Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1670 (2004) 56–68
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Nuclear localization of tetrahydrobiopterin biosynthetic enzymes

Lina Elzaouk[a,1], Stephanie Laufs[b,1], Dirk Heerklotz[a], Walter Leimbacher[a], Nenad Blau[a], Annette Résibois[c], Beat Thöny[a,*]

[a] *Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland*
[b] *German Cancer Research Center, E120, Heidelberg, Germany*
[c] *Laboratoire de Chimie Biologique et de la Nutrition, Faculté de Médecine, Université Libre de Bruxelles, Belgium*

Received 6 August 2003; received in revised form 1 October 2003; accepted 17 October 2003

Abstract

Biosynthesis of the tetrahydrobiopterin (BH_4) cofactor, essential for catecholamines and serotonin production and nitric oxide synthase (NOS) activity, requires the enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR). Upon studying the distribution of GTPCH and PTPS with polyclonal immune sera in cross sections of rat brain, prominent nuclear staining in many neurons was observed besides strong staining in peri-ventricular structures. Furthermore, localization studies in transgenic mice expressing a *Pts-LacZ* gene fusion containing the N-terminal 35 amino acids of PTPS revealed β-galactosidase in the nucleus of neurons. In contrast, PTPS-β-galactosidase was exclusively cytoplasmic in the convoluted kidney tubules but nuclear in other parts of the nephron, indicating again that nuclear targeting may occur only in specific cell categories. Furthermore, the N terminus of PTPS acts as a domain able to target the PTPS-β-galactosidase fusion protein to the nucleus. In transiently transfected COS-1 cells, which do not express GTPCH and PTPS endogenously, we found cytoplasmic and nuclear staining for GTPCH and PTPS. To further investigate nuclear localization of all three BH_4-biosynthetic enzymes, we expressed Flag-fusion proteins in transiently transfected COS-1 cells and analyzed the distribution by immunolocalization and sub-cellular fractionation using anti-Flag antibodies and enzymatic assays. Whereas 5–10% of total GTPCH and PTPS and ~1% of total SR were present in the nucleus, only GTPCH was confirmed to be an active enzyme in nuclear fractions. The in vitro studies together with the tissue staining corroborate specific nuclear localization of BH_4-biosynthetic proteins with yet unknown biological function.

Keywords: Tetrahydrobiopterin; Nuclear localization; Monoamine neurotransmitter; Aromatic amino acid monooxygenase; NOS

1. Introduction

The naturally occurring cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4) is essential for catalysis of diverse monooxygenases, including all three types of nitric oxide synthases (NOS) [1], the aromatic amino acid hydroxylases, phenylalanine-, tyrosine-, and tryptophan-hydroxylase [2], and the glyceryl-ether monooxygenase [3]. Besides various additional but biochemically less defined roles of BH_4, including growth proliferation, neuroprotection and neurotransmitter release, the cofactor is a limiting factor and thus controls enzyme activities via its availability. For instance, BH_4-cofactor insufficiency due to genetic defects leads to monoamine neurotransmitter depletion and reduced nitric oxide metabolites in cerebrospinal fluid, and the phenotypical spectrum of disease reaches from mild cofactor reduction to severe and potentially lethal deficiency [4,5].

Three biosynthetic, two recycling, and one regulatory protein are responsible for the cellular metabolism of the BH_4-cofactor. This includes the GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR) for biosynthesis, pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) for recycling, and the GTPCH feedback regulatory protein (GFRP) for regulation. Their genetic organization, protein structure and modification, and enzymatic function and regulation have been thoroughly studied [6]. However, very little is known so far about transport and availability of BH_4 and its pterin derivatives, which are probably major determinants controlling physiology but

* Corresponding author. Tel.: +41-1-266-7622; fax: +41-1-266-7169.
E-mail address: Beat.Thony@kispi.unzh.ch (B. Thöny).
[1] This author contributed equally to this work.

0304-4165/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2003.10.015

also pathophysiology and disease development (see, e.g. Ref. [7]).

