The Polypyrimidine Tract-binding Protein (PTB) Is Involved in the Post-transcriptional Regulation of Human Inducible Nitric Oxide Synthase Expression

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Human inducible nitric oxide synthase (iNOS) expression is regulated by transcriptional and post-transcriptional mechanisms. We have recently shown that the multifunctional RNA-binding proteins KH-type splicing regulatory protein and tristetraprolin are critically involved in the post-transcriptional regulation of human iNOS expression. Several reports have shown that KH-type splicing regulatory protein colocalizes with the polypyrimidine tract-binding protein (PTB), and both RNA-binding proteins seem to interact with the same mRNAs. Therefore we analyzed the involvement of PTB in human iNOS expression. In human DLD-1 cells, cytokine incubation necessary to induce iNOS expression did not change PTB localization or expression. However, intracellular binding of PTB to the human iNOS mRNA increased after cytokine stimulation. Overexpression of PTB resulted in enhanced cytokine-induced iNOS expression. Accordingly, small interfering RNA-mediated knock down of PTB reduced cytokine-dependent iNOS expression. Recombinant PTB displayed binding to an UC-rich sequence in the 3′-untranslated region of the human iNOS mRNA. Transfection experiments showed that PTB mediates its effect on iNOS expression via binding to this region. The underlying mechanism is based on a modulation of iNOS mRNA stability. In summary, human iNOS is the first example of a human pro-inflammatory gene regulated by PTB on the level of mRNA stability.

Post-transcriptional mechanisms represent an important part of the regulation of gene expression. In particular, genes whose expression has to be controlled precisely (e.g. oncogenes, pro-inflammatory genes) are regulated at the post-transcriptional level, mostly by modulation of mRNA stability (1, 2). Inherently unstable mRNAs that code for cytokines, transcription factors, proto-oncogenes, and pro-inflammatory mediators often contain AU-rich elements (AREs) in their 3′-untranslated regions (3′-UTRs). These AREs are targets for trans-acting proteins regulating mRNA stability and translation (1, 3). The family of ARE-binding proteins includes the embryonic lethal abnormal vision protein family members (most importantly HuR), the ARE/poly(U)-binding/degradation factor 1 (AUF-1, also named hnRNP D), the KH-type splicing regulatory protein (KSRP), tristetraprolin, the T cell-restricted intracellular antigen (TIA-1), and the T cell-restricted intracellular antigen-related protein (1, 3). In mammalian cells, the ARE-sequences mediate mRNA decay mainly by recruitment of the exosome (a multisubunit particle with 3′ to 5′ nuclease activity) to the mRNAs, thereby promoting their rapid degradation. However, the mammalian exosome does not seem to recognize the ARE-containing RNAs on its own but requires certain ARE-binding proteins (like KSRP or tristetraprolin) for this interaction (4, 5).

The polypyrimidine tract-binding protein (PTB), also known as hnRNP I, is a major hnRNP protein with multiple roles in mRNA metabolism, including regulation of alternative splicing (6), internal ribosome entry site-driven translation (7), hepatitis C virus replication (8), mRNA localization (9), and polyadenylation (10). Recent reports also showed stabilization of the CD154, insulin, and vascular endothelial growth factor mRNA by PTB (11–15). PTB has strong RNA binding activity since it contains a large number of RNA-binding sites that are preferably involved in ARE-containing RNAs (16). However, the mammalian exosome does not seem to recognize the ARE-containing RNAs on its own but requires certain ARE-binding proteins (like KSRP or tristetraprolin) for this interaction (4, 5).

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5 □ The abbreviations used are: ARE, AU-rich element; 3′-UTR, 3′-untranslated region; CM, cytokine mixture; Dox, doxycyclin; DRB, 6-dichloro-1-ribofuranosyl/benzimidazol; hnRNP, heteronuclear ribonucleoprotein; KSRP, KH-type splicing regulatory protein; NO, nitric oxide; iNOS, inducible NO synthase; PTB, polypyrimidine tract-binding protein; qRT-PCR, quantitativa ro-real-time reverse transcription-PCR; shRNA, short hairpin RNA; siRNA, small interfering RNA; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Luc, luciferase.

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the post-transcriptional regulation of human iNOS expression in a complex manner (22–25).

We were interested in analyzing the involvement of PTB in the regulation of human iNOS expression because KSRP seems to colocalize with PTB and binding of both proteins to the same mRNA has been described (18, 19, 26). Moreover, in the murine system, an interaction of PTB with the iNOS mRNA has been reported (27).

