GTP cyclohydrolase I mRNA: novel splice variants in the slime mould *Physarum polycephalum* and in human monocytes (THP-1) indicate conservation of mRNA processing

Georg GOLDERER*, Ernst R. WERNER*, Christine HEUFLE†, Wolfgang STROHMAIER‡, Peter GRÖBNER* and Gabriele WERNER-FELMAYER*¹

*Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria, †Department of Dermatology, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria, and ‡Ludwig Boltzmann Institute of Experimental and Clinical Traumatology, Donauerschingerstrasse 13, A-1200 Vienna, Austria

GTP cyclohydrolase I (EC 3.5.4.16) is the first enzyme in the biosynthesis of tetrahydrobiopterin [(6R)-5,6,7,8-tetrahydro-L-biopterin, H₄-biopterin] in mammals and of folic acid in bacteria. Here we have characterized the GTP cyclohydrolase I gene structure and two mRNA species from *Physarum polycephalum*, an acellular slime mould that synthesizes H₄-biopterin and metabolites of the folic acid biosynthetic pathway. Its GTP cyclohydrolase I gene consists of seven exons, and the two GTP cyclohydrolase I cDNA species isolated from *Physarum* encode for proteins with 228 (25.7 kDa) and 195 (22.1 kDa) amino acids. Furthermore, we identified two previously undescribed mRNA species in interferon- γ -treated human myelomonocytoma cells (THP-1) in addition to the cDNA coding for the fully functional 250-residue (27.9 kDa) protein, which is identical with that in human pheochromocytoma cells. One of the new splice variants codes for a 233-residue (25.7 kDa) protein, whereas the other codes for the full-length protein but is alternatively spliced within the 3'-untranslated region. In heterologous expression, the shorter proteins of *Physarum* as well as of THP-1 cells identified here are degraded by proteolysis. Accordingly, only the 27.9 kDa protein was detectable in Western

blots from THP-1 cell extracts. Quantification of GTP cyclohydrolase I mRNA species in different human cell types with and without cytokine treatment showed that in addition to the correct mRNA the two splice variants isolated here, as well as the two splice variants known from human liver, are strongly induced by cytokines in cell types with inducible GTP cyclohydrolase I (THP-1, dermal fibroblasts), but not in cell types with constitutive GTP cyclohydrolase I expression (SK-N-SH, Hep-G2). As in human liver, splicing of the new mRNA variant found in THP-1 cells occurs at the boundary of exons 5 and 6. Strikingly, the 195-residue protein from *Physarum* is alternatively spliced at a homologous position, i.e. at the boundary of exons 6 and 7. Thus alternative splicing of GTP cyclohydrolase I at this position occurs in two species highly distant from each other in terms of evolution. It remains to be seen whether variant proteins encoded by alternatively spliced GTP cyclohydrolase I mRNA transcripts do occur *in vivo* and whether they participate in regulation of enzyme activity.

Key words: exon–intron structure, splicing, tetrahydrobiopterin.

INTRODUCTION

GTP cyclohydrolase I (EC 3.5.4.16) catalyses the conversion of GTP into 7,8-dihydroneopterin triphosphate, the precursor for several biologically active pteridine compounds (reviewed in [1,2]). These comprise enzyme cofactors such as (6R)-5,6,7,8-tetrahydro-L-biopterin (H₄-biopterin) that are essential for aromatic amino acid hydroxylases, ether lipid oxidase and nitric oxide synthases (reviewed in [3,4]) and 7,8-dihydrofolate, whose derivatives catalyse one-carbon transfer reactions. Furthermore, pteridines derived from this first reaction serve as eye pigments in *Drosophila melanogaster* [5] and might be involved in photo-reception in fungi and plants (reviewed in [6]). Additional biological roles of H₄-biopterin are a still unidentified effect in cell proliferation and differentiation [7,8], the regulation of melanin biosynthesis [9], and the stimulation of neurotransmitter release [10].

For higher eukaryotes, H₄-biopterin is the predominant pteridine formed, whereas folic acid, which is produced by bacteria, fungi and plants, is a vitamin [1,2]. In contrast, *Escherichia coli* does not form H₄-biopterin [11], and for the protozoan *Crithidia fasciculata* biopterin is an essential growth factor [1]. Cyanobacteria instead produce biopterin glucoside, which serves as a protective agent against UV-A radiation [12]. The acellular slime mould *Physarum polycephalum* seems to occupy an intermediate position because it produces H₄-biopterin as well as 6-hydroxymethyl-7,8-dihydropterin, a metabolite of folate biosynthesis produced by 7,8-dihydroneopterin aldolase [13]. The co-existence of the two pathways was also observed in *Neurospora crassa*, *Phycomyces blakesleanus* and *Euglena gracilis* [6].

In mammals, the biosynthesis of H₄-biopterin *de novo* is regulated by GTP cyclohydrolase I activity, which is influenced by certain hormones and cytokines (reviewed in [1,4]). The first

Abbreviations used: H₄-biopterin, (6R)-5,6,7,8-tetrahydro-L-biopterin; IFN- γ , interferon γ ; IPTG, isopropyl β -D-thiogalactoside; LPS, lipopolysaccharide; TNF- α , tumour necrosis factor α .

¹ To whom correspondence should be addressed (e-mail gabriele.werner-felmayer@uibk.ac.at).

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DNA sequence available was isolated from *E. coli* [14]. Subsequently, cDNA sequences were identified from various species, including rat [15], human [16,17], mouse [18] and the cellular slime mould *Dictyostelium discoideum* [19]. Genomic structures were characterized for *D. melanogaster* [5], human and mouse [20,21], *Dictyostelium discoideum* [19] and *Saccharomyces cerevisiae* [22]. The mammalian gene is organized in six exons containing introns of several kilobases [20] and the *Drosophila* gene in five exons [5], whereas the *Saccharomyces* gene consists only of one exon [22] and the *Dictyostelium* gene of two exons separated by a 101 bp intron [19].

