Low tetrahydrobiopterin biosynthetic capacity of human monocytes is caused by exon skipping in 6-pyruvoyl tetrahydropterin synthase

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Biosynthesis of (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4-biopterin), an essential cofactor for aromatic amino acid hydroxylases and NO synthases, is effectively induced by cytokines in most of the cell types. However, human monocytes/macrophages form only a little H_4-biopterin, but release neopterin/7,8-dihydroneopterin instead. Whereas 6-pyruvoyl tetrahydropterin synthase (PTPS) activity, the second enzyme of H_4-biopterin biosynthesis, is hardly detectable in these cells, PTPS mRNA levels were comparable with those of cell types containing intact PTPS activity. By screening a THP-1 cDNA library, we identified clones encoding the entire open reading frame (642 bp) as well as clones lacking the 23 bp exon 3, which results in a premature stop codon. Quantification of the two mRNA species in different cell types (blood-derived cells, fibroblasts and endothelial cells) and cell lines showed that the amount of exon-3-containing mRNA is correlated closely to PTPS activity. The ratio of exon-3-containing to exon-3-lacking PTPS mRNA is not affected by differential mRNA stability or nonsense-mediated mRNA decay. THP-1 cells transduced with wild-type PTPS cDNA produced H_4-biopterin levels and expressed PTPS activities and protein amounts comparable with those of fibroblasts. We therefore conclude that exon 3 skipping in transcription rather than post-transcriptional mechanisms is a major cause of the low PTPS protein expression observed in human macrophages and related cell types.

Key words: fibroblast, lymphocyte, myeloid cell, pteridine, 6-pyruvoyl tetrahydropterin synthase, RNA splicing.

INTRODUCTION

(6*R*)-5,6,7,8-Tetrahydro-L-biopterin (H_4-biopterin) is an essential cofactor in catecholamine, serotonin and NO formation and in diethyl ether lipid hydroxylation. Hence, biosynthesis of this pteridine is vital for a vast number of biological processes, including neurotransmission, vasorelaxation and immune response [1–3]. H_4-biopterin is formed from GTP by three enzymic steps, i.e. GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase. Besides being expressed constitutively at the major sites of aromatic amino acid hydroxylation, i.e. the liver and the central nervous system, this pathway is induced by hormones, cytokines and certain immune stimuli, such as bacterial lipopolysaccharide (LPS) in numerous mammalian tissues and cell types via transcriptional up-regulation of GTP cyclohydrolase I [3].

In general, GTP cyclohydrolase I is the rate-limiting step of H_4-biopterin biosynthesis. However, in human monocytes and macrophages, PTPS activity is very low [4] and cannot always be detected [5]. Thus PTPS becomes rate-limiting in these cells on up-regulation of GTP cyclohydrolase I by cytokines [4]. As a result, human monocytes and macrophages produce very little H_4-biopterin, but large amounts of 7,8-dihydroneopterin/neopterin. These metabolites stem from 7,8-dihydroneopterin triphosphate, the first intermediate of H_4-biopterin biosynthesis which is cleaved by intracellular phosphatases [4]. Neopterin is a well-established clinical marker for immune activation in various diseases [6] and, more recently [7], it was shown that 7,8-dihydroneopterin, similar to other reduced pterins, interferes with the redox status of cells, leading to apoptosis and activation of redox-sensitive transcription factors.

Patients with H_4-biopterin deficiency, due to H_4-biopterin metabolism-inherited disorders, suffer from severe monoamine neurotransmitter deficiency, and in most cases also from hyperphenylalaninaemia [8] and impaired NO production in the brain [9]. Defects of the PTS gene resulting in PTPS deficiency are the major cause of inherited H_4-biopterin deficiency [8]. This, however, is not related to the localized PTPS and, hence, to H_4-biopterin deficiency observed in human monocytes/macrophages. In the present study, we found that the degree of cell-type-specific exon 3 skipping in the PTPS pre-mRNA leading to a premature stop codon is crucial for this pteridine metabolism peculiarity in humans.

EXPERIMENTAL

Culture techniques

THP-1 myelomonocytoma cells (TIB-202), HL-60 acute promyelocytic leukaemia cells (CCL-240), Jurkat clone E6-1

Abbreviations used: CHX, cycloheximide; CSF, colony-stimulating factor; H_4-biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; HUVEC, human umbilical-vein endothelial cells; IFN-γ, interferon-γ; LPS, lipopolysaccharide; ORF, open reading frame; PBMC, peripheral blood mononuclear cells; PTPS, 6-pyruvoyl tetrahydropterin synthase; TNF-α, tumour necrosis factor-α.