In DLD-1 cells, cytokine incubation did not change PTB expression or localization. However, intracellular binding of PTB to the human iNOS mRNA was markedly increased after cytokine stimulation. Overexpression or siRNA-mediated down-regulation of PTB demonstrated that PTB enhances cytokine-dependent iNOS induction. In vitro binding studies revealed an interaction of PTB with an UC-rich sequence in the 3′-UTR of the human iNOS mRNA. We could show that PTB exerts its effect on iNOS expression via this binding by enhancing iNOS mRNA stability.

EXPERIMENTAL PROCEDURES

Cell Culture, Cytokine Treatment, and RNA Isolation—Human alveolar epithelial A549/8 cells and human colon carcinoma DLD-1 cells were grown in Dulbecco’s modified Eagle’s medium with 2 mM l-glutamine, penicillin, and streptomycin (all from Sigma, Deisenhofen, Germany) and 5 or 10% heat-inactivated fetal bovine serum (from PAN-Systems, Nürnberg, Germany), respectively. Eighteen hours before cytokine induction, the cells were washed with phosphate-buffered saline and incubated with Dulbecco’s modified Eagle’s medium containing 2 mM l-glutamine in the absence of serum and phenol red. Inos expression in cells was induced with a cytokine mixture (CM) containing human interferon-γ (100 units/ml), interleukin-1β (50 units/ml), and tumor necrosis factor-α (10 ng/ml, all cytokines from Strathmann, Hannover, Germany) for the corresponding time periods depending on the experiment. Afterward, supernatant of the cells (50 µl) was used to measure NO_2 by the Sievers NOA 280 nitric oxide analyzer (ADInstruments, Spechbach, Germany), and cells were processed for RNA isolation by guanidinium thiocyanate/phenol/chloroform extraction as described (22) or for protein extraction as described below.

Establishment of Cell Lines Expressing an EGFP-PTB Fusion Protein—To generate DLD-1 cells overexpressing an EGFP-PTB fusion protein, cells were transfected with 5 µg of pEGFP-PTB (28) with FuGENE (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s recommendations. Stable transfectants (DLD-1-EGFP-PTB) were selected with G418 (1 mg/ml, Calbiochem, Bad Soden, Germany). As a control, DLD-1 cells stably transfected with the pEGFP-C1 vector (InVitrogen, Groningen, The Netherlands) were generated (DLD-1-EGFP-C1) as well. The G418-resistant cell pools were normalized by those of GAPDH mRNA. The relative amount of iNOS mRNA at 0 h DRB was set at 100%, and the percentage of iNOS or PTB mRNA expression was calculated.

DRB Experiments—To analyze the effect of experimental interventions on iNOS mRNA stability, cells were incubated as indicated, and iNOS expression was induced by cytokines for 4 h. Then 25 µg/ml 6-dichloro-1-ribofuranosylbenzimidazole (DRB, Sigma) was added, and RNAs were prepared 0–4 h thereafter. Relative iNOS and GAPDH mRNA amounts were determined by qRT-PCR, and iNOS mRNA was normalized to GAPDH mRNA. The relative amount of iNOS mRNA at 0 h DRB was set at 100%. Curve fittings of the resulting DRB time curves were performed by non-linear regression using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Analysis of Human iNOS Promoter Activity in Stably Transfected Cells—To investigate the effect of PTB overexpression on cytokine-induced iNOS promoter activity, DLD-1-EGFP-PTB or DLD-1-EGFP-C1 cells were transiently transfected by lipofection with FuGENE according to the manufacturer’s recommendations. After overnight incubation, cells were incubated with or without CM. Then the cells were lysed in 1× passive lysis buffer (Promega, Heidelberg, Germany), and firefly and Renilla luciferase activities were determined. The light units of the firefly luciferase were normalized by those of Renilla luciferase after subtraction of extract backgrounds.

Western Blot Experiments—To study protein expression in DLD-1 or A549/8 cells, total cell proteins or cytosolic and nuclear proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes by semidry electrophotocopy. All further steps were performed as described (22). For the detection of KSRP, iNOS, PTB, TATA box-binding factor, or β-tubulin monoclonal antibodies (anti-iNOS, R&D Systems, Wiesbaden, Germany; anti-PTB, Zymed Laboratories) were used. Mouse monoclonal anti-iNOS (R&D Systems, Wiesbaden, Germany) also reacts with rat iNOS and thus is a valid control for the rat experiments described in this study. The goat anti-rabbit conjugate (Zymed Laboratories, Munich, Germany) was used to detect KSRP and PTB. The anti-PTB monoclonal antibody to iNOS and PTB (Zymed Laboratories, Munich, Germany) was used to detect PTB. The anti-iNOS antibody is highly specific to iNOS in both rat and human tissues.

qRT-PCR was performed with the manufacturer’s recommendations using the oligonucleotides listed (all from MWG-Biotech, Ebersberg, Germany).

