β-actin is a suitable housekeeping gene. The primers used and their properties are as follows:

Gene	Forward primer	Reverse primer	Size (bp)	Annealing temp. (°C)
GAPDH	ACCACAGTCC ATGCCATCAC	TCCACCACCC TGTTGCTGTA	452	57
β-Actin	GCTCTGGCT CCTAGCACC	CCACCGATCCAC ACAGAGTACTTG	76	55
PTB	CCTAATGTCC ATGGAGCCTT	ACATCACCGTA GACGCCGAA	190	59
Insulin	CCATCAGCA AGCAGGTCA	CCACACACCAG GTAGAGAGC	161	59

The PCR products were analyzed on a 2% agarose gel to ensure that the fragments had the correct sizes.

To ensure that cDNA samples from the siRNA experiment were not contaminated with residual siRNA that might interfere with the real time PCR analysis, the cDNA was treated with RNase A and RNase T1 (from *Aspergillus oryzae*) both from Boehringer–Mannheim. Ten milligram per milliliters of RNase A and 30,000 U/ml RNase T1 were boiled for 10 min before being added to the cDNA samples, which were then incubated at 37 °C for 60 min. The RNase treated samples were diluted to 100 μl and the cDNA was extracted by the chloroform/phenol method. To precipitate the DNA 2 volumes of 95% ethanol and 1/10 volume of sodium acetate (3 M, pH 5.3) were added. The pellet was washed once in 70% ethanol and resuspended in water for real time PCR.

PTB immunoblot analysis. Cells were washed once with PBS and then lysed with SDS sample buffer; 2% SDS, 5% β-mercaptoethanol, 100 mM Tris–HCl, pH 6.8, 10% glycerol and bromophenol blue. The protein samples were boiled for 5 min and if necessary sonicated before separation on a 9% SDS–PAGE together with a Broad Range Marker (Biolabs). The separated proteins were electrophoretically transferred to Hybond-P membranes (Amersham Biocscience), which were then blocked for 1 h using a 2.5% milk protein solution. After washing the membranes in PBS–Tween (0.1% Tween) the membranes were hybridized with monoclonal mouse anti-PTB antibody (Zymed) and monoclonal rabbit anti-ERK antibody (Cell Signaling) for 1 h. Before the secondary antibody was added, the membranes were washed four times in PBS–Tween. The horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies was used as secondary antibodies and was detected with the ECL system (Amersham Bioscience). The resulting bands were then digitalized and their optical density measured.

Results

Effects of IL-1β or glucose on insulin and PTB mRNA levels

We first investigated whether PTB-mRNA levels were affected by IL-1β or glucose, both stimulators of β-cell insulin gene expression [1,12,13]. As expected, a 24 h culture period in the presence of 25 U/ml of IL-1β resulted in an almost 3-fold increase in the βTC-6 content of insulin mRNA (Fig. 1A), and a high glucose