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T-cell leukaemia cells (TIB-152) and HuT 78 cells (TIB-161) were obtained from A.T.C.C. (Rockville, MD, U.S.A.) and cultured according to manufacturer's instructions. MOLT-4 T-cell leukaemia cells (clone 8) were a gift from G. Kraus (Department of Microbiology and Immunology, University of Miami, FL, U.S.A.). HL-60 cells were differentiated into monocytes after 6 days treatment with 100 nM calcitriol or into granulocytes after 6 days treatment with 1 μM vitamin A. Primary monocytes were purified from the donated blood using Lymphoprep (Nycomed, Technoclone GmbH, Vienna, Austria) and subsequent adherence to plastic with washing after 2 h. Adherent cells were harvested after 24 h of culture [4]. For determination of PTPS activity, CD14+ cells were selected from peripheral blood mononuclear cells (PBMC) using Dynabeads (Dynal A/S, Oslo, Norway) and used for enzyme assays without prior cultivation. Primary macrophages were obtained from peripheral blood-derived monocytes (purified by combined Ficoll/Percoll centrifugation; Amersham Biosciences, Vienna, Austria) adhered for 7 days as described previously [4]. Dendritic cells were differentiated by granulocyte/monocyte colony-stimulating factor (CSF) and interleukin-4 from CD14+ cells isolated from human peripheral blood as described previously [10]. T-lymphocytes were obtained from PBMC by rosetting with neuraminidase-treated sheep red blood cells and subsequent treatment with 100 units/ml interleukin-2 (Sigma). CD34+ haematopoietic progenitor cells were purified from human umbilical-cord blood mononuclear cells using Dynabeads M-450 CD34 and DETACHaBEAD CD34 (Dynal). Alternatively, CD34+ cells from healthy donors (five donors) after 6 days treatment with granulocyte CSF were isolated from leukapheresis material using the CliniMACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD34-specific antibody was QBEND/10 (Miltenyi Biotec). Purity of the cell preparation (> 90 %) was assessed by FACS analysis using an FITC-labelled HPCA-2 antibody from Pharmingen (Becton Dickinson, Palo Alto, CA, U.S.A.). Dermal fibroblasts were grown from skin biopsies of healthy persons (five individuals) and cultured as described previously [4]. Passages 2–15 were used for experiments. Human umbilical-vein endothelial cells (HUVEC) were isolated and cultured as described previously [11]. The isolation of various primary cell types from human material was approved by the Ethics Committee of the Universities of Jena and Innsbruck. For comparison, RAW264.7 mouse macrophages were tested (A.T.C.C. TIB-71). Culture medium and foetal calf serum with low endotoxin quality was obtained from Biochrom (Berlin, Germany) or BioWhittaker (HUVEC; Apen, Germany). In some experiments, cells were treated with human recombinant interferon-γ (IFN-γ; generously provided by F.E. Rentschler, Biotechnologie GmbH, Laupheim, Germany) alone or in combination with human recombinant tumour necrosis factor-α (TNF-α; Sigma) or LPS from *Escherichia coli* O55:B5 (L9023; Sigma), with 25–100 μg/ml cycloheximide (CHX; Sigma) or with 5 μg/ml actinomycin D (Sigma).

Pteridine and nitrite determination and GTP cyclohydrolase I and PTPS enzyme assays

Intracellular pteridines, GTP cyclohydrolase I and PTPS activities were determined as described previously [11,12]. Briefly, biopterin derivatives were determined after iodine oxidation at acidic or alkaline pH, and subsequent fluorescence detection after separation via HPLC. Pteridine levels of cells were expressed in pmol/mg of protein. Nitrite in supernatants was determined using the Griess method. To determine GTP cyclohydrolase I and PTPS activities, cytosolic protein fractions from cell lysates were used. For GTP cyclohydrolase I, these were incubated with GTP, and the amount of 7,8-dihydroneopterin triphosphate was quantified as neopterin after iodine oxidation at acidic pH and cleavage of the triphosphate by phosphatase. For PTPS, protein fractions were incubated with 7,8-dihydroneopterin triphosphate in the presence of NADPH, Mg^{2+} and an excess of recombinant mouse sepiapterin reductase (the expression plasmid was kindly provided by I. Ziegler, GSF Research Center, Munich, Germany) to convert the reaction product 6-pyruvoyl-H_4-pterin into H_4-biopterin, which was then determined after iodine oxidation at acidic pH as the fluorescent biopterin by using HPLC. The PTPS substrate 7,8-dihydroneopterin triphosphate was prepared freshly using GTP cyclohydrolase I (an *E. coli* GTP cyclohydrolase I expression plasmid; kindly provided by A. Bacher, Technical University, Munich, Germany). Enzyme activities are given as pmol of neopterin $\cdot$ (mg of protein)$^{-1}$ $\cdot$ min^{-1} or pmol of biopterin $\cdot$ (mg of protein)$^{-1}$ $\cdot$ min^{-1} respectively. Protein determination was done as described by Bradford [12a] using the Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Munich, Germany) with BSA as a standard. Neopterin levels in culture supernatants were determined by ELISA (ELItest Screening, Brahms Diagnostica GmbH, Berlin, Germany).

RNA preparation, cDNA probe, Northern-blot analysis, library screening, conventional PCR and sequencing

Total RNA was isolated using the TRIzol® reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. A 647 bp *Bam*HI–*Eco*RI PTPS cDNA fragment containing the open reading frame (ORF) and flanking sequences, which was amplified initially from isolated human PTPS cDNA [13] using primers PTPS11/14 [14] and cloned into pUC18, as well as a 578 bp probe for human GTP cyclohydrolase I [15], were used as probes. For Northern-blot analysis, total RNA (20 μg) was resolved in 1 % (w/v) agarose/6 % (v/v) formaldehyde gels, blotted on to Duralon-UV nylon membranes (Stratagene, La Jolla, CA, U.S.A.) by means of vacuum and cross-linked by UV irradiation (Stratalinker; Stratagene). Blots were hybridized overnight with 10^6 c.p.m./ml [^{32}P]dCTP probe at 65 °C. After washing, blots were exposed either to Bio-Max MS (Kodak, Rochester, NY, U.S.A.) autoradiography films at −80 °C with intensifying screens or to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and then scanned using a Storm 849 scanner (Amersham Biosciences). For a control, blots were also hybridized with a 1.3 kb human glyceraldehyde-3-phosphate dehydrogenase probe. The PTPS probe was also used for screening a cDNA library generated from THP-1 cells treated for 7 h with IFN-γ [15]. PTPS mRNA variants were amplified from cDNA, reverse-transcribed (Superscript II RNase H$^-$ reverse transcriptase; Invitrogen) from total RNA using *Thermus aquaticus* DNA polymerase (Sigma) and TaqStart antibody (ClonTech Laboratories, Palo Alto, CA, U.S.A.), thermowell tubes (Corning, Corning, NY, U.S.A.) and a PerkinElmer GeneAmp PCR system 2400 or 9600 (Applied Biosystems, Vienna, Austria). After a 2 min denaturation step at 94 °C, PTPS was amplified with 30 cycles of 30 s at 94 °C, 1 min at 57 °C and 1 min at 72 °C, with an extra cycle of 7 min at 72 °C at the end. The primer sequences used were as follows: ptpsex2, 5′-CAATCCAAATGGCCATGGGC-3′; ptpsex3, 5′-TGGTGACAGTACATGGAGAG-3′; ptpsex 6, 5′-TGGGCTTTGTGCAATGCTAAC-3′ (human PTPS); muptpsex2, 5′-CAACAATCCGAATGGCCAG-3′; muptpsex3, 5′-TGGTGACAGTCCATGGAGAG-3′; and muptpsex6, 5′-GTGCCTGTCTACAATAGCAAC-3′ (mouse PTPS for RAW264.7 cells). Primer synthesis and sequencing of PCR products were performed by Microsynth (Windisch, Switzerland).