Recent work analysed 5812 bp of the 5'-flanking region of rat GTP cyclohydrolase I and identified basal and cAMP response elements [23]. So far, however, it is not clear how interferon γ (IFN- γ) and other cytokines regulate the activity of GTP cyclohydrolase I in mammalian cells. Although the 5'-flanking region of the mouse and human GTP cyclohydrolase I gene contains a number of transcription-factor-binding motifs [20,21], no element mediating the effect of IFN- γ could be identified within 2.6 kb upstream of the transcription start site [21]. Another phenomenon that is still not understood is the occurrence of multiple mRNA forms of human liver GTP cyclohydrolase I [16].

Here we report on the cloning and characterization of previously undescribed GTP cyclohydrolase I mRNA variants from a cDNA library from IFN- γ -treated human myelomonocytoma cells (THP-1) as well as from the primitive eukaryote *P. polycephalum*. Although biogenetically distant, mRNA variants of both species occur owing to splicing at an equivalent position, i.e. at the exon 5/exon 6 boundary of the human GTP cyclohydrolase I gene corresponding to the exon 6/exon 7 boundary in *Physarum*.

EXPERIMENTAL

Culture techniques

Cultivation of *P. polycephalum* (strain M₃b, a Wis 1 isolate) was done as described [13]. In brief, microplasmodia were grown in submersed shake-culture in presence of 0.013% (w/v) haemoglobin and macroplasmodia were obtained by coalescence of exponentially growing microplasmodia. The growth temperature was 25 °C. Mitosis (telophase) was determined in ethanol-fixed smears under phase contrast.

For spherulation, microplasmodia grown for 24 h were seeded into a nutrient-free salts solution of pH 3.8 and shaken at 25 °C in the dark for a further 24–36 h until encystment into spherules was completed [24].

THP-1 human myelomonocytoma cells, originally obtained from the American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.), were grown in RPMI 1640 (Biocrom, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biocrom) and 2 mM L-glutamine. For the induction of GTP cyclohydrolase I, cells were seeded at a density of 10⁶/ml and treated with IFN- γ [Gammaferon, human recombinant, specific activity 2 × 10⁷ i.u./mg of protein, generously provided by F. E. Rentschler (Biotechnologie GmbH, Laupheim, Germany)], lipopolysaccharide (LPS) from *E. coli* O55:B5 (L9023; Sigma, Vienna, Austria), tumour necrosis factor α (TNF- α , human recombinant; Sigma) or combinations of IFN- γ plus LPS or IFN- γ plus TNF- α .

Human dermal fibroblasts from normal skin, the human liver cell line Hep G2 and the human neuroblastoma cell line SK-N-SH (SK-N-SH and Hep-G2 were obtained from A.T.C.C.) were cultured in Dulbecco's modified Eagle's medium (human dermal fibroblasts, Hep G2) or minimal essential medium with Earle's salts base (SK-N-SH). Both media (obtained from Biocrom)

were supplemented as outlined above for RPMI with a further addition of 1 mM pyruvic acid (Sigma) in the case of SK-N-SH.

cDNA libraries

Total RNA was isolated from IFN- γ -treated (250 i.u./ml, 7 h) THP-1 cells by the acid guanidinium isothiocyanate method [25]. For *Physarum*, the RNeasy Plant Mini kit from Qiagen (Hilden, Germany) was used. mRNA was isolated with the Oligotex mRNA kit from Qiagen. Oligo(dT)-primed cDNA was ligated into the Lambda ZAPIII vector (digested with *Eco*RI and dephosphorylated) with the Gigapack II packaging kit from Stratagene (La Jolla, CA, U.S.A.). The amplified libraries had a titre of 2.7 × 10⁹ plaque-forming units/ml for *Physarum* and 1.0 × 10⁹ plaque-forming units/ml for THP-1.

cDNA probes and library screening

By using degenerate primers 3 (sense) and 8 (anti-sense) used for homology cloning of GTP cyclohydrolase I from various species [26], a 282 bp product was generated by PCR with reverse-transcribed (SuperScript RNase H⁻ reverse transcriptase; Life Technologies, Vienna, Austria) poly(A)⁺ RNA from *Physarum* S-phase macroplasmodia. PCR conditions were 30 cycles of 30 s at 94 °C, 1 min at 55 °C, 1 min at 72 °C, with an initial denaturation step at 94 °C for 2 min. For IFN- γ -treated THP-1 cells, a 578 bp product was generated by PCR with 5'-AGCGAGGAGGATAACGAGCTG-3' as the sense primer and 5'-CCTGATGAGTGTGAGGAAGTCTG-3' as the anti-sense primer. These primers are directed to consensus sequences from rat [15], human [16] and mouse [18] liver and to *E. coli* [14]. PCR conditions were as detailed above. Having verified that these PCR products were specific for GTP cyclohydrolase I, they were used for screening the cDNA libraries of *Physarum* and THP-1 cells. Nucleotide sequencing of PCR products and cDNA clones as well as primer synthesis were performed by a custom service (Microsynth, Balgach, Switzerland).