| Oligonucleotide 1     | PTB sense antisense probe |
|-----------------------|---------------------------|
| iNOS                   | TGGCAAGCACGTCGTTACTCC     |
|                        | TGGTACGCAGCTAGCGGATG      |
| GAPDH                  | CTGCCACCAAACACTGTACCCACCC|

| Oligonucleotide 2     | PTB sense antisense probe |
|-----------------------|---------------------------|
|                | CTGCCACCAAACACTGTACCCACCC|

| Oligonucleotide 3     | PTB sense antisense probe |
|-----------------------|---------------------------|
|                | CTGCCACCAAACACTGTACCCACCC|
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Inc., San Francisco, CA; anti-TATA box-binding factor, BD Transduction Laboratories, Heidelberg, Germany; anti-β-tubulin, Sigma) were used. The immunoreactive proteins on the blots were visualized by the enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Freiburg, Germany).

Analysis of mRNA Stability Using a Tetracycline-inducible Expression Vector—DLD-1-TR7 cells constitutively expressing a tetracycline repressor were a kind gift of Dr. M. Weitering, Utrecht, The Netherlands. Two different pcDNA4/TO-based luciferase expression vectors with or without the human iNOS 3′-UTR sequence were generated. DNA sequences of the clones were determined using the dye-chain termination method with a sequencing kit from Amersham Biosciences.

To analyze the effect of PTB on the expression of these luciferase reporter mRNAs, DLD-1-TR7 cells were transiently transfected by lipofection with FuGENE according to the manufacturer’s recommendations. To induce luciferase expression, cells were incubated with 10 ng/ml doxycycline for 24 h. Then cells were lysed in 1× passive lysis buffer, and firefly and Renilla luciferase activities were determined as described above.

Purification of GST-PTB Proteins—A procaroytic expression vector (pGEX2T-PTB) coding for a GST-PTB fusion protein was generated. Purified GST or GST-PTB fusion proteins were prepared using the plasmids pGEX2T (Amersham Biosciences) and pGEX2T-PTB as described (22). The yield of the purification procedure was determined by comparison with the BSA standard on Coomassie Blue-stained SDS-PAGE.

UV Cross-linking Experiments—cDNAs encoding subfragments of the human iNOS 3′-UTR have been described previously (22, 25). To generate radiolabeled iNOS 3′-UTR sense probes for RNA binding experiments, 0.5–1 μg of DNA (linearized plasmids, PCR fragments, or double-stranded oligonucleotides) was in vitro transcribed, and UV cross-linking experiments were performed as described (24, 25).

Down-regulation of PTB Expression by RNA Interference—To generate an expression vector enabling intracellular short hairpin RNA (shRNA) synthesis, a double-stranded oligonucleotide (5′-AAGGAAAATCCATCCATCCAGAAGAATTTGC- TTATTTCTTGAAAGAATGGAGATGTTGCTATAGTGA-3′; sequence of the siRNA repeats directed against the human PTB mRNA underlined) was cloned into the BbsI sites of psiRNA-hH1-GFPzeo (InvivoGen, San Diego, CA) to generate psiRNA-hH1-GFPzeo-PTB. The DNA sequence of the construct was determined using the dye-chain termination method with a sequencing kit from Amersham Biosciences. To generate A549/8 cells stably expressing shRNAs directed against the human PTB mRNA, cells were transfected with psiRNA-hH1-GFPzeo-PTB by lipofection with FuGENE according to the manufacturer’s recommendations. Stable transfecnts were selected with zeocin (200 μg/ml, InvivoGen) and for GFP expression by fluorescence-activated cell sorting. As a control, A549/8 cells stably transfected with the psiRNA-hH1-GFPzeo vector were generated as well.

Immunoprecipitation-qRT-PCR Assay—For determination of intracellular protein-RNA interactions, DLD-1 cells were incubated for 4 h with or without the cytokine mixture. All further steps were performed as described before (24).

RESULTS

Cytokine Incubation Does Not Change PTB Expression or Localization in DLD-1 Cells—In previous studies, we demonstrated that KSRP is critically involved in the post-transcriptional regulation of human iNOS expression in interplay with other RNA-binding proteins (24, 25). In murine and human cells, KSRP colocalizes with PTB (18, 19), and opposite functions of both proteins in hnRNA splicing have been described (18). Therefore we were interested in whether PTB as well as KSRP fulfills a role in the regulation of iNOS expression.

Induction of iNOS in human cells requires stimulation with a complex CM containing interleukin-1β, interferon-γ, and tumor necrosis factor-α (23). First we analyzed the influence of this cytokine mixture on the expression of PTB. Therefore DLD-1 cells were incubated with or without CM for 2–24 h, and RNA and total cell proteins were isolated. As shown before for KSRP (24), cytokine incubation did not affect PTB protein (Fig. 1A) or mRNA expression (Supplemental Fig. 1).