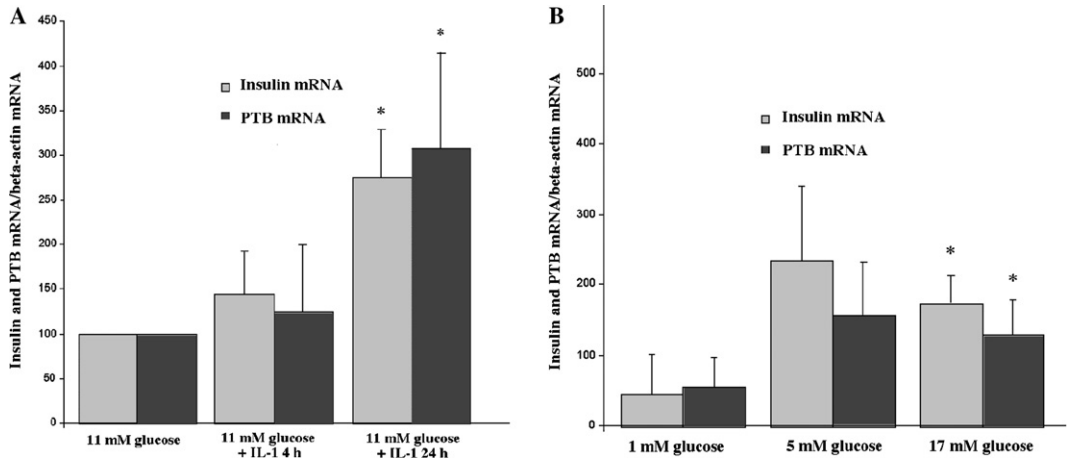

Fig. 1. Effects of IL-1β (A) and glucose (B) on insulin and PTB mRNA levels in βTC-6 cells. Cells were cultured for 24 h in RPMI 1640 medium containing 4 mM L-glutamax and 100 U/ml benzylpenicillin and 10% FCS supplemented with 25 U/ml IL-1β or different glucose concentrations. One group of cells received IL-1β for only 4 h. Light gray bars represent insulin mRNA and dark gray bars represent PTB mRNA. Results are normalized against β-actin and expressed as percent of the 11 mM glucose culture condition. Bars are means ± SD for three different experiments, each performed in duplicate. *Denotes $p < 0.05$ compared to the control, using Student's paired t test.

concentration (17 mM) increased insulin mRNA almost 4-fold as compared to a low glucose concentration (Fig. 1B). Interestingly, PTB mRNA was similarly increased in response to the 24 h stimulation with IL-1β or glucose (Figs. 1A and B). No effect was observed following a 4 h exposure to IL-1β (Fig. 1A). IL-1β did not increase production of nitric oxide from βTC-6 cells in the presence of 10% FCS (results not shown).

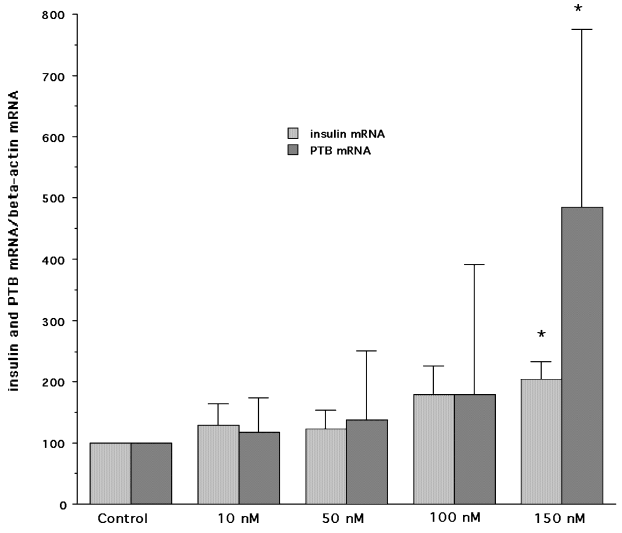

Fig. 2. Effects of PTB-siRNA on insulin and PTB mRNA levels in βTC-6 cells. βTC-6 cells were transfected with siRNA targeted against PTB using a liposome reagent, Lipofectamine (Invitrogen). Transfection was carried out for 3 h using 10 μg Lipofectamine and 10, 50, 100 or 150 nM siRNA. The control contained 100 nM of non-targeting siRNA (Dharmacon). Cells were harvested after 24 h and analyzed for PTB and insulin mRNA using real-time PCR. Light gray bars represent insulin mRNA and dark gray bars represent PTB mRNA. Results are normalized against β-actin and expressed as percent of the control. Bars are means ± SD of five different experiments in duplicate. *Denotes $p < 0.05$ compared to the control, using Student's unpaired t test.

Effects of PTB-siRNA on PTB mRNA and insulin mRNA

To establish a direct role of PTB in the control of insulin mRNA stability, we next attempted to down-regulate PTB in βTC-6 cells using the RNAi technique. Transfection was carried out using 10 μg Lipofectamine and 10, 50, 100 or 150 nM siRNA. Surprisingly, we observed that the highest concentrations of PTB siRNA, 150 nM, resulted in a significant increase in PTB mRNA levels (Fig. 2). This increase was paralleled by a 2-fold increase in insulin mRNA (Fig. 2). To ascertain that the increase in PTB mRNA resulted in an increased level of PTB, immunoblot analysis of PTB was performed. Indeed, we observed a specific increase in PTB 24 h after treatment with 150 nM PTB siRNA (Fig. 3).