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PCR products were separated by electrophoresis over 2 % agarose (15 cm × 20 cm gel columns) for 16–20 h at 50 V. Control PCRs without cDNA performed in parallel did not produce any detectable signal. For sequence verification, PCR products were cloned into the Topo TA cloning vector (Invitrogen). Sequence analysis was performed using the Wisconsin Analysis Package version 9.1 from the Genetics Computer Group (Madison, WI, U.S.A.).

Real-time PCR

Abundance of PTPS mRNA variants in different human cell types was quantified using real-time PCR (Abi Prism 7700 Sequence Detector; Applied Biosystems). A human PTPS probe (5′-6-carboxyfluorescein and 3′-6-carboxy-tetramethyl-rhodamine-labelled) and primers, selected with the Primer Express software (Applied Biosystems) and synthesized by Microsynth, had the following sequences. For probe: 5′-TTCATAACCATTCCCGTAGCAGGGTCAA-3′; for primers: hptsex2, 5′-TTGGGAAATGCAACAATCCAA-3′; hptsex3, 5′-AAGTTGTGGTGACAGTACATGGAGA-3′; and hptsex4, 5′-CCATATATTTTTTGAGATCAGCCAGA-3′. Random primed cDNA species were prepared (Superscript II; Invitrogen) from 500 ng of total RNA from different cell types and amplified using the Brilliant Quantitative PCR Core kit (Stratagene) and Microamp plasticware from Applied Biosystems. For a control, a probe for 18 S rRNA was applied to cDNAs diluted to 1:1000. Plasmid DNA (750 fg/reaction) encoding PTPS cDNA was used to ensure that both exon-2- and exon-3-specific primers and probes bound with the same effectivity.

Transduction of THP-1 cells with full-length PTPS mRNA

A 650 bp fragment containing the human PTPS cDNA was used to generate the retroviral vector construct HSY32 [16]. As a control, the BAG vector expressing *E. coli* β-galactosidase was used [16]. These vectors were used to produce retroviral particles and to infect wild-type THP-1 cells as described previously [16]. The resulting cell lines, transduced stably either with human full-length PTPS or with β-galactosidase, were termed THP-1/HSY and THP-1/BAG respectively.

Western blotting

Cell extracts were prepared by repeated freeze–thaw cycles. Total protein (100 μg) was separated by SDS/PAGE (15 % gel), blotted on to a nitrocellulose membrane and subjected to standard Western-blot analysis. A rabbit antiserum to human PTPS (F3878) was used at a dilution of 1:25 000. For detection, a goat anti-rabbit IgG alkaline phosphatase conjugate was used.

RESULTS

PTPS mRNA levels are abundant in THP-1 cells

In the initial experiments, we tested THP-1 myelomonocytoma cells for their ability to express PTPS mRNA and enzyme activity in comparison with dermal fibroblasts of a healthy donor. To stimulate pteridine biosynthesis via induction of GTP cyclohydrolase I, cells were treated with cytokines (IFN-γ and TNF-α). The results summarized in Figures 1(A) and 1(B) show that PTPS mRNA is abundant in THP-1 cells, whereas PTPS activity is hardly detectable in this cell type. Accordingly, H_4-biopterin levels in cytokine-treated THP-1 (1.2 ± 0.4 pmol/mg) remained two orders of magnitude below those of cytokine-treated fibroblasts (187 ± 45 pmol/mg). On the other hand, no neopterin derivatives were detected in cytokine-treated fibroblasts, whereas THP-1 cells accumulated 313 ± 27 pmol/mg neopterin derivatives (all values are means ± S.D. for three experiments). As shown in Figure 1(B), GTP cyclohydrolase I activity of fibroblasts treated with IFN-γ and TNF-α remained lower than the PTPS activity, with the ratio being approx. 1:2.2. Interestingly, an approx. 2-fold increase in PTPS activity was observed in cytokine-treated fibroblasts (Figure 1B), yet PTPS mRNA levels appeared to be unaffected by this treatment (Figure 1A). In contrast with fibroblasts, GTP cyclohydrolase I activity of cytokine-treated THP-1 cells largely exceeded PTPS activity, the ratio being approx. 1:0.007 for cells treated with IFN-γ and TNF-α (Figure 1B). The very low PTPS activities of THP-1 cells cannot be explained by transcriptional down-regulation of PTPS expression.