We have been interested in several aspects of disease due to BH_4 deficiency, including diagnosis and differentiation of the various defects, mutations in genes encoding cofactor metabolizing enzymes, and disease treatment. In the course of analyzing and mapping the expression pattern of BH_4-biosynthesis enzymes in rats, we repeatedly observed a complex but apparently specific subcellular localization of GTPCH and PTPS, including a sometimes prominent and/or exclusive nuclear localization in various tissues or cells. Here we document the unprecedented nuclear localization with unknown function of GTPCH, PTPS, and SR in various mammalian tissues and cells.

2. Materials and methods

2.1. Immune sera against GTPCH and PTPS

Primary antiserum against GTPCH was raised in chicken against a maltose binding protein-mouse GTPCH fusion protein and used in the form of IgY purified from egg yolk by the dextran sulfate precipitation method [8] at dilutions of 1:7500 together with immunoperoxidase. Negative controls for anti-GTPCH as well as rat brain distribution of GTPCH obtained with this immune serum have been described elsewhere [9].

Primary antisera against PTPS were raised in rabbits against a maltose-binding protein-rat PTPS fusion protein ('SZ30') or against a maltose-binding protein-human PTPS fusion protein ('SZ28'). Both sera gave identical results and were used either native as 1:1000 or 1:2000 dilutions, or after affinity purification as 1:100 or 1:200 dilutions. Controls for immunolabeling were made on adjacent sections and included rabbit serum before immunization and diluted immune serum preincubated overnight with 10 μg of fusion protein, purified rat or human PTPS, or maltose-binding protein. The *Escherichia coli* maltose-binding protein does not occur in eukaryotic cells but preincubation of the immune sera with the *E. coli* protein was necessary to rule out any cross-reactivity of irrelevant eukaryotic proteins.

To compare the intracellular distribution of GTPCH and PTPS immunoreactivities with the distribution of a related purely cytoplasmic protein, adjacent brain sections were stained with the mouse monoclonal clone 2/40/15 against tyrosine hydroxylase (TH) (Boehringer Mannheim) as a 1:200 dilution.

2.2. Immunohistochemistry

Ten male Wistar rats, 6 to 8 weeks old, were anesthetized by intraperitoneal nembutal injection. They were then perfused through the ascending aorta with 0.1 M phosphate-buffered 4% formaldehyde freshly prepared from paraformaldehyde. The brain was removed, post-fixed for 4 h at 4 °C, rinsed in several changes of phosphate-buffered saline (PBS, 0.1 M; pH 7.4) and cryoprotected in 30% sucrose. Frozen 30-μm coronal sections were processed free-floating. After quenching of the endogenous peroxidases, the sections were incubated with the primary antibodies for 2 days at 4 °C. For anti-GTPCH immune serum, we used biotinylated anti-chicken gamma globulin (Vector) and avidin–biotin–peroxidase complex (Vector). Anti-PTPS immune serum was revealed by anti-rabbit gamma globulin (Nordic) followed by peroxidase–antiperoxidase complex (Dako). Anti-TH was revealed by anti-mouse gamma-globulin (Nordic) followed by peroxidase–antiperoxidase complex (Dako). The fixed peroxidase was visualized using diamonibenzidine in the presence of nickel-ammonium sulfate [10].

2.3. β-Galactosidase activity and X-gal staining

A transgenic mouse with the *Pts* allele (encoding PTPS) replaced by an in-frame insertion in exon 2 with the *lacZ* gene was used [11]. β-Galactosidase activity in mice tissues was determined using a commercially available kit (Promega) with the *o*-nitrophenyl-β-D-galactopyranoside substrate (ONPG). We defined 1 U of β-galactosidase activity as 1 OD at 420 nm per minute at pH 7.5 at 37 °C. We used newborn and adult mice heterozygous for the *Pts-LacZ* allele ($Pts^{+/-}$), and newborn mice homozygous for the *Pts-LacZ* allele ($Pts^{-/-}$). For X-gal staining, thick sections of brain and kidney were fixed for 2 h in 4% freshly prepared paraformaldehyde, rinsed three times in PBS 0.1 M and processed for staining in a solution containing 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$, 2 mM $MgCl_2$, 0.02% NP-40 (Igepal, Sigma) and 2 mg/ml X-gal at 37 °C for 24 h. After 2-h post-fixation in paraformaldehyde, the sections were rinsed in PBS, quickly dehydrated in acetone and toluol, and embedded in paraffin. Slices of 5-μm thin sections were very quickly rehydrated, counterstained with cresyl violet or haematoxylin for accurate identification of the labeled cell groups and intracellular compartiments, very quickly dehydrated in acetone, and mounted for microscopic examination.