Discussion

We have presently investigated the effects of glucose on insulin and PTB mRNA levels in βTC-6 cells. As insulin mRNA levels are mainly controlled by post-transcriptional mechanisms over a 24 h period [3], it is likely that the presently observed effects reflect changes in insulin mRNA stability. In a previous study, we observed that PTB levels in isolated rat islets were not affected by a 60 min glucose stimulation [3]. The incubation period was therefore increased to 24 h in this study. At this time point, both insulin and PTB mRNA

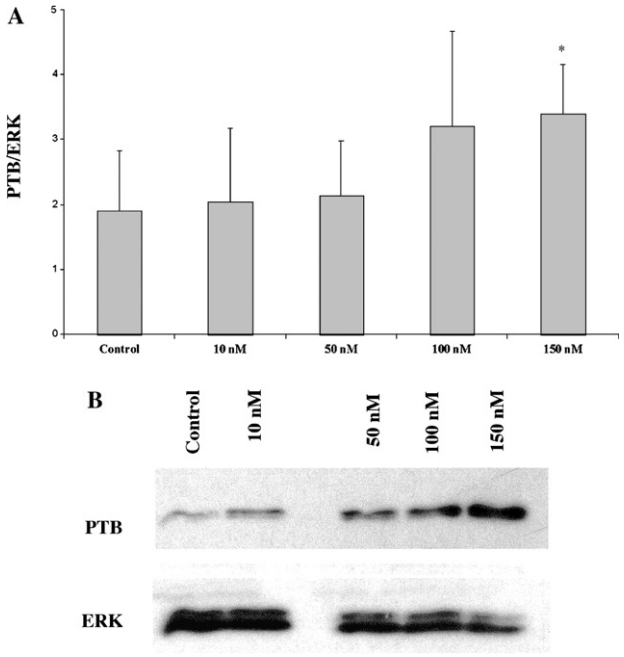

Fig. 3. PTB protein levels in βTC-6 cells transfected with siRNA targeted against PTB. (A) Transfection was carried out for 3 h using 10 μg Lipofectamine and 10, 50, 100 or 150 nM siRNA. The control contained 100 nM of non-targeting siRNA (Dharmacon). Cells were harvested after 24 h and analyzed for PTB and ERK using immunoblot analysis. PTB-bands are normalized against ERK and expressed as percent of the control. Bars are means ± SD for three different experiments in duplicates. *Denotes $p < 0.05$ compared to the control, using Student's unpaired t test. (B) One representative immunoblot is shown.

levels were increased in response to glucose, which supports the notion that PTB enhances insulin mRNA stability [3,7]. It has previously been proposed that PTB, in the short-term, promotes stabilization of insulin mRNA when phosphorylated and translocated from the nucleus to the cytosol [7,10]. The present observations add the possibility of a second control mechanism, i.e., long-term regulation of insulin production mediated by increased PTB gene expression. To our knowledge, the regulation of PTB gene transcription has not been studied, and it is therefore not known how glucose increases PTB gene expression. However, possible candidates as mediators of increased PTB mRNA levels are mTOR, a protein kinase thought to promote PTB binding to insulin mRNA [14], the PAS kinase, which was recently demonstrated to mediate glucose-induced insulin gene transcription [15], and AMPK, a protein kinase that negatively regulates insulin gene expression [16].

Also IL-1β augmented PTB and insulin mRNA levels after a 24 h incubation period. IL-1β is known to increase insulin mRNA in β-cells that are not inhibited by iNOS expression and nitric oxide production [12,13]. These findings suggest that IL-1β-induced signaling promotes increased insulin production by enhancing PTB gene expression. Interestingly, stimulation of INS-1E cells with IL-1β and IFN-γ for only 2 h increased PTB mRNA in microarray analysis 7-fold [17]. The early response observed in the INS-1E cells may be the consequence of IFN-γ-induced modulation of the IL-1β signal.

In the recent study by Knoch et al. [7] silencing of PTB in INS-1 cells, using the RNAi technique, resulted in depletion of secretory granules. The present attempt to silence PTB produced an increase, rather than a decrease, in PTB mRNA and protein levels. There are two possible explanations to this unexpected finding; either the siRNA molecule induces stability as it attaches to the PTB mRNA, by covering a degradation site, or the siRNA molecule modulates PTB mRNA splicing so that a more stable mRNA is generated [18]. In any case, the increase in PTB mRNA and protein was paralleled by higher insulin mRNA levels, which support a role of PTB gene expression in the control of insulin production. Further studies are warranted for the elucidation of the control of PTB gene expression in β-cells.

Acknowledgments

This study was supported in part by grants from the Swedish Research Council, the Swedish Diabetes Association, and the Family Ernfors Fund.