Exon-intron organization of *Physarum* GTP cyclohydrolase I

Exon/intron boundaries for *Physarum* GTP cyclohydrolase I were determined by PCR with primers constructed to putative boundaries as deduced from GTP cyclohydrolase I from man [20] and *Drosophila* [5]. Genomic DNA was purified from *Physarum* microplasmodia with the DNeasy plant mini kit from Qiagen. DNA was sheared and analysed with the following primer combinations (ph means *Physarum*; numbers refer to exons from the human gene; f and r refer to forward and reverse, respectively; the expected size of the cDNA is given in parentheses): ph1f 5'-GTGGACGAAGCTTCCGAGGAGATG-3' and ph2r 5'-CTACAACCATCTCATTGTGGTCTC-3' (315 bp); ph2f 5'-GGACCACAATGAGATGGTTGTAGTG-3' and ph3r 5'-CTCAGCCCCAACACTTTTCCATTG-3' (126 bp); ph3f 5'-CAAATGGAAAAGTGTGTTGGGGCTGAG-3' and ph4r 5'-GCAGCCGACGTGCAAAGATCTCTG-3' (65 bp); ph4f 5'-AGATCTTTGCACGTCGGCTGCAAG-3' and ph5r 5'-TTCTTCGAGAGCCAGGCAATCTG-3' (67 bp); ph5f 5'-CCTGGCTCTCGAAGAAGCAATTCG-3' and ph6r 5'-CCTCGTGTCTCGAGTCTGTTG-3' (155 bp). Further combinations tested were ph1f/ph3r (419 bp), ph2f/ph4r (166 bp), ph3f/ph5r (112 bp), and ph1f/ph6r (665 bp). In addition, primers close to the position of intron 1 from *D. melanogaster* [5] were used: ph1af 5'-CAGCTCCAGTCCATTGCTGACAGCAC-3' and ph1ar 5'-GTGCTGTGCAATGGACTGGAGCTG-3'. Tested combinations were ph1f/ph1ar

(150 bp) and ph1af/ph2r (196 bp). PCR conditions were varied between 30 and 40 cycles of 30 s at 94 °C, between 1 and 3 min at 58–62 °C (depending on primer combinations and expected size), 1 min at 72 °C, with a final step of 7 min at 72 °C. *Taq* polymerase was either of standard (Promega, Madison, WI, USA) or long-distance [Advantage 2 polymerase mix (Clontech, Palo Alto, CA, U.S.A.) and Expand Long Template PCR System (Roche Diagnostics, Vienna, Austria)] quality. PCR products were either sequenced directly after gel purification (PCR extraction kit; Qiagen) or they were cloned with the Topo TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced (Microsynth).

PCR analysis of GTP cyclohydrolase I mRNA variants in THP-1 cells

For the detection of various GTP cyclohydrolase I mRNA variants, a PCR analysis of cDNA was performed from either untreated THP-1 cells or THP-1 cells treated for 7 h with 1250 i.u./ml of human recombinant IFN- γ plus 250 i.u./ml of human recombinant TNF- α , a maximal stimulus for GTP cyclohydrolase I induction [4]. The following primer combinations were applied: F4/R5, F4/R6, F4/R7. For primer sequences see [27]. Primer F4 binds to exon 5, primer R5 is specific for GTP cyclohydrolase I type 3 mRNA [16], primer R6 for type 2 mRNA [16], R7 for type 1 mRNA [16], i.e. functional GTP cyclohydrolase I [28]. R7 also binds to type 4 and type 5 mRNA isolated in this work. An additional primer R8 binding to type 1 and type 4 mRNA was also applied in combination with F4. The sequence of R8 was 5'-AGAAGAAAGTAGAG-GGCTCAACCC-3'. PCR conditions were 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C [27].

Quantification of GTP cyclohydrolase I mRNA variants in THP-1 cells

Quantitative PCR analysis was performed with real-time PCR (Abi Prism 7700 sequence detector; Applied Biosystems, Vienna, Austria). Sequences for probes (FAM label) and primers (synthesized by Microsynth) specific for various GTP cyclohydrolase I mRNA variants were selected with the Primer Express software (Applied Biosystems): type 1 mRNA, 5'-CATTAGG-AGCTGAGCTTCATTCAG-3' (sense), 5'-CAGACAGACAA-TGCTACTGGCAGT-3' (anti-sense), 5'-CGATCGGCAACC-AACGCACACA-3' (probe); type 2 mRNA, 5'-GGTTGAAG-CAACGTCTGCAG-3' (sense), 5'-TCCACCGTCAGTTCATT-CTGTG-3' (anti-sense), 5'-CCATAGCTTCCACGCACCTGA-ACGA-3' (probe); type 3 mRNA, 5'-CGGCCTGCTGGAGT-CG-3' (sense), 5'-CTTCTAGTGACCATTTATGACGTTACT-3' (anti-sense), 5'-TTGAAGCAACGTAAGTCTGCATCTGC-CTT-3' (probe); type 4 mRNA, 5'-GGCAATATTAAGTGA-ACTGCTAATAGTG-3' (sense), 5'-CACCGTCAGTTCATTC-TGTGCT-3' (anti-sense), 5'-TGCAGAACCATAGCTTCCAC-GCACCT-3' (probe); type 5 mRNA, 5'-GCCCAAGAGTTCC-TGACTCTCA-3' (sense), 5'-GGTGCCTGGAAGCTATGGT-T-3' (anti-sense), 5'-AGCTGAGCTTCATTCAAGTGTGTGTC-TGCA-3' (probe). For PCR, the TaqMan PCR master mix from Applied Biosystems was used. Random primed cDNA species were prepared (Superscript II RNase H⁻ reverse transcriptase; Life Technologies) from total RNA isolated from THP-1 cells, human dermal fibroblasts, SK-N-SH cells and Hep G2 cells (for culture conditions see above) either untreated or treated for 7 h with 1250 i.u./ml IFN- γ in combination with 250 i.u./ml TNF- α .

Sequence analysis

Sequence analysis was performed with the Wisconsin Analysis Package version 9.1 from the Genetics Computer Group (GCG, Madison, WI, U.S.A.).