Then the cellular localization of PTB and KSRP was determined by Western blot experiments using nuclear and cyto-
These analyses showed a PTB-dependent enhancement of human iNOS expression. In DLD-1-EGFP-PTB cells, we detected a similar increase of cytokine-induced iNOS mRNA (Fig. 3B) and protein expression (Fig. 3C) as well as iNOS-mediated NO production (Fig. 3D) when compared with control cells (DLD-1-EGFP).

Down-regulation of PTB Reduces Cytokine-induced iNOS Expression in A549/8 and DLD-1 Cells—To confirm these results, we tested whether down-regulation of endogenous PTB expression using the RNA interference technique resulted in reduced iNOS expression. Therefore A549/8 cells (known to be able to express iNOS after cytokine incubation (30)) were stably transfected with an expression vector (psiRNA-hH1-GFPzeo-PTB) leading to constitutive intracellular expression of shRNAs directed against the PTB coding region (indicated on figures as siPTB). As a control, cells stably transfected with the expression vector (indicated on figures as siGFP) were generated as well. These cell pools were incubated in the presence or absence of the cytokine mixture, and total protein and RNA were isolated. NO production was determined as described above.

In A549/8 cells stably transfected with psiRNA-hH1-GFPzeo-PTB (siPTB) PTB protein (Fig. 4A) and mRNA (Supplemental Fig. 2), expression was significantly reduced when compared with control cells (siGFP). Analysis of cytokine-induced iNOS mRNA in these cells showed that down-regulation of PTB by siPTB clearly reduced iNOS mRNA expression (Fig. 4B). Also, cytokine-induced iNOS protein expression (Fig. 4C) and iNOS-dependent NO production (Fig. 4D) were reduced to a similar degree in these cells. Transient transfection of anti-PTB shRNAs into DLD-1 cells resulted in an analogous effect (see Supplemental Fig. 3). In summary, the data indicate that PTB up-regulates cytokine-induced human iNOS expression.

PTB Binds to the 3'-UTR of the Human iNOS mRNA—The human iNOS 3'-UTR sequence contains several putative PTB-binding sites (Fig. 5A, UCUIII_1 to UCUIII_3). To investigate the definite binding site of PTB in the human iNOS 3'-UTR, recombinant GST-PTB fusion protein was incubated with 32p-labeled transcripts comprising different nucleotide regions (Fig. 5A). Then PTB-RNA interaction was assayed by UV cross-linking experiments. We detected complex formation between recombinant GST-PTB protein and the whole 3'-UTR transcript. This binding activity was not observed with the GST protein (Fig. 5B, panel I, 3'-UTR). To localize the PTB-binding site within the iNOS 3'-UTR, the region was first dissected into two subfragments. One was the non-AU fragment without AU repeats containing one UCUII sequence (UCUII_1). The other was the AU fragment containing the AU repeats and two UCUII sequences (UCUII_2 and UCUII_3). Only the AU subfragment interacted with the GST-PTB protein (Fig. 5B, panel I). Subsequently, the AU fragment was dissected into three subfragments: subfragment A (232–329, with the UCUII_2 sequence), subfragment B (327–428, with the UCUII_3 sequence), and subfragment C (387–477, with the UCUII_3 sequence). As shown in Fig. 5B (panel II), GST-PTB displayed a marked binding activity to both fragment B and fragment C. These results suggested that the PTB-binding site in the human iNOS mRNA 3'-UTR is located in the overlapping sequence of fragments B and C (Fig. 5A, B_C_overlap). Also, these data
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**Figure 3.** Overexpression of an EGFP-PTB fusion protein enhances cytokine-induced iNOS mRNA expression and iNOS-dependent NO production. A plasmid construct (pEGFP-PTB) allowing high level expression of an EGFP-PTB fusion protein was stably transfected into DLD-1 cells (EGFP-PTB). Cells transfected with the pEGFP-C1 vector backbone (EGFP) were used as controls. For analysis of iNOS expression, pools of stable transfected cells were preincubated for 24 h in medium without fetal calf serum and phenol red. Then cells were incubated with CM or expression or PTB and EGFP vector backbone (sion and iNOS-dependent NO production.