2.4. Cell culturing and immunofluorescent staining

COS-1 monkey kidney cells (CRL 1650) and SK-N-BE human neuroblastoma cells (CRL 2271) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (cDMEM; Life Technologies, Inc.).

For immunofluorescent staining, cells were plated 48 h post transfection on sterile glass cover slips in cDMEM at 37 °C. After attachment, cells were washed three to

four times with DMEM, fixed in 3.7% formaldehyde for 15 min at 37 °C, washed twice with DMEM containing 5% horse serum, permeabilized in ice-cold methanol for 10 min, and washed again twice with DMEM containing 5% horse serum. The subsequent steps were performed in DMEM containing 5% horse serum. After incubation with the anti-Flag M2 monoclonal antibody (diluted 1:300; F3165, Sigma) for 60 min at 37 °C, cells were washed and incubated for 60 min with the Cy3 anti-mouse antibody (diluted 1:200; Sigma). Subsequently cells were washed with PBS and the cover slips were mounted on glass slides, embedded with Mowiol (Hoechst) for examination on a Zeiss fluorescent microscope equipped with the appropriate fluorescent filters.

For indirect immunofluorescence of transfected COS-1, cells were briefly washed in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and fixed on coverslips for 30 min with freshly prepared 3.7% paraformaldehyde (Calbiochem-Novabiochem) in PBS. After being washed in PBS, cells were permeabilized by a 15-min treatment with 0.5% Triton X-100 in PBS. After blocking for 30 min in PBS containing 1% bovine serum albumin (Sigma-Aldrich), cells were incubated for 3 h with polyclonal anti-FLAG antibodies (Sigma-Aldrich) diluted 1:200 in PBS containing 1% bovine serum albumin. After being washed twice with PBS and stained with fluorochrome-coupled goat anti-rabbit secondary antibodies, samples were mounted for microscopic inspection in mounting solution (100 mM Tris–HCl, pH 8.5, 24% glycerol, 9.6% mowiol 4-88 (Calbiochem-Novabiochem), 2.5% 1,4-diazabicyclo-(2.2.2)-octane). Confocal laser scan micrographs were captured using a TCS4D microscope (Leica Lasertechnik, Germany) and Imaris software (Bitplane, Switzerland). Captured images were resized and combined using Photoshop 6.0 software (Adobe Systems, USA).

2.5. Plasmid constructions and cell transfection

The pcDNA3 plasmid vector (Stratagene) was used to express human BH_4-biosynthetic enzymes containing at the N or C terminus the eight-amino-acid-spanning 'Flag' tag (amino acid sequence DYKDDDDK encoded by the synthetic sequence 5′-GACTACAAGGACGACGATGACAAG-3′). The constructs were generated by PCR using different human cDNAs as templates: GTPCH [12,13], PTPS [14], and SR [15] (the cDNA for GTPCH was kindly provided by M. Gütlich, the cDNA for SR by H. Ichinose). Table 1 lists all primer sequences used for insert generation by PCR. GTPCH was cloned as an *Eco*RI–*Xba*I fragment, whereas all PTPS and the SR fragments were cloned as *Bam*HI–*Xba*I inserts into pcDNA3. This resulted in plasmids pHGCH9 and pHSR6, expressing the N-terminal Flag-fusion proteins Flag-GTPCH and Flag-SR, respectively. For PTPS, an N- and C-terminal Flag-fusion construct was generated (pHSY45 and pHSY46), plus different mutant PTPS with an N-terminal Flag-tag and the following amino acid alterations or deletions: C10A (pHSY59), R16C (pHSY57), R25Q (pHSY60), K143A (pHSY67), Δ1–11 (pHSY62), Δ143–145 (pHSY68), Δ119–145 (pHSY69). Plasmid pHSY80 contains the wild-type PTPS with an N-terminal nuclear localization signal (NLS, amino acid sequence DPKKKRKV) followed by the Flag tag sequence. All constructs were confirmed by DNA sequence analysis.