Northern analysis

RNA was isolated from *Physarum* macroplasmodia at different times during the cell cycle or from spherulating microplasmodia. Samples frozen in liquid nitrogen were homogenized with the RNeasy Plant Mini kit from Qiagen. RNA from THP-1 cells, either untreated control cells or cells treated with IFN- γ or LPS, was isolated by extraction with acid guanidinium isothiocyanate [25]. Total RNA (20 μ g) was resolved in 1 % (w/v) agarose/6 % (v/v) formaldehyde gels, blotted to Duralon-UV nylon membranes (Stratagene) by means of vacuum and cross-linked by UV irradiation (Stratalinker; Stratagene). Blots were hybridized overnight with 10⁶ c.p.m./ml of [³²P]dCTP-labelled probes (see above) at 65 °C in accordance with standard protocols and exposed to Bio-Max MS or X-OMAT autoradiography films (Kodak) at -80 °C with intensifying screens. To control for RNA levels applied to the gel, a 207 bp PCR fragment of the 19 S small subunit of *Physarum* rRNA (GenBank accession no. X13160, 1964 bp [29]) was used. For THP-1 cells, a cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was used, giving a signal at approx. 1.3 kb.

GTP cyclohydrolase I assay

GTP cyclohydrolase I activity was detected as detailed previously [13,30]. Samples from *Physarum* were homogenized with a hand-held dispenser (Ultra-Turrax T8; Ika Labortechnik, Staufen, Germany) and cell debris was removed by ultracentrifugation at 350 000 *g* for 10 min at 4 °C (TLA 120.2 fixed-angle rotor, Beckman tabletop ultracentrifuge). Samples from THP-1 cells were homogenized by three cycles of freeze-thawing in a small volume of distilled water (500 μ l per 10⁷ cells) and cleared by subsequent centrifugation at 10 000 *g* for 10 min at 4 °C. Eluates free of low-molecular-mass compounds were prepared by Sephadex G25 chromatography (NAP-5; Amersham Pharmacia Biotech, Vienna, Austria). GTP (2 mM) (Serva, Heidelberg, Germany) was used as a substrate. Incubations were performed at 25 °C for *Physarum* or 37 °C for THP-1 cells. GTP cyclohydrolase I generates 7,8-dihydroneopterin triphosphate; this was oxidized by iodine in HCl and cleaved by alkaline phosphatase to neopterin. Neopterin was detected by fluorescence after HPLC. The protein content of eluates was determined by the Bradford method [31] with the protein-dye reagent from Bio-Rad (Vienna, Austria) and BSA as a standard. GTP cyclohydrolase I activity is expressed as the amount of neopterin formed per min per mg of protein.

Recombinant expression of *Physarum* and human GTP cyclohydrolase I

Because the expression of *Physarum* GTP cyclohydrolase I in *E. coli* with the use of pET16b – which should yield native protein and pET32a, leading to N-terminal His-tag, S-tag and thioredoxin-tag fusion [both vectors from Novagen (Madison, WI, U.S.A.); detection of S-tag by S-tag peptide antibody conjugated to alkaline phosphatase (Novagen)] – failed, both the 732 bp cDNA clone (GenBank accession no. AF177165) encoding the type 1 228-residue protein and the 647 bp cDNA

clone (GenBank accession no. AF177164) encoding the type 2 195-residue protein were cloned into the pFASTBAC vector, transposed into baculovirus and transfected into *Spodoptera frugiperda* Sf9 cells using the Bac-to-Bac Baculovirus expression system from Gibco BRL (Life Technologies). GTP cyclohydrolase I activity was determined in homogenates from transiently transfected Sf9 cells.

cDNA clones from THP-1 cells [GenBank accession no. U66097 (2909 bp) and GenBank accession no. U66095 (1906 bp)] were expressed in *E. coli* BL21DE3 cells with the pET16b vector (Novagen). Because human GTP cyclohydrolase I contains seven arginine residues with AGG or AGA codons, two triplets being translated in *E. coli* by the rare tRNA^{Arg4}, a product of the *argU* gene, this gene was supplemented by co-transforming BL21DE3 cells with the helper plasmid pUBS520, which was kindly provided by Professor R. Mattes (University of Stuttgart, Stuttgart, Germany) [32]. The alternatively spliced 1897 cDNA was also cloned into pET32a (Novagen), leading to the formation of an N-terminal His-tagged fusion protein. However, this protein still had no enzyme activity but could be purified via the His-tag in accordance with the manufacturer's protocol. The expression of all pET plasmids in *E. coli* was induced by 0.5 mM isopropyl β -D-thiogalactoside (IPTG; Serva).

Purification of recombinant GTP cyclohydrolase I from THP-1, preparation of antisera and Western blotting

Functional native GTP cyclohydrolase I from THP-1 was purified with DEAE-cellulose, hydroxyapatite [both from Merck (Darmstadt, Germany)] and chromatofocusing columns (Amersham Pharmacia Biotech) as modified from [33]. Highly active fractions showing a major band at 30 kDa after SDS/PAGE [30,33] were used for the preparation of rabbit antisera by injecting 50 μ g of purified protein and subsequently two boost injections with 50 μ g of protein each (with incomplete Freund's adjuvant). Western blots were performed with standard procedures by using PVDF nylon membranes and the Supersignal detection reagent (Pierce, Rockford, IL, U.S.A.). Primary antisera were diluted 1:5000 in PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween 20. A monoclonal anti-(rabbit IgG)-horseradish peroxidase conjugate (Sigma A-1949), diluted 1:50000, was used for detection.