A, Western blots for the analysis of PTB, EGFP, and β-tubulin protein expression in DLD-1-EGFP or DLD-1-EGFP-PTB cells using monoclonal anti-PTB and β-tubulin antibodies. The positions of β-tubulin, PTB, and EGFP-PTB are indicated. This blot is representative of four other blots showing similar results. B, a summary of 12 qRT-PCR analyses is shown using RNAs from DLD-1-EGFP-C1 (EGFP) or DLD-1-EGFP-PTB (EGFP-PTB) cells. Data (means ± S.E.) represent relative iNOS mRNA levels (***, p < 0.001 versus CM-treated pEGFP-C1 cells). C, analysis of iNOS and β-tubulin (β-Tub) protein expression in extracts from stably transfected DLD-1-EGFP or DLD-1-EGFP-PTB cells by Western blot using monoclonal anti-iNOS and β-tubulin antibodies. The positions of β-tubulin and iNOS are indicated. This blot is representative of three other blots showing similar results. D, a summary of 11 nitrite analyses using supernatants from DLD-1-EGFP-C1 (EGFP) or DLD-1-EGFP-PTB (EGFP-PTB) cells is shown. The nitrite values in the supernatants of untreated cells were subtracted from those of the CM-treated cells. Data (means ± S.E.) represent relative nitrite levels (100% corresponds to 4200 pmol/ml/24 h; ***, p < 0.001; ns, not significant versus CM-treated pEGFP-C1 cells).

 imply that the UCUU_2 sequence is not important for the binding of PTB to the human iNOS 3'-UTR. In accordance, direct mutation of the UCUU_2 sequence (UCUU → AGAA) did not result in reduced binding to the human iNOS 3'-UTR (data not shown). As shown in Fig. 5B (panel III), deletion of the UCUU_3 sequence (indicated as AU-ΔNco) in the context of the AU fragment or direct mutation of this sequence (UCUU → AGAA; data not shown) did not modify the binding of GST-PTB. However, deletion of the B_C-overlap sequence in the context of the AU fragment (Fig. 5, panel III, AU-ΔBsrGI) resulted in a complete loss of the binding of GST-PTB.

All the data described above showed that none of the UCUU sequences are important for the binding of PTB to the human iNOS 3'-UTR. However, the UC-rich sequence 5'-UAACACCC- AGUCGUUUCCCCCAUGG-3' (positions 389–412 of the 3'-UTR; Fig. 5A F_PTB s) seems to be essential for this PTB-RNA interaction.

PTB Enhances the Expression of a Luciferase mRNA Containing the Human iNOS mRNA 3'-UTR—To analyze whether the enhancing effect of PTB on human iNOS mRNA expression in intact cells results from its binding to the 3'-UTR, we performed Tet-On analyses. We generated expression vectors with (pCDNA4/TO-Luc-3'-UTR; indicated on figures as Luc-UTR) or without (pCDNA4/TO-luc; indicated on figures as Luc) the iNOS 3'-UTR cloned behind the firefly luciferase reporter gene under the control of a tetracycline responsible promoter. These vectors together with pEGFP-C1 or pEGFP-PTB and pRL-SV40 (Renilla luciferase expression vector) were transiently transfected into DLD-1-TR7 cells constitutively expressing a tetracycline repressor. To induce luciferase expression, cells were incubated for 24 h with doxycycline (Dox). Then cells were lysed, and relative luciferase activities were determined. As shown in Fig. 6, Dox incubation resulted in a marked enhancement of firefly luciferase activity (compare columns 1 or 2 with columns 5 and 6, respectively). The presence of the 3'-UTR of the human iNOS mRNA 3' to the firefly luciferase stop codon markedly reduced luciferase expression (comparison of Luc with Luc-UTR in pEGFP-C1-transfected cells; column 5 versus column 6). Overexpression of PTB in this system resulted in clearly enhanced firefly luciferase activity in cells transfected with pCDNA4/TO-Luc-UTR (comparison of Luc-UTR in pEGFP-C1 and pEGFP-PTB-transfected cells; column 6 versus column 8). Therefore the effect of PTB on the human iNOS mRNA is mediated by its binding to the human iNOS 3'-UTR sequence.