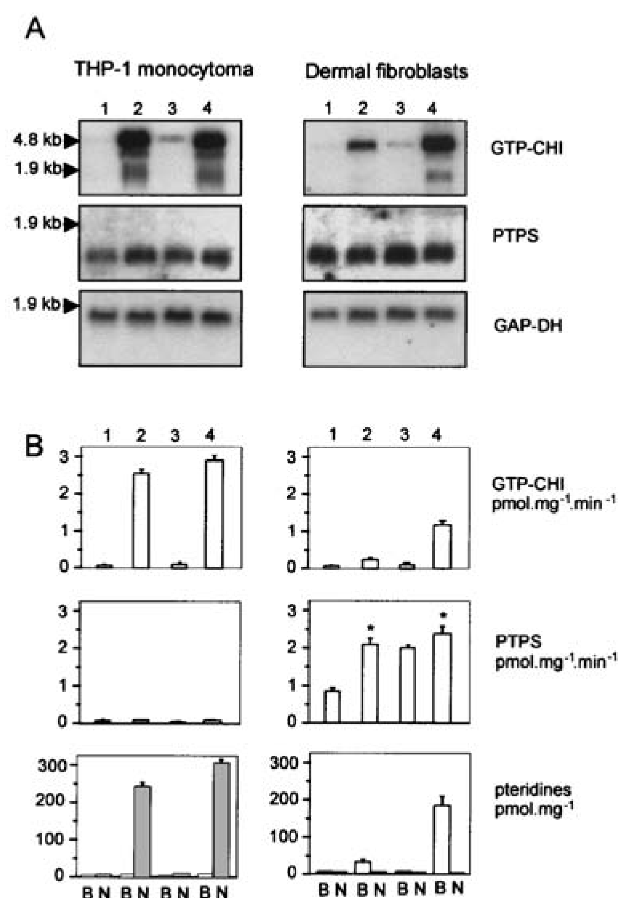

Figure 1 Pteridine biosynthesis in THP-1 monocytoma cells compared with dermal fibroblasts

(**A**) mRNA expression of GTP cyclohydrolase I (GTP-CHI) and PTPS. GAP-DH, glyceraldehyde 3-phosphate dehydrogenase. (**B**) Enzymic activities of GTP cyclohydrolase I and PTPS as well as intracellular levels of biopterin (B) and neopterin (N). 1, Untreated controls; 2, 1250 units/ml IFN-γ; 3, 100 units/ml TNF-α; and 4, IFN-γ and TNF-α. Incubation time periods were 7 h (for RNA isolation) and 24 h (for enzyme activity and pteridine levels) respectively. Means ± S.D. for triplicate determinations from one of five similar experiments are shown. According to Student's *t* test, *$P < 0.002$.

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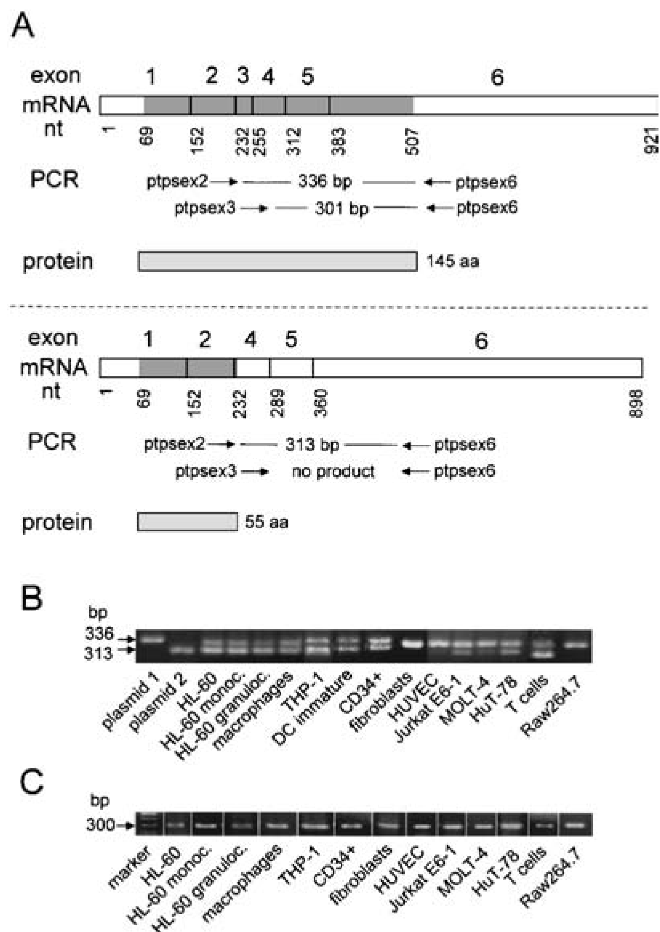

Figure 2 PTPS mRNA transcripts

(**A**) Schematic representation of PTPS mRNA containing exon 3 (upper panel) and lacking exon 3 (lower panel) and the resulting proteins. The gene structure was taken from reference [33], GenBank® accession no. L76259. The ORF is shown in grey. 1–6, exons and nt, nucleotide positions of the exons and the ORF. Sequences of PCR primers, ptpsex2, ptpsex3 and ptpsex6, shown as arrows, are described in the Experimental section. (**B**) PCR products were obtained in different cell types with primer pair ptps2/ptps6 and separated overnight on large agarose gels. CD34+ cells were from cord blood. DC, dendritic cells. (**C**) PCR products were obtained with primer pair ptpsex3/ptpsex6 in different cell types indicating that full-length PTPS mRNA was detectable in several of the tested cells.