RESULTS AND DISCUSSION

We have shown previously that *P. polycephalum* has biosynthetic activities for the formation of H₄-biopterin and precursors of folic acid [13]; it is thus one of the few known species able to produce both pteridine cofactors [6]. The first and key enzyme of these pteridine biosynthetic pathways is GTP cyclohydrolase I. Having generated a cDNA probe for *Physarum* GTP cyclohydrolase I, we first checked for mRNA expression during the cell cycle and spherulation. As can be seen from Figure 1, GTP cyclohydrolase I mRNA is evenly expressed over the cell cycle and is effectively down-regulated within the first 6 h of spherulation. The size of *Physarum* GTP cyclohydrolase I was estimated to be approx. 1 kb. GTP cyclohydrolase I activities parallel GTP cyclohydrolase I mRNA levels (Figure 1). Spherulation, the equivalent *in vitro* for the sclerotization occurring in Nature, is achieved by switching environmental conditions from growth promotion to starvation, leading to the formation of an encysted resting stage, i.e. the diploid spherule or sclerotium respectively. In a recent study we found that GTP cyclohydrolase I is also down-regulated during sporulation, a more complex and finally irreversible differentiation process

leading to the formation of haploid spores. Furthermore, glucose, which promotes growth and keeps macroplasmidia from sporulation, strongly induced the expression of GTP cyclohydrolase I mRNA (G. Golderer, E. R. Werner, P. Gröbner and G. Werner-Felmayer, unpublished work). Although the molecular mechanisms regulating GTP cyclohydrolase I expression during growth or differentiation into resting stages remain to be clarified, it is conceivable that GTP cyclohydrolase I itself is involved in switching from the dormant stage to growth and vice versa.

A cDNA analysis of *Physarum* S-phase macroplasmidia identified two clones: a 732 bp clone encoding a 228-residue protein with a molecular mass of 25747 Da and an isoelectric point of 7.00 (type 1) and a 648 bp clone encoding for a 195-residue protein with an estimated molecular mass of 22134 Da and an isoelectric point of 5.92 (type 2). Among GTP cyclohydrolase I proteins from different species, the identity of the *Physarum* amino acid sequences is highest (65.75%) with that found for the cellular slime mould *Dictyostelium discoideum*. Several amino acids involved in catalysis and/or substrate binding are conserved in the 228-residue type 1 *Physarum* protein (Figure 2) in comparison with several other species [3,34]. The first 185 amino acids of the two types of *Physarum* GTP cyclohydrolase I are identical (results not shown).

A phylogenetic tree analysis of various eukaryotic and prokaryotic GTP cyclohydrolase I sequences by the PILEUP program places GTP cyclohydrolase I from *Physarum* and *Dictyostelium* on an individual branch that is equidistant from human GTP cyclohydrolase I and the sequences from yeast (results not shown). However, the genomic structure of GTP cyclohydrolase I varies considerably between these species: whereas the *Saccharomyces cerevisiae* gene consists of only one exon [22] and the *Dictyostelium discoideum* gene of two exons interrupted by a 101 bp intron [19], the exon-intron structure of *Physarum* resembles those found for *Drosophila* [5] and mammals [20]. As can be seen from Figure 2, *Physarum* GTP cyclohydrolase I is structured into seven exons. The six introns are 1830, 90, 650, 379, 180 and 807 bp long respectively, as determined by PCR analysis (see the Experimental section for details). *Physarum* introns 1, 3 and 6 are at positions homologous with those of *Drosophila* introns 1 (lacking in the human gene), 2 (corresponding to human intron 1) and 4 (corresponding to human intron 5). *Physarum* intron 4 is at a homologous position to the only *Dictyostelium* intron (Figure 2). *Physarum* introns 2 and 5 have no corresponding introns in the other species studied so far (for a summary of the exon-intron structures of various species see [19]).

Recent work characterized the exon-intron organization of *Physarum* by using the 14 complete and 5 partial coding sequences available from GenBank, which contained 64 introns [35]. Whereas consensus sequences at exon/intron boundaries of this small group of *Physarum* genes resemble those of other species, long introns never occupied the 5'-most position; a tendency for introns of longer than 200 bp to lie between longer exons but for short introns to be flanked by short exons has been observed [35]. For *Physarum* GTP cyclohydrolase I, these two latter features cannot be seen. On the contrary, the longest intron (intron 1) is positioned at the 5' end and there is no obvious correlation between the lengths of introns and exons. As in *Physarum*, and also in *Drosophila*, man and mouse, the first and 5'-most intron of the GTP cyclohydrolase I gene is much longer than the other introns [5,20].

Our results also shed some light on the evolutionary relationship of *Physarum* (acellular or plasmodial slime moulds, Myxomycota) and *Dictyostelium* (cellular slime moulds): whereas work based on the analysis of ribosomal RNA suggests that

Figure 1 GTP cyclohydrolase I expression during cell cycle and spherulation of *P. polycephalum*

Upper panels: GTP cyclohydrolase I (GTP-CHI) mRNA expression, with 19 S ribosomal RNA levels as a control, and enzyme activity were determined at various times during the cell cycle of *P. polycephalum* macroplasmidia. Mitosis 2 took place at zero time and mitosis 3 occurred 560 min afterwards, as determined microscopically. Northern blots were performed with 20 μ g of total RNA isolated from *Physarum*. GTP cyclohydrolase I activities (each point is the mean of triplicate incubations) were measured as defined in the Experimental section. For details of Northern blotting and enzyme detection see the Experimental section. One representative experiment of three is shown. Lower panels: GTP cyclohydrolase I (GTP-CHI) mRNA expression, with 19 S ribosomal RNA levels as a control, and enzyme activity were determined at various times during the spherulation of *Physarum* microplasmidia. For Northern blots 20 μ g of total RNA was applied. More details on methods are given in the Experimental section. One representative experiment of three is shown.

Figure 2 Protein sequence and exon structure of *P. polycephalum* GTP cyclohydrolase I

Upper panel: the protein sequence of functional type 1 GTP cyclohydrolase I from *P. polycephalum* is shown. Exon boundaries as determined by PCR (see the Experimental section for more details) are indicated by vertical lines. Amino acids marked in black are identical with those in eukaryotic GTP cyclohydrolase I (consensus of human type 1 GTP cyclohydrolase I, *D. melanogaster* (U01118), *Caenorhabditis elegans* (Z72509) and *Dictyostelium discoideum* (Z49706); amino acids marked in grey are similar to this consensus sequence. Arrows indicate amino acids involved in enzyme catalysis and/or substrate binding (reviewed in [3]), which are all conserved in *Physarum* GTP cyclohydrolase I. Lower panel: for comparison, a scheme of the exon organization of GTP cyclohydrolase I from *Dictyostelium discoideum* [19], *P. polycephalum*, *D. melanogaster* [5], and man [20] indicating the amino acids near the exon boundaries is shown.

these two species stem from early-branching lineages, trees based on actin, β -tubulin and elongation factor-1 α favour the view that *Physarum* and *Dictyostelium* are of monophyletic origin, i.e. that they belong to a single coherent group of organisms [36].

Figure 3 GTP cyclohydrolase I expression induced by IFN- γ in human THP-1 cells

Total RNA and cell homogenates were obtained from THP-1 cells at different times after the addition of 250 i.u./ml human IFN- γ (detailed in the Experimental section). For Northern blots (upper panel), 20 μ g of RNA was applied and probed with human GTP cyclohydrolase I and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. One representative Northern blot of three is shown. Enzyme activities are means $\pm$ S.D. for triplicate incubations of four independent experiments.

GTP cyclohydrolase I is a perfect example of this dichotomy. Whereas the homology of protein sequences from *Physarum* and *Dictyostelium* is highest in several other species (see above),

Figure 4 Splice variants of GTP cyclohydrolase I mRNA in THP-1 cells

Upper panel: a schematic representation of the five types of GTP cyclohydrolase I mRNA identified so far (types 2 and 3, see [16]; types 4 and 5, the present paper) indicates the following: (1) the position of the deduced proteins and their molecular mass; (2) the boundary of exons 5 (dotted part) and 6 at 779 bp; (3) the different positions of splicing within exon 6, which occurs at 1045, 1142, 1868, 2168 and 2909 bp of type 1 GTP cyclohydrolase I; (4) the positions of various primers (4–8) used for identifying different variants (expected sizes of PCR products specific for various mRNA types are summarized in the tabulation); (5) the positions of TaqMan probes (dotted hexagonal boxes above the bars) used for the quantification of several mRNA variants. Lower panel: total RNA was isolated from THP-1 cells, human dermal fibroblasts (HFB), neuroblastoma cells (SK-N-SH) and liver cells (Hep-G2) either untreated (hatched bars) or treated with 1250 i.u./ml IFN- γ in combination with 250 i.u./ml TNF- α for 7 h (open bars). Sequences of primers and probes for various GTP cyclohydrolase I mRNA variants are outlined in the Experimental section. Real-time PCR was performed and GTP cyclohydrolase I mRNA was quantified in relation to 18 S ribosomal RNA. Values are means $\pm$ S.D. for three determinations.

organization of this gene varies considerably and places *Physarum* much closer than *Dictyostelium* to higher eukaryotes.

In mammals, GTP cyclohydrolase I is constitutively expressed in liver and neuronal tissues. Besides being regulated by phosphorylation [37,38], GTP cyclohydrolase I transcription is induced by various stimuli, including IFN- γ and/or LPS (reviewed in [4,23]). This induction is also observed in cell types [4] and tissues [39] that otherwise express GTP cyclohydrolase I at only very low or undetectable levels. cDNA clones isolated for human GTP cyclohydrolase I all stem from sources constitutively expressing the enzyme, i.e. liver [16,28] or phaeochromocytoma cells [17].

A typical time course of GTP cyclohydrolase I expression in human myelomonocytoma (THP-1) cells treated with IFN- γ is shown in Figure 3. The size of GTP cyclohydrolase I mRNA was

estimated to be approx. 3 kb; this is slightly smaller than previously assumed [40], but corresponds well to the size of full-length GTP cyclohydrolase I cDNA from phaeochromocytoma cells, which is 2921 bp [17]. In contrast, and as in *Physarum*, GTP cyclohydrolase I mRNA from rodents is approx. 1 kb owing to a shorter 3'-untranslated region than that of human GTP cyclohydrolase I [15,18,20].

To clarify whether a hypothetical, distinct, cytokine-induced transcript exists, we searched a cDNA library from IFN- γ -treated human myelomonocytoma (THP-1) cells for GTP cyclohydrolase I. Two cDNA variants could be isolated, namely a 2909 bp clone having a sequence identical with that of the 2921 bp clone isolated from human phaeochromocytoma cells [17] and encoding the 250-residue (27.9 kDa) protein identified in human liver [16,20], and a 1906 bp clone encoding a 233-residue

(25.7 kDa) variant of GTP cyclohydrolase I that is as yet unidentified. Previously, two mRNA variants of GTP cyclohydrolase I encoding 213-residue (23.5 kDa) and 209-residue (23.2 kDa) proteins were isolated from human liver [16]. Alternative splicing of the mRNA encoding the 25.7 kDa protein isolated here occurs at a position identical with that found for type 2 and type 3 GTP cyclohydrolase I mRNA species, i.e. the boundary of exons 5 and 6 (Figure 4, upper panel). PCR analysis showed that, in addition to the cloned new mRNA variant termed type 4, there are also type 2 and type 3 mRNA variants from human liver [16] detectable in THP-1 cells (Figure 4, upper panel). In addition, an unexpected type 5 variant, giving a 317 bp PCR band, occurred with primer combination F4/R7. This PCR product turned out to be a partial sequence of the 250-residue protein-encoding sequence being spliced within the 3'-untranslated region of exon 6. Thus the boundary of exons 5 and 6 is a predominant but obviously not exclusive position for alternative splicing of GTP cyclohydrolase I mRNA.