Overexpression of PTB Modulates Human iNOS mRNA Stability—To test whether the enhancing effect of PTB on iNOS expression resulted from PTB-mediated changes in the stability of the human iNOS mRNA, we performed experiments using DRB to block the RNA polymerase II-dependent transcription. DLD-1-EGFP-C1 (indicated on figures as EGFP) or DLD-1-EGFP-PTB (indicated on figures legends as EGFP-PTB) cells were incubated with CM for 4 h. Then DRB (25 µg/ml) was added to stop transcription, and RNA was iso-
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FIGURE 4. Down-regulation of PTB expression by siRNAs reduces cytokine induced iNOS expression. AsA549/8 cells were stably transfected with psiRNA-hH1-GFPzeo-PTB (siPTB). In these cells, shRNAs were generated that were directed against the human PTB mRNA. Stable transfectants were selected for zeocin resistance and GFP expression. As a control, A549/8 cells stably transfected with the psiRNA-hH1-GFPzeo vector were generated as well (siGFP). The cells were preincubated for 18 h in medium without fetal calf serum and phenol red before being exposed to cytokines (CM) for 6 or 8 h. Then total RNA or protein extracts were prepared. To determine iNOS-mediated NO production, cells were incubated for 24 h with or without CM, and the supernatant of the cells was analyzed for nitrite content. A, analysis of PTB and β-tubulin (β-Tub) protein expression in extracts from A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP) by Western blot using monoclonal anti-PTB and β-tubulin antibodies. The positions of β-tubulin and PTB are indicated. This blot is representative of three other blots showing similar results. B, statistical analysis of five qRT-PCR experiments using RNA isolated from stably transfected A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP). C, analysis of iNOS and β-tubulin protein expression in extracts from stably transfected A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP) by Western blot using monoclonal anti-iNOS and β-tubulin antibodies. The positions of β-tubulin and iNOS are indicated. This blot is representative of three other blots showing similar results. D, a summary of eight nitrite analyses using supernatants from stably transfected A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP) is shown. The nitrite values in the supernatants of untreated cells were subtracted from those of the CM-treated cells. Data (means ± S.E) represent relative nitrite levels (***, p < 0.001 versus psiRNA-hH1-GFPzeo-PTB cells incubated for 6 h with CM). As a control, A549/8 cells were incubated for different time periods in the presence of cytokine mixture, and nitrite levels were analyzed in extracts of these cells. As shown in Fig. 7B, the human iNOS mRNA and protein expression increased in a time-dependent manner in both cell types. As a control, A549/8 cells stably transfected with the psiRNA-hH1-GFPzeo vector were generated as well (siGFP). The cells were preincubated for 18 h in medium without fetal calf serum and phenol red before being exposed to cytokines (CM) for 6 or 8 h. Then total RNA or protein extracts were prepared. To determine iNOS-mediated NO production, cells were incubated for 24 h with or without CM, and the supernatant of the cells was analyzed for nitrite content. A, analysis of PTB and β-tubulin (β-Tub) protein expression in extracts from A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP) by Western blot using monoclonal anti-PTB and β-tubulin antibodies. The positions of β-tubulin and PTB are indicated. This blot is representative of three other blots showing similar results. B, statistical analysis of five qRT-PCR experiments using RNA isolated from stably transfected A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP). C, analysis of iNOS and β-tubulin protein expression in extracts from stably transfected A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP) by Western blot using monoclonal anti-iNOS and β-tubulin antibodies. The positions of β-tubulin and iNOS are indicated. This blot is representative of three other blots showing similar results. D, a summary of eight nitrite analyses using supernatants from stably transfected A549/8-psiRNA-hH1-GFPzeo-PTB or -psiRNA-hH1-GFPzeo cells (siPTB or -siGFP) is shown. The nitrite values in the supernatants of untreated cells were subtracted from those of the CM-treated cells. Data (means ± S.E) represent relative nitrite levels (100% corresponds to 4200 pmol/ml/24 h; ***p < 0.001; *p < 0.05 versus CM-treated A549/8-psiRNA-hH1-GFPzeo-PTB cells).

Section 4. Down-regulation of PTB expression by siRNAs reduces cytokine induced iNOS expression. We analyzed whether increase of PTB expression changes human iNOS expression (24, 25). The 3'-UTR of the human iNOS mRNA contains five AREs (Fig. 5A) and has been shown to destabilize reporter mRNAs (22). Of several proteins known to bind to such AREs, the embryonic lethal abnormal vision protein HuR and KSRP bind to this 3'-UTR sequence and regulate the stability of the human iNOS mRNA (22, 24).

Beyond this, several other RNA-binding proteins have been characterized that are involved in the regulation of RNA metabolism. One of them is the PTB also known as hnRNP I. PTB has initially been described as a splicing repressor that affects splicing of many alternative exons (6). However, recent evidence suggests that PTB is a major hnRNP protein with multiple roles in mRNA metabolism, including the stabilization of mRNAs like the CD154, insulin, and vascular endothelial growth factor mRNA (11–15). Moreover, PTB has been implicated in the regulation of the murine iNOS expression (27, 37).

Previous reports have shown that PTB interacts and colocalizes with KSRP in murine and human cells (18, 19). As we have demonstrated that KSRP is critically involved in regulation of human iNOS expression (24, 25), we analyzed the expression and cellular localization of PTB and KSRP in DLD-1 cells. As shown by Hall et al. (19) for HeLa cells, we could detect a very similar distribution of PTB and KSRP in the nucleus and the cytoplasm of DLD-1 cells by Western blot (Fig. 1B) and immunofluorescence analyses (data not shown). The localization and expression of both proteins did not change by cytokine treatment of the cells (Fig. 1A) (24).
In contrast to the unchanged localization and expression of PTB and KSRP, cytokine incubation of DLD-1 cells resulted in a markedly enhanced intracellular binding of PTB to the human iNOS mRNA (Fig. 2). As published before (24), the KSRP binding was reduced in the same cells. As both proteins are described to have contrary roles in the splicing of the c-Src mRNA (18) and PTB has been shown to enhance the stability of different mRNAs (12, 14, 15, 26, 38), we hypothesized that PTB presumably regulates human iNOS expression in an opposite manner than KSRP.