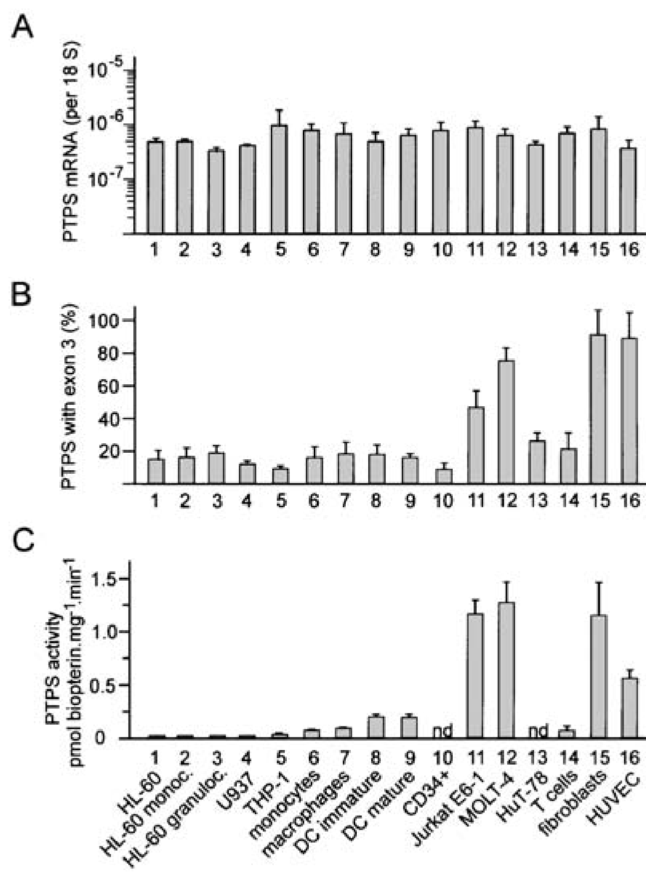

Figure 3 PTPS expression in different cell types

Quantification of total PTPS mRNA (**A**) and the percentage of exon-3-containing PTPS mRNA (**B**), both determined by Taqman analysis, as well as PTPS enzyme activities (**C**) of different cell types are shown. Results are shown as means ± S.D. for 3–5 experiments. nd, not determined. Number of donors (primary cells): 4 monocytes, 5 macrophages, 3 dendritic cells (DC), 4 T-cells, 5 CD34+ [mobilized by granulocyte CSF treatment; similar results were obtained with CD34+ cells from umbilical vein blood of three donors (results not shown)], 5 fibroblasts and 4 HUVEC. Data shown for cell lines are the means ± S.D. for 3–5 experiments. Nd, not determined.

Blood-derived cell types and related cell lines contain PTPS mRNA with and without exon 3

Screening a cDNA library of THP-1 cells revealed eight clones that encoded the entire ORF and were identical with the liver PTPS cDNA sequence [13], as well as three clones lacking the 23 bp exon 3. This leads to a premature stop codon encoding for 55-amino-acid PTPS protein instead of the full-length functional 145-amino-acid protein (Figure 2A, lower panel). PCR performed using the primer combination ptpsex2/ptpsex6 (Figure 2A) with cDNA from different cell types showed that several human blood-derived cell types and related cell lines contained two signals corresponding to the 336 bp fragment of PTPS containing exon 3 as well as to the 313 bp fragment of PTPS lacking exon 3 (Figure 2B). In contrast, exon-3-lacking PTPS cDNA was not detected by this conventional PCR approach in dermal fibroblasts or in mouse macrophage cell line RAW264.7, and only a weak signal was seen in HUVEC. On the other hand, several cell types including THP-1 cells and primary macrophages also contained clearly detectable full-length PTPS mRNA, as confirmed by PCRs performed with primer pair ptpsex3/ptpsex6 (Figure 2C).

Quantification of PTPS mRNA species reveals correlation of the amount of exon-3-containing mRNA with PTPS activity

Using real-time PCR, we then quantified PTPS mRNA levels, the percentage of full-length, i.e. exon-3-containing PTPS mRNA as well as PTPS enzyme activities of different human cell types. As shown in Figure 3(A), total levels of PTPS mRNA are within the same order of magnitude in several cell types tested. Values ranged between $(2.26 \pm 0.34) \times 10^{-7}$ PTPS mRNA per 18 S RNA for HL-60-derived granulocytes (means ± S.D. for three experiments) and $(9.41 \pm 2.61) \times 10^{-7}$ for THP-1 cells (means ± S.D. for five experiments). Values for fibroblasts from five different donors (± S.D.) were $(8.15 \pm 5.54) \times 10^{-7}$. Quantification of exon-3-containing PTPS mRNA (Figure 3B), however, revealed that monocytes and related cell lines, macrophages, dendritic cells, CD34+ progenitor cells and primary T-cells express only a low percentage (9–22 %) of their PTPS mRNA

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with exon 3 (Figure 3B). PTPS activities observed in primary T-cells (0.07 ± 0.04 pmol·mg^{-1}·min^{-1}; means ± S.D. for four donors) were in the same range as those in monocytes, i.e. CD14+ cells isolated from PBMC (two donors; 0.09 and 0.036 pmol·mg^{-1}·min^{-1} respectively), macrophages (0.12 ± 0.05 pmol·mg^{-1}·min^{-1}; means ± S.D. for four donors) and dendritic cells (0.20 ± 0.02 and 0.19 ± 0.03 pmol·mg^{-1}·min^{-1} for mature and immature dendritic cells respectively; means ± S.D. for three donors) (Figure 3C). In contrast, fibroblasts, HUVEC and also the T-cell leukaemia cell lines, Jurkat E6-1 and MOLT-4, expressed approx. three times more of exon-3-containing PTPS than primary T-cells. Accordingly, these cell types also had relatively high PTPS activities [1.16 ± 0.13 and 1.27 ± 0.19 pmol·mg^{-1}·min^{-1} respectively versus 0.07 ± 0.04 pmol·mg^{-1}·min^{-1} for primary T-cells (four donors); Figure 3C]. The correlation between exon-3-containing mRNA levels and PTPS activity was highly significant [Spearman rank correlation coefficient of 0.817 and $P = 0.007$ for percentage of exon-3-containing PTPS mRNA versus PTPS activity, and correlation coefficient of 0.767 and $P = 0.016$ for exon-3-containing PTPS mRNA levels versus PTPS activity, calculated using Statistical Package for the Social Sciences from SPSS (Chicago, IL, U.S.A.)].