References

[1] J. Brunstedt, S.J. Chan, Direct effect of glucose on the preproinsulin mRNA level in isolated pancreatic islets, Biochem. Biophys. Res. Commun. 106 (1982) 1383–1389.
[2] M. Welsh, D.A. Nielsen, A.J. MacKrell, D.F. Steiner, Control of insulin gene expression in pancreatic β-cells and in an insulin-producing cell line, RINm-5F cells, J. Biol. Chem. 260 (1985) 13590–13594.
[3] L. Tillmar, C. Carlsson, N. Welsh, Control of insulin mRNA stability in rat pancreatic islets, J. Biol. Chem. 277 (2002) 1099–1106.
[4] B. Wicksteed, T.P. Hebert, C. Alarcon, M.K. Lingohr, L.G. Moss, C.J. Rhodes, Cooperativity between the preproinsulin mRNA untranslated region is necessary for glucose-stimulated translation, J. Biol. Chem. 276 (2001) 22553–22558.
[5] W.R. Paulding, M.F. Czyzyk-Krzeska, Regulation of tyrosine hydroxylase mRNA stability by protein-binding, pyrimidine-rich sequence in the 3′-untranslated region, J. Biol. Chem. 274 (1999) 2532–2538.
[6] L. Tillmar, N. Welsh, Hypoxia may increase rat insulin mRNA levels by promoting binding of the polypyrimidine tract-binding protein (PTB) to the pyrimidine-rich insulin mRNA 3′-untranslated region, Mol. Med. 8 (2002) 263–272.
[7] K.-P. Knoch, H. Bergert, B. Borgonovo, H.-D. Saeger, A. Altkrüger, P. Verkade, M. Solimena, Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis, Nat. Cell Biol. 6 (2004) 207–214.
[8] Y.L. Oh, B. Hahm, Y.K. Kim, H.K. Lee, J.W. Lee, O. Song, K. Tsukiyama-Kohara, M. Kohara, A. Nomoto, S.K. Jang, Determination of functional domains in polypyrimidine-tract-binding protein, Biochem. J. 331 (1998) 169–175.

[9] E.J. Wagner, M.A. Garcia-Blanco, Polypyrimidine tract binding protein antagonizes exon definition, Mol. Cell. Biol. 21 (2001) 3281–3288.
[10] J. Xie, J.-A. Lee, T.L. Kress, K.L. Mowry, D.L. Black, Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein, Proc. Natl. Acad. Sci. USA 100 (2003) 8776–8781.
[11] G.C. Webb, M.S. Akbar, C. Zhao, D.F. Steiner, Expression profiling of pancreatic β cells: glucose regulation of secretory and metabolic pathway genes, Proc. Natl. Acad. Sci. USA 97 (2000) 5773–5778.
[12] G.A. Spinas, B.S. Hansen, S. Linde, W. Kastern, J. Molvig, T. Mandrup-Poulsen, C.A. Dinarello, J.H. Nielsen, J. Nerup, Interleukin1 dose-dependently affects the biosynthesis of (pro)insulin in isolated rat islets of Langerhans, Diabetologia 30 (1987) 474–480.
[13] D.L. Eizirik, Interleukin-1 beta induces an early decrease in insulin release, (pro)insulin biosynthesis and insulin mRNA in mouse pancreatic islets by a mechanism dependent on gene transcription and protein synthesis, Autoimmunity 10 (1991) 107–113.
[14] L. Tillmar, N. Welsh, Glucose-induced binding of the polypyrimidine tract-binding protein (PTB) to the 3′-untranslated region of the insulin mRNA (ins-PRS) is inhibited by rapamycin, Mol. Cell. Biochem. 260 (2004) 85–90.
[15] G. da Silva Xavier, J. Rutter, G.A. Rutter, Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose, Proc. Natl. Acad. Sci USA 101 (2004) 8319–8324.
[16] G. da Silva Xavier, I. Leclerc, I.P. Salt, B. Doiron, D.G. Hardie, A. Kahn, G.A. Rutter, Role of AMP-activated protein kinase in the regulation by glucose of islet beta cell gene expression, Proc. Natl. Acad. Sci. USA 97 (2000) 4023–4028.
[17] B. Kutlu, A.K. Cardozo, M.I. Darville, M. Kruhoffer, N. Magnusson, T. Orntoft, D.L. Eizirik, Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells, Diabetes 52 (2003) 2701–2719.
[18] M.C. Wollerton, C. Gooding, E.J. Wagner, M.A. Garcia-Blanco, C.W. Smith, Autoregulation of polypyrimidine tract binding protein by alternative splicing leading to nonsense-mediated decay, Mol. Cell 13 (2004) 91–100.