Indeed, our data confirmed this hypothesis. In contrast to KSRP, which destabilizes iNOS mRNA (24), PTB up-regulates iNOS expression as shown by overexpression (Fig. 3) or siRNA-mediated down-regulation of PTB (Fig. 4). As the effect of PTB on iNOS mRNA equals those on iNOS protein and iNOS-dependent NO production, PTB seems to influence mainly iNOS mRNA expression. The effect of PTB on iNOS mRNA translation seems of minor importance but cannot be excluded absolutely by the data presented. Since these effects were observed in DLD-1 and A549/8 cells, PTB-mediated modulation of iNOS expression appears to be a more general feature of human cells.

PTB possesses a strong RNA binding activity, and in vitro studies determined UCUU flanked by pyrimidines as a preferred PTB-binding site (17). Analysis of the human iNOS 3'-H11032-UTR sequence showed the existence of three of these motifs (Fig. 5A, UCUU_1 to UCUU_3).

Analysis of PTB interaction with the human iNOS mRNA 3'-H11032-UTR surprisingly showed that PTB binding does not seem to depend on one of these UCUU sequences. Our data strongly indicate that PTB binds to the sequence 5'-UAACACCCAGUCGUGUCCCCAUGG-3' (positions 389–412 of the human iNOS 3'-UTR, indicated in Fig. 5A as F_PTB_s). This sequence is also relatively UC-rich but does not contain the “classical” UCUU-motif. In summary, besides the ARE sequences already proven to be essential for iNOS mRNA stability (22–25), these data reveal a second important sequence motif necessary for the post-transcriptional regulation of human iNOS expression.
To prove the importance of PTB binding to the iNOS 3′-UTR in intact cells, we analyzed the effect of PTB on the expression of a luciferase reporter mRNA with (indicated as Luc-UTR) or without (indicated as Luc) the iNOS 3′-UTR under control of a tetracycline-inducible promoter. With this method, we detected the formerly described (22) destabilizing effect on human iNOS expression, PTB also enhances expression of an unrelated reporter mRNA containing the human iNOS 3′-UTR. Combined with our immunoprecipitation-qRT-PCR and UV cross-linking experiments, this indicates that the PTB effect on human iNOS expression is mediated by the PTB-binding site (Fig. 5A, F_PTB_s) located in the 3′-UTR.

All the data shown above support our hypothesis that PTB post-transcriptionally affects human iNOS expression. Indeed, as shown in Fig. 7, PTB enhanced human iNOS mRNA stability but did not change human iNOS promoter activity at any time point analyzed.

The above data indicate that after cytokine incubation, PTB increases the stability of the human iNOS mRNA by enhanced binding to its 3′-UTR. In this way, PTB contributes to the cytokine-dependent induction of human iNOS expression and iNOS-mediated NO production.

In the RNA-binding protein network regulating iNOS mRNA stability, KSRP and PTB display antagonistic effects,
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which parallels the described opposite roles of both proteins in c-Src-mRNA splicing (18). As published before, KSRP and HuR also regulate iNOS mRNA expression in an opposite manner by competing for the same binding site (24). In contrast, the binding site of PTB in the iNOS 3’-UTR differs from that of KSRP. Thus the antagonistic effect of both proteins does not depend on direct competition for the same RNA sequence. Finally, PTB plays an essential role in the post-transcriptional regulation of human iNOS expression and therefore could also be important for the regulation of other pro-inflammatory human genes whose expression is controlled by KSRP.