Comparison of the stability of exon-3-containing and - lacking PTPS mRNA in THP-1 cells and fibroblasts

Using actinomycin D, we assessed the stability of PTPS mRNA transcripts in THP-1 cells and in fibroblasts. As shown in Figure 4(A), there was no significant difference in the half-life of PTPS mRNA in the two cell types. Also, the percentage of exon-3-containing PTPS mRNA within a given cell type remained unchanged by the inhibition of transcription (results not shown). We then examined the effect of stabilizing mRNA in the two cell types by inhibiting translation–elongation using CHX. As shown in Figures 4(B) and 4(C), stabilization of PTPS mRNA transcripts was observed in both cell types after 4 h, albeit it was less pronounced in fibroblasts (Figure 4C) when compared with THP-1 cells (Figure 4B). After 24 h, a 3–4-fold increase in mRNA levels was observed in both cell types. The ratio of exon-3-containing to exon-3-lacking PTPS mRNA transcripts, however, was not altered by CHX treatment in either cell type.

Overexpression of exon-3-containing PTPS mRNA in THP-1 cells yields intact PTPS protein at levels comparable with fibroblasts

To verify that THP-1 cells are capable of expressing intact PTPS enzyme provided that sufficient exon-3-containing PTPS mRNA transcript is present, THP-1 cells were transduced with a retroviral vector containing the full-length PTPS cDNA. The resulting cell line was termed as THP-1/HSY. As a control, the same vector containing β-galactosidase cDNA was used, yielding the cell line THP-1/BAG. PTPS mRNA expression, the amount of exon-3-containing PTPS mRNA, as well as the low PTPS activity and low capacity to form H_4-biopterin were identical in THP-1/BAG cells and THP-1 wild-type cells (results not shown; for THP-1 wild-type cells, refer to Figures 1 and 3). Furthermore, the extent of GTP cyclohydrolase I induction by cytokines was not altered in the transduced cell lines THP-1/BAG and THP-1/HSY as compared with wild-type THP-1 cells (results not shown). In comparison with THP-1/BAG control cells, THP-1/HSY cells expressed similar amounts of the 0.65 kb PTPS mRNA signal and no alteration of the PTPS mRNA signal was observed in response to cytokine treatment (Figure 5A, left panel). In addition, two

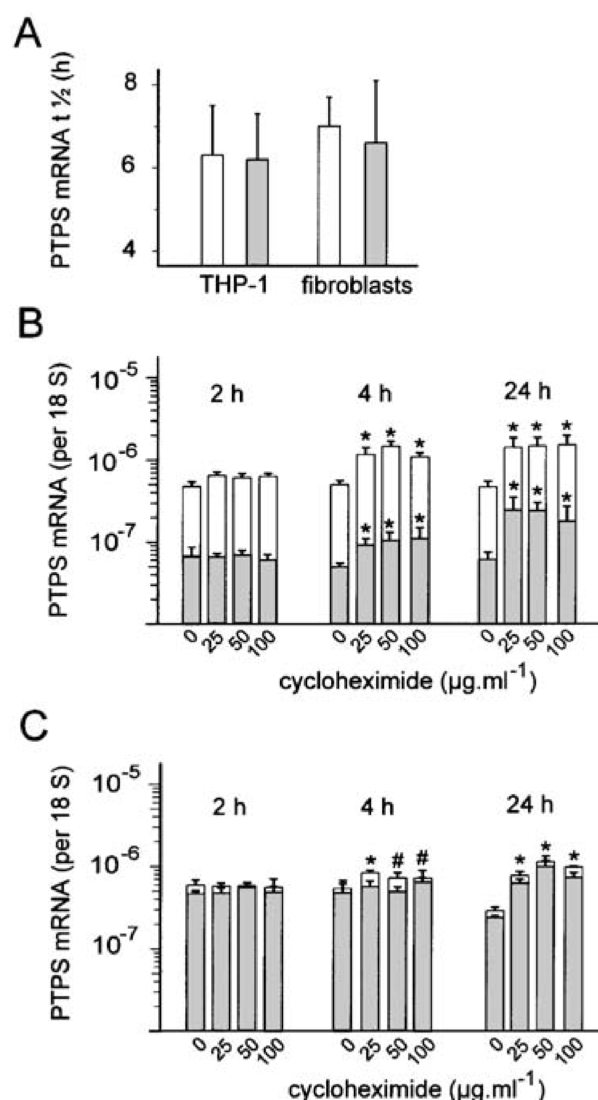

Figure 4 PTPS mRNA half-life and mRNA stabilization by inhibition of translation with CHX

(**A**) PTPS mRNA half-life in THP-1 cells and in fibrolasts. Stabilization of mRNA by inhibition of translation with CHX is shown for THP-1 cells (**B**) and fibroblasts (**C**). mRNA was quantified by Taqman analysis. The amount of exon-3-containing PTPS mRNA (grey bars) versus total PTPS mRNA (open bars) is shown. One of two similar experiments (means ± S.D.) with duplicate RNA preparations and triplicate Taqman analysis is shown. Statistical significance according to Student's t test, $^{\#}P < 0.05$, $^{*}P < 0.001$.

larger bands of approx. 1.3 and 4.8 kb were detected with the PTPS probe. The two bands stem from retroviral transcripts. Since there is no transcription stop 3′ to the PTPS cDNA, the 4.8 kb band quite probably corresponds to a full-length transcript containing PTPS-neo-ori-polyadenylated mRNA, whereas the 1.3 kb band fits with an mRNA harbouring a transcriptional stop downstream of the PTPS ORF.