Transient transfections of COS-1 (and SK-N-BE and HeLa) cells with the pcDNA3-derivatives were carried out by using a modified lipofectamine protocol (Life Technologies). Cells were grown to a confluency of up to 80–100%. Vector DNA (10 μg) was mixed with DMEM up to 1/10 of the final volume (for 10 cm plates 5 ml total volume), and 100 μl of the lipofectamine reagent was diluted with DMEM up to 1/10 of the final volume. The DNA- and the lipofectamine solutions were combined and mixed gently. After incubation for 30 min at room temperature, 4 ml of DMEM was added, and the mix was slowly dropped onto the plates containing the cells. After 6 h of incubation at 37 °C, the cells were washed with PBS and 10 ml of cDMEM was added. Forty-eight hours after transfection, the cells were either stained for immunodetection or harvested for protein analysis. Transient co-transfections with the pSVβgal plasmid, expressing the *E. coli* β-galactosidase from the SV40 promoter, were used to determine the transfection efficiency [16].

2.6. Cell fractionation, Western blot analysis, and enzymatic assays

For cell fractionation into cytoplasmatic and nuclear extracts, transiently transfected COS-1 cells were harvested by trypsinization, washed twice with PBS, and resuspended in lysis buffer containing 10 mM HEPES, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 5 mM DTT, 1% NP-40. After briefly vortexing and centrifugation at $1700 \times g$ for 5 min (Hettich centrifuge, rotor 5034), the supernatant was used as 'cytoplasmatic' fraction. In order to remove detergents and residual cytoplasmatic contents, the pellet fraction containing the nuclei was washed again with lysis buffer without NP-40 by briefly vortexing and centrifugation at $1700 \times g$ for 5 min. The nuclear pellet was resuspended for lysis in extraction buffer containing 25 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5% Triton X-100. After vortexing, the lysed nuclei were centrifuged in a SS34 rotor in a Sorvall RB-C5 centrifuge at 4 °C for 30 min at 16,000 rpm ($30{,}000 \times g$), and the supernatant was saved as the soluble 'nuclear' extract. To examine the extent of cross-contamination, both fractions were probed with the anti-SV40 large T-antigen monoclonal antibody (4 μg/ml; DP02, Calbiochem) for a nuclear marker protein and lactate dehydrogenase activity (LDH; Beckmann kit no. 442655) for a cytoplasmatic marker enzyme.

Table 1
PCR primers used to generate eukaryotic expression plasmids for GTPCH, PTPS, and SR Flag-fusion proteins

Construct plasmid[a]	Amino acid aberration	5′ primer[b]	3′ primer[b]
Flag-GTPCH pHGCH9	wild-type	GCH-203 5′-CG*GAATTC*GCCACCATG*GACTACAAGGACG ACGATGACAAG*GAGAAGGGCCCTGTGCGG	GCH-202 5′-GC*TCTAGA*TCAGCTCCTAATGAGAGTCAG
Flag-PTPS pHSY45	wild-type	PTPS-34 5′-CG*GGATCC*GCCACCATG*GACTACAAGGACGA CGATGACAAG*AGCACGGAAGGTGGTGGCCAC	PTPS-35 5′-GC*TCTAGA*CTATTCTCCTTTATAAAC
PTPS-Flag pHSY46	wild-type	PTPS-36 5′-CG*GGATCC*GCCACCATGAGCACGGAAGGT GGTGGC	PTPS-37 5′-GC*TCTAGA*CTA*CTTGTCATCGTCGTCCTTG TAGTC*TTCTCCTTTATAAACCAC
Flag-PTPS pHSY59	C10A	PTPS-34	PTPS-35
Flag-PTPS pHSY57	R16C	PTPS-34	PTPS-35
Flag-PTPS pHSY60	R25Q	PTPS-34	PTPS-35
Flag-PTPS pHSY67	K143A	PTPS-34	PTPS-45 5′-GC*TCTAGA*CTATTCTCCGGCAT
Flag-PTPS pHSY62	Δ1–11	PTPS-42 5′-CG*GGATCC*GCCACCATG*GACTACAAGGAC GACGATGACAAG*GCACAAGTGTCCCGCCGC	PTPS-35
Flag-PTPS pHSY68	Δ143–145	PTPS-34	PTPS-46 5′-GC*TCTAGA*CTAATAAACCACAATATTATT
Flag-PTPS pHSY69	Δ119–145	PTPS-34	PTPS-47 5′-GC*TCTAGA*CTACTGGAGGTTGTCCCAGATA
NLS-Flag-PTPS pHSY80	wilt-type	PTPS-61 5′-CG*GGATCC*GCCACCATG*GACCCAAAAAAGA AGAGAAAGGTA*GACTACAAGGACGACGATGAC	PTPS-59 5′-GATCAGCGAGCTCTAGCATTTAGGTGACAC
Flag-SR pHSR6	wild-type	SR-214 5′-CG*GGATCC*GCCACCATG*GACTACAAGGACG ACGATGACAAG*GAGGGCGGGCTGGGGCGT	SR-215 5′-GC*TCTAGA*TTATTTGTCATAGAAGTCCAC