With the use of real-time PCR, the abundances of GTP cyclohydrolase I mRNA variants of types 1 to 5 were assessed in different cell types, namely THP-1, human dermal fibroblasts, neuroblastoma cells (SK-N-SH) and hepatoma cells (Hep-G2). As can be seen from Figure 4 (lower panel), all five mRNA types were identified in the four cell lines. In THP-1 cells and in human dermal fibroblasts, the amounts of several GTP cyclohydrolase I mRNA transcripts were strongly induced by treatment with 1250 i.u./ml IFN- γ in combination with 250 i.u./ml TNF- α , a stimulus known to induce maximum levels of GTP cyclohydrolase I activity in various cell types [4]. In the two cell lines, SK-N-SH and HepG2, which constitutively express GTP cyclohydrolase I activity, no increase in mRNA transcripts, or only a much smaller one, was observed.

As shown previously, the proteins encoded by GTP cyclohydrolase I type 2 and type 3 mRNA species from human liver [16] have no catalytic activity [28]. In addition, the new variant type 4 isolated here had no catalytic activity when expressed in *E. coli* with the pET16b vector (0.011 ± 0.005 nmol/min per mg compared with 0.013 ± 0.005 nmol/min per mg for cells transformed with pET16b without insert, both in the presence of IPTG; values are means $\pm$ S.D. for three independent experiments). For comparison, homogenates from *E. coli* transformed with the type 1 GTP cyclohydrolase I clone isolated from THP-1 cells had 0.74 ± 0.11 nmol/min per mg (compared with 0.025 ± 0.009 nmol/min per mg in the absence of IPTG; values are means $\pm$ S.D. for five experiments). Purification of type 1 GTP cyclohydrolase I from *E. coli* homogenates by sequential chromatographic steps (see the Experimental section) yielded a specific activity of 49.2 ± 9.8 nmol/min per mg (mean $\pm$ S.D. for three purification runs). This activity is approx. one-sixth of the previously reported activity obtained for purified maltose-binding protein 2-GTP cyclohydrolase I fusion protein [28], but is similar to that observed for purified rat liver GTP cyclohydrolase I, which was 45 nmol/min per mg [33]. On SDS/PAGE, this purified recombinant human type 1 GTP cyclohydrolase I was visible as a major band of approx. 30 kDa (results not shown). Similar molecular masses were observed under these conditions for rat and human GTP cyclohydrolase I [28,33]. An antiserum prepared against purified type 1 GTP cyclohydrolase I stained a single 30 kDa band in homogenates from *E. coli* overexpressing human GTP cyclohydrolase I (Figure 5). Unlike type 2 and type 3 GTP cyclohydrolase I, which could be expressed as intact but inactive native or fusion proteins [28], the fusion of variant type 4 to an N-terminal His-tag did not permit the recovery of intact protein (expected molecular mass 42.8 kDa). Instead, multiple bands were observed that reacted

Figure 5 Western blots of recombinantly expressed human GTP cyclohydrolase I type 1 and type 2 and induction of GTP cyclohydrolase I protein in stimulated THP-1 cells

GTP cyclohydrolase I was detected with a rabbit antiserum generated against purified recombinant human type 1 GTP cyclohydrolase I diluted 1:5000 and an anti(rabbit IgG)–horseradish peroxidase conjugate diluted 1:50000 with the use of chemiluminescent detection (see the Experimental section). Lanes 1–3, recombinantly expressed GTP cyclohydrolase I. Lane 1, GTP cyclohydrolase I type 1 (2909 bp cDNA) was expressed in *E. coli* BL21DE3 by using the pET16b vector in the presence of the helper plasmid pUBS520 (see the Experimental section); 0.5 μ g of protein from a bacterial lysate was applied. Lane 2, GTP cyclohydrolase I type 2 (1906 bp) expressed in the same system; 2.5 μ g of protein from bacterial lysate. Lane 3, GTP cyclohydrolase I type 2 expressed as pET32a plasmid (fusion protein with N-terminal His-tag); 10 μ g of protein from bacterial lysate. Lanes 4–12, GTP cyclohydrolase I induced in THP-1 cells: untreated control (lane 4), 50 i.u./ml IFN- γ (lane 5), 250 i.u./ml IFN- γ (lane 6), 1250 i.u./ml IFN- γ (lane 7), 1 μ g/ml LPS (lane 8), LPS plus 50 i.u./ml IFN- γ (lane 9), LPS plus 250 i.u./ml IFN- γ (lane 10), LPS plus 1250 i.u./ml IFN- γ (lane 11), 2.5 μ g of protein from lysate of bacteria recombinantly expressing GTP cyclohydrolase I as a positive control (lane 12). The loading of cellular protein from THP-1 cells was 20 μ g in each lane.

with the specific antiserum against GTP cyclohydrolase I (Figure 5). No enzyme activity was observed (0.013 ± 0.002 nmol/min per mg compared with 0.010 ± 0.0008 nmol/min per mg for cells transformed with pET32a without insert, both in the presence of IPTG; values are means $\pm$ S.D. for three experiments). These observations suggest that the GTP cyclohydrolase I type 4 variant might be rapidly degraded by proteolysis. It remains to be determined whether this is related to heterologous recombinant expression or whether it also takes place in human cells. In homogenates from IFN- γ -treated THP-1 cells at least, a single band of approx. 30 kDa was observed but there was no indication of smaller or degraded GTP cyclohydrolase I bands (Figure 5).