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REFERENCES
1. Hollams, E. M., Giles, K. M., Thomson, A. M., and Leedman, P. J. (2002) Neurochem. Res. 27, 957–960
2. Moore, M. J. (2005) Science 309, 1514–1518
3. Barreau, C., Paillard, L., and Osborne, H. B. (2005) Nucleic Acids Res. 33, 7138–7150
4. Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Rajimakers, R., Pruijn, G. J., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001) Cell 107, 451–464
5. Gherzi, R., Lee, K. Y., Briata, P., Wegmuller, D., Moroni, C., Karin, M., and Chen, C. Y. (2004) Mol. Cell 14, 571–583
6. Wagner, E. J., and Garcia-Blanco, M. A. (2001) Mol. Cell. Biol. 21, 3281–3288
7. Cornelis, S., Tinton, S. A., Schepens, B., Bruynooghe, Y., and Beyaert, R. (2005) Nucleic Acids Res. 33, 3095–3108
8. Domitrovich, A. M., Diebel, K. W., Ali, N., Sarker, S., and Siddiqui, A. (2005) Virology 335, 72–86
9. Cote, C. A., Gautreau, D., Denegre, J. M., Kress, T. L., Terry, N. A., and Mowry, K. L. (1999) Mol. Cell 4, 431–437
10. Castelo-Branco, P., Furger, A., Wollerton, M., Smith, C., Moreira, A., and Proudfoot, N. (2004) Mol. Cell. Biol. 24, 4174–4183
11. Tillmar, L., Carlsson, C., and Welsh, N. (2002) J. Biol. Chem. 277, 1099–1106
12. Coles, L. S., Bartley, M. A., Bert, A., Hunter, J., Polyak, S., Diamond, P., Vadas, M. A., and Goodall, G. J. (2004) Eur. J. Biochem. 271, 648–660
13. Kosinski, P. A., Laughlin, J., Singh, K., and Covey, L. R. (2003) J. Immunol. 170, 979–988
14. Hamilton, B. J., Genin, A., Cron, R. Q., and Rigby, W. F. (2003) Mol. Cell. Biol. 23, 510–525
15. Fred, R. G., and Welsh, N. (2005) Biochem. Biophys. Res. Commun. 328, 38–42
16. Perez, I., McAfee, J. G., and Patton, J. G. (1997) Biochemistry 36, 11881–11890
17. Perez, I., Lin, C. H., McAfee, J. G., and Patton, J. G. (1997) RNA 3, 764–778
18. Markovtsov, V., Nikolic, J. M., Goldman, J. A., Turck, C. W., Chou, M. Y., and Black, D. L. (2000) Mol. Cell. Biol. 20, 7463–7479
19. Hall, M. P., Huang, S., and Black, D. L. (2004) Mol. Biol. Cell 15, 774–786
20. Forstermann, U., Gath, I., Schwarz, P., Closs, E. I., and Kleinert, H. (1995) Biochem. Pharmacol. 50, 1321–1332
21. Korhonen, R., Lahti, A., Kankaanranta, H., and Moilanen, E. (2005) Curr. Drug Targets Inflamm. Allergy 4, 471–479
22. Rodriguez-Pascual, F., Hausding, M., Ihrig-Biedert, I., Furneaux, H. R., Levy, A. P., Forstermann, U., and Kleinert, H. (2000) J. Biol. Chem. 275, 26040–26049
23. Kleinert, H., Pauitz, A., Linker, K., and Schwarz, P. M. (2004) Eur. J. Pharmacol. 500, 255–266
24. Linker, K., Pauitz, A., Fechir, M., Hubrich, T., Geye, J., and Kleinert, H. (2005) Nucleic Acids Res. 33, 4813–4827
25. Fechir, M., Linker, K., Pauitz, A., Hubrich, T., Forstermann, U., Rodriguez-Pascual, F., and Kleinert, H. (2005) Mol. Pharmacol. 67, 2148–2161
26. Irwin, N., Baekelandt, V., Goritchenko, L., and Benowitz, L. I. (1997) Nucleic Acids Res. 25, 1281–1288
27. Soderberg, M., Raffalli-Mathieu, F., and Lang, M. A. (2002) Mol. Pharmacol. 62, 423–431
28. Hallm, B., Cho, O. H., Kim, J. E., Kim, Y. K., Kim, J. H., Oh, Y. L., and Jang, S. K. (1998) FEBS Lett. 425, 401–406
29. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
30. Kleinert, H., Echenhofer, C., Ihrig-Biedert, I., and Forstermann, U. (1996) Mol. Pharmacol. 49, 15–21
31. Hall, M. P., Weber, A., and Levins, D. (2000) Nucleic Acids Res. 28, 4558–4565
32. Katahira, M., Miyanoiri, Y., Enokizono, Y., Matsuda, G., Nagata, T., Ishikawa, F., and Uesugi, S. (2001) J. Mol. Biol. 311, 973–988
33. Fiset, S., and Chabot, B. (2001) Nucleic Acids Res. 29, 2268–2275
34. Donoe, R. M., Doneva, T. A., Bowen, W. R., and Sheer, D. (2002) Mol. Cell. Biochem. 233, 181–185
35. de Vera, M. E., Shapiro, R. A., Nüssler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1054–1059
36. Misquitta, C. M., Iyer, V. R., Werstiuk, E. S., and Grover, A. K. (2001) Mol. Cell 6, 423–431
37. Soderberg, M., Raffalli-Mathieu, F., and Lang, M. A. (2007) Mol. Immunol. 44, 434–442
38. Tillmar, L., and Welsh, N. (2002) Mol. Med. 8, 263–272