Quantification of exon-3-containing PTPS mRNA by Taqman analysis (Figure 5A, right panel) showed that THP-1/HSY cells contained approx. 20-fold more of total PTPS mRNA when compared with THP-1/BAG cells and confirmed that 82 ± 12 % (means ± S.D. of triplicate determinations from two independent experiments) of this PTPS mRNA contained exon 3, whereas only 8 ± 0.8 % of PTPS mRNA of THP-1/BAG cells contained exon 3

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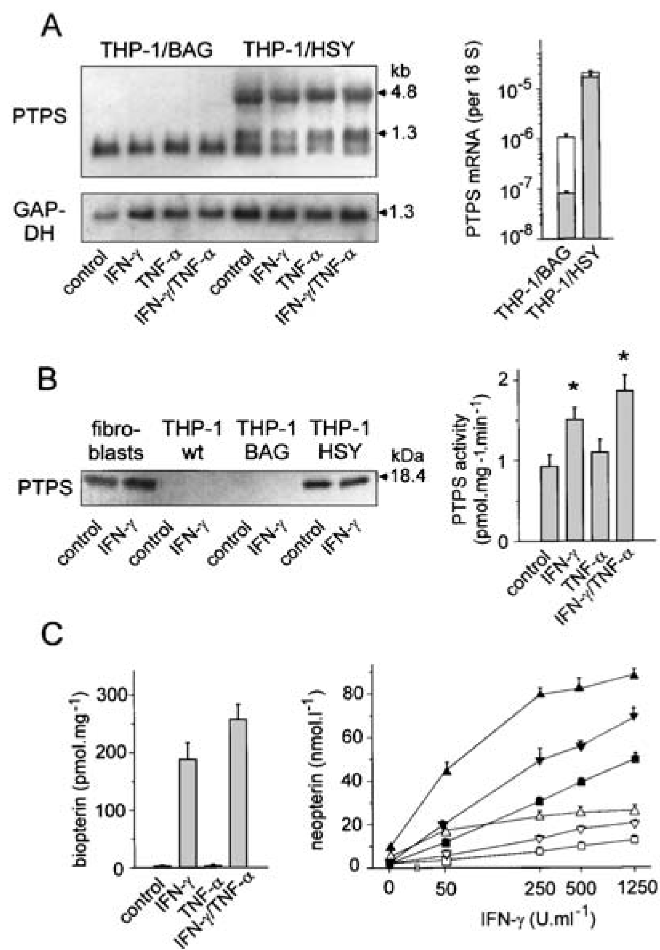

Figure 5 PTPS expression in THP-1/HSY cells (transduced with full-length PTPS cDNA) compared with THP-1 controls (THP-1/BAG) or fibroblasts

(**A**) Left, PTPS mRNA expression (Northern-blot analysis) on cytokine treatment (for 7 h; 1250 units/ml IFN-γ and 100 units/ml TNF-α); right, quantification of total (open bars) and exon-3-containing (grey bars) PTPS mRNA in THP-1/HSY compared with THP-1/BAG. (**B**) Left, Western-blot analysis of cell extracts (100 μg/lane) from fibroblasts and THP-1 cells (either untreated or treated with 1250 units/ml IFN-γ for 24 h); right, PTPS activity of THP-1/HSY (means $\pm$ S.D. for four experiments with triplicate incubations; Student's *t* test, $^*P < 0.001$; cytokine treatment for 24 h). (**C**) Left, total biopterin levels found in THP-1/HSY cells in response to cytokines after 24 h; right, neopterin in supernatants of THP-1/HSY (open symbols) compared with THP-1/BAG (filled symbols) treated with various doses of IFN-γ alone (□, ■), with IFN-γ plus 100 units/ml TNF-α (▽, ▼), and with IFN-γ plus TNF-α and 1 μg/ml *E. coli* LPS (△, ▲). The means $\pm$ S.D. of neopterin levels of 8 wells for one of three similar experiments are shown.

(Figure 5A, right panel). In Western-blot analyses, PTPS protein was detectable clearly in THP-1/HSY cells but not in THP-1/BAG or wild-type cells. Its expression level was comparable with fibroblasts and was not influenced by IFN-γ (Figure 5B, left panel). PTPS enzyme activity of THP-1/HSY cells (Figure 5B, right panel) was comparable with that found in fibroblasts (Figure 1B). Similar to fibroblasts (Figure 1), treatment of THP-1/HSY cells with IFN-γ alone or in combination with TNF-α increased PTPS activities approx. 1.5–2-fold (Figure 5B, right panel). TNF-α alone had no such effect in THP-1/HSY cells (Figure 5B), whereas it stimulated PTPS activity of fibroblasts (Figure 1B). As expected from the PTPS activity, THP-1/HSY cells were also capable of forming biopterin after the induction of GTP cyclohydrolase I by cytokines (Figure 5C, left panel) to a similar extent as fibroblasts (Figure 1B). Approximately 90 % of biopterin occurred as redox-active H_4 derivative (results not shown). Furthermore, the capacity of THP-1/HSY cells to release neopterin into the supernatant in response to IFN-γ, IFN-γ and TNF-α or at maximal GTP cyclohydrolase I induction with IFN-γ/TNF-α and LPS was decreased to approx. 25 % of that observed in THP-1/BAG cells (Figure 5C, right panel) or THP-1 wild-type cells (results not shown). Despite the induction of H_4-biopterin biosynthesis, THP-1/HSY cells were not able to induce NO synthase, as determined by measuring nitrite in supernatants, on various combined cytokine stimuli (results not shown).