Although type 1 *Physarum* GTP cyclohydrolase I shares 61.9% amino acid identity with human type 1 GTP cyclohydrolase I, it could not be functionally expressed in *E. coli* as native (vector pET16b) or His-tagged fusion (vector pET32a) protein. Again, degradation products of various sizes smaller than the expected 42.8 kDa fusion protein were detected by staining the S-tag part of the fusion protein (results not shown). However, expression in baculovirus-transfected insect cells confirmed that *Physarum* GTP cyclohydrolase I type 1 is catalytically active (2.64 ± 0.24 pmol/min per mg compared with 0.31 ± 0.04 pmol/min per mg for negative controls, i.e. cells transfected with vector without insert; values are means $\pm$ S.D. for three experiments). Type 2 *Physarum* GTP cyclohydrolase I was inactive in this expression system (0.28 ± 0.02 pmol/min per mg; mean $\pm$ S.D. for three experiments).

As can be seen from Figure 6, alternative splicing of the newly identified GTP cyclohydrolase I variants from THP-1 cells and from *Physarum* occurs at a homologous position, i.e. the boundaries of exons 5 and 6 and of exons 6 and 7 respectively. Splicing at this position also yields type 2 and type 3 GTP cyclohydrolase I from human liver (compare with Figure 2). Four out of 19 residues known to be involved in catalysis and/or substrate binding are lacking in all of the identified GTP cyclohydrolase I variants. This is caused either by a premature stop (type 3 from

Figure 6 C-termini of various human GTP cyclohydrolase I mRNA species in comparison with two types of *P. polycephalum* GTP cyclohydrolase I mRNA

The C-termini of several GTP cyclohydrolase I mRNA variants were aligned. Identical and similar amino acids are highlighted by black and grey backgrounds respectively. The stop of the amino acid chain is marked by an asterisk. The positions of amino acids involved in the function of GTP cyclohydrolase I (arrowheads) and the positions of regions important for the correct folding of the enzyme are indicated.

human liver) or by alternative splicing that results in alternative C-termini in type 2 from human liver, type 4 from THP-1 cells and type 2 from *Physarum*. Structural crystallography of *E. coli* GTP cyclohydrolase I showed that the protein is composed of two pentamers. Each subunit of these pentamers contains α -helices and β -sheets permitting the formation of a 20-stranded anti-parallel β -barrel with 10 equivalent active sites (reviewed in [3]). Several splice variants of GTP cyclohydrolase I lack the β -4 sheet and the α_6 -helix in addition to the four residues mentioned above, which might explain the lack of catalytic activity of these proteins.

Evidence obtained in this study, namely the observation of a single band in stimulated THP-1 cells and enhanced protease sensitivity of type 4 THP-1 GTP cyclohydrolase I and type 2 *Physarum* GTP cyclohydrolase I (Figure 5), suggests that the variant proteins encoded by the alternatively spliced mRNA species are rapidly degraded and are therefore by-products of erroneous splicing. However, this conclusion might be premature, especially if one considers that some of the alternatively spliced mRNA species, such as type 2 and/or type 3 GTP cyclohydrolase I, are quite abundant in comparison with type 1 mRNA (see Figure 4). At present the possibility cannot be excluded that the expression of such variant proteins occurs and might interfere with the formation of active decameric enzyme and might thus be a tool for regulation. Interestingly, a patient with hereditary progressive dystonia with marked diurnal fluctuation/dopa-responsive dystonia had a C $\rightarrow$ A transversion in GTP cyclohydrolase I. This led to a Thr¹⁸⁶ $\rightarrow$ Lys mutation and resulted in a splice variant lacking exon 5 and part of exon 6 [27]. Results obtained by quantifying GTP cyclohydrolase I mRNA types 1 to 3 from human liver and the patient-specific splice variant in correlation with GTP cyclohydrolase I activity supported the hypothesis that mutant subunits disturb the activity of normal subunits [27]. Further work, employing bicistronic recombinant expression or co-expression of plasmids containing different types of GTP cyclohydrolase I mRNA, will be needed to clarify the biological significance of these splice variants.

In summary, our work indicates that human GTP cyclohydrolase I is an extensively spliced gene with a preferential site for variation at the boundary of exons 5 and 6. The occurrence of two splice variants reported for human liver [16] as well as two new splice variants described here is not restricted to a certain cell type but seems to be a general phenomenon. The expression of several GTP cyclohydrolase I mRNA species identified so far is induced by cytokine treatment (Figure 4). As shown here, alternative splicing at this position is obviously not restricted to humans but can also be observed in a phylogenetically very distant organism, namely *P. polycephalum*, in which this position

corresponds to the boundary of exons 6 and 7. Analysis of the genomic structure of *Physarum* GTP cyclohydrolase I revealed organization into seven exons, with some of the boundaries being identical with those in the mammalian [20] and *Drosophila* [5] genes. Although the 3'-most boundary, i.e. the exon 5/6 boundary of mammals and the exon 6/7 boundary of *Physarum*, exists in *Drosophila* (Figure 2), no alternative splicing at this position has yet been reported for this organism; however, it cannot be excluded for methodological reasons [5]. Conversely, two alternatively spliced transcripts resulting from two different transcription start sites, of which one is located within intron 1, are observed in *Drosophila* [5]. At present the possibility cannot be excluded that *Physarum*, which contains an intron at a homologous position, also contains 5' splice variants analogous to the *Drosophila* transcripts. As discussed above, the biological significance of alternative splicing of GTP cyclohydrolase I mRNA observed in *Physarum* as well as in humans is not yet clear. Provided that variant proteins that were inactive when expressed recombinantly ([28], and the present study) are synthesized *in vivo*, these proteins might be introduced into decameric GTP cyclohydrolase I and thus could potentially interfere with enzyme activity.

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