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Increased expression of polypyrimidine tract binding protein results in higher insulin mRNA levels

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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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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