DISCUSSION

The purpose of the present study was to investigate the molecular background of the localized PTPS deficiency found in human monocytes of healthy individuals, which leads to impaired H_4-biopterin biosynthesis but increased neopterin formation in this cell type when pteridine biosynthesis is induced by cytokines or LPS. Human monocytes express PTPS mRNA levels comparable with dermal fibroblasts or HUVEC, two cell types with high PTPS activity. In contrast with fibroblasts, however, in human monocytes only a small proportion of this mRNA contains the 23 bp exon 3 and encodes the full-length active PTPS protein. According to our findings, this pattern is not restricted to monocytes/macrophages but occurs also in CD34+ progenitor cells, in primary T lymphocytes, in dendritic cells as well as in myeloid cell lines. In contrast with primary T lymphocytes, T-cell lines, Jurkat E6-1 cells and MOLT-4 cells deviate from this pattern. They express a higher percentage of exon-3-containing PTPS mRNA and also have higher PTPS activities (Figure 3). This is consistent with previous work showing that MOLT-4 cells contain approx. ten times more biopterin than primary T-cells or other T-cell lines [5,17–19].

The fact that biopterin, albeit at very low levels, was detectable in T lymphocytes and at higher levels in T-cell leukaemia cell lines, e.g. MOLT-4, but remained undetectable in primary macrophages, the promyelocytic cell line HL-60 and the histiocytoma cell line U937 [5] suggested a principal difference in pteridine biosynthesis of macrophages and T-cells. However, we did show several years ago that primary human macrophages express PTPS activity and synthesize H_4-biopterin, which was possible due to increased sensitivity of biopterin detection as well as the use of highly concentrated cell extracts, obtained by lysing the cells in small volumes when still adherent [4]. In the present study, we found that the levels of exon-3-containing and total PTPS mRNA as well as the PTPS activity present in monocytes and macrophages match those observed in dendritic cells and T lymphocytes as well as CD34+ progenitor cells, the common precursor of cells of the myeloid and lymphoid lineage. Thus pteridine biosynthesis is quite comparable in both macrophages (and related cell types) and T-cells, except for inducibility of GTP cyclohydrolase I, which is increased up to 50-fold by IFN-γ in monocytes/macrophages, whereas in T-cells this enzyme activity remains comparatively low even after allogenic stimulation or treatment with phytohaemagglutinin [21]. Moreover, the peculiar pattern of increased exon 3 skipping in PTPS mRNA appears to be conserved in haematopoiesis and remains unaffected by differentiation into various cell types.

The hypothesis that skipping of exon 3 is a major cause of the decreased ability of certain cell types to express functional PTPS is substantiated further by transduction experiments with intact PTPS mRNA, which showed that THP-1, a cell type with very low endogenous PTPS activity, is capable of expressing high PTPS activities once the complete mRNA is introduced into the

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cells. Although cytokines did not alter expression of PTPS at the mRNA or protein level (see Figure 5), PTPS activity was increased approx. 1.5–2-fold by IFN-γ in THP-1/HSY cells and by IFN-γ as well as TNF-α in fibroblasts (see Figures 1 and 5). It remains to be investigated whether altered phosphorylation [26] and/or other post-translational mechanisms are responsible for this effect. Previous investigations with HUVEC also suggested an up-regulation of PTPS mRNA in response to cytokines, determined by conventional PCR [27]. From our work with fibroblasts and THP-1 cells, there is no experimental evidence for transcriptional regulation of PTPS expression by cytokines. Nevertheless, the possibility of cell-type-specific differences remains overt. Furthermore, PTPS mRNA was induced 3–4-fold in adrenal glands of reserpine-treated rats [28], indicating that PTPS is also regulated at the transcriptional level under certain conditions.

The molecular basis of the observed cell-type-specific degree of exon 3 skipping is unclear at present. It is, however, not caused by differential mRNA decay since half-lives of exon-3-containing and -lacking PTPS mRNA were comparable in THP-1 cells and fibroblasts. In addition, exon-3-lacking PTPS mRNA is obviously not a target for nonsense-mediated mRNA decay, a powerful mechanism to destroy mRNA species that contain premature stop codons [22–24], since inhibition of translation caused the expected stabilization of PTPS mRNA [22,23], but caused no shift in the ratio of the two mRNA species in either of the cell types investigated (see Figure 4). It remains to be seen whether skipping of exon 3 in certain cell types is caused by its short length alone or whether regulated splice site selection plays a role herein, as reported previously for a c-*src* exon that is transcribed only in neuronal cells but is skipped in non-neuronal cells [25]. Genomic organization as well as the nucleotide sequence of the PTS gene of mice [29] is highly similar to humans, but no exon 3 skipping is observed in mouse macrophages, which express much higher levels of PTPS activities than their human counterparts [4,20]. This may argue for a regulated mechanism rather than a simple inefficiency in the splicing machinery of human monocytes and related cell types.

In summary, we have been able to demonstrate that skipping of exon 3 in PTPS mRNA, which causes a premature stop of translation, is prominent in a number of blood-derived human cell types that have low PTPS activity, but not in human fibroblasts or endothelial cells that express much higher PTPS activities. Since supply of complete PTPS cDNA rendered THP-1 cells capable of producing abundant H_4-biopterin, skipping of exon 3 appears to be a major cause of impaired PTPS activity of certain cell types. Considering that macrophages, dendritic cells and T lymphocytes are highly professional cells, it is intriguing that their H_4-biopterin biosynthetic capacities are impaired compared with human fibroblasts or endothelial cells, or compared with mouse macrophages. Understanding these cell-type- and species-specific differences in gene expression with regard to pteridine synthesis may contribute to defining the background of differential expression of related pathways, such as formation of NO. The well-known difficulties in inducing NO production in human macrophages under controlled conditions *in vitro* [30–32], however, seem to be caused by ways not yet understood of regulation of NO synthase expression rather than by restricted H_4-biopterin supply due to low PTPS activity.

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