[a] Except for plasmid pHSY46 (construct 'PTPS-Flag') all Flag sequences are N-terminally fused; NLS, nuclear localization signal (see text for details).
[b] Italics, restriction endonucleases recognition sites (*GGATCC* for *Bam*HI, *GAATTC* for *Eco*RI, and *TCTAGA* for *Xba*I); underlined, coding sequence for the eight-amino-acid Flag fusion (NLS for primer PTPS-61 is underlined and in italics).

For Western blot analysis, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [17]. Proteins were boiled in sample buffer (100 mM Tris–HCl, pH 6.8, 5% glycerin, 10% SDS, 10% β-mercaptoethanol, 0.04% bromophenol blue), separated on 12.5% acrylamide-SDS gel, and transferred to Pure Nitrocellulose Membrane (0.45 μm, BioRad 162-014) by standard electroblotting. Filters were first probed with an anti-Flag M2 monoclonal antibody (diluted 1:3000 or 1:4000; F3165, Sigma), and second with a goat anti-mouse IgG alkaline phosphatase conjugate human IgG (diluted 1:3000; BioRad). Immunostaining was carried out by the colorimetric method for alkaline phosphatase using the NBT/BCIP solution (Roche).

To identify the location and distribution of the enzymes GTPCH, PTPS, and SR, the fractions were monitored by immunoblotting with either the anti-FLAG M2 monoclonal antibody (F3165 from Sigma, diluted 1:4000), or the anti-human PTPS polyclonal antibody F3867 (diluted 1:20,000; Leimbacher and Thöny, unpublished).

Enzymatic assays for GTPCH, PTPS and SR have been described elsewhere [18–20]. Analysis of the converted metabolites neopterin, pterin, and biopterin was performed by reversed-phase HPLC separation and subsequent fluorescent detection and quantification as described [21,22]. For determination of protein concentration, the Bradford reagent and bovine plasma gamma globulin as standard were used (BioRad). One unit of enzymatic activity produces 1 μmol of product per minute (i.e. neopterin for GTPCH, and biopterin for PTPS and SR).

3. Results

3.1. Nuclear immunoreactivity of GTPCH and PTPS in rat brain

The distribution of GTPCH-immunoreactivity in rat brain was described in an earlier communication with the

same polyclonal antibody as was used here [9]. However, the intracellular distribution of GTPCH was not reported in detail. In some brain regions with a particularly strong GTPCH immunoreactivity, the whole cell body was labeled, with a nucleus similarly or more strongly labeled than the surrounding cytoplasm (Fig. 1a). In these neuron groups, a few weakly stained dendrites could generally be seen close to the cell body but no contrast was observed at a distance and no terminal field was visible. Axons were never visible and no positive nerve bundles were seen. However, most brain cell groups were only weakly stained by GTPCH immune serum, and in these cells, the nucleus was often not distinguishable from the positive pericaryon of the neuron [9]. Conversely tyrosine hydroxylase (Fig. 1b) was present in the whole cytoplasm including processes and terminal field but never observed within the nucleus.

Consistent with the GTPCH distribution, PTPS immunoreactivity was present in all the catecholaminergic (Fig. 1c) and serotoninergic (Fig. 1d) neuron groups. In addition, many cells were also labeled outside of these cell groups, mainly in the hypothalamus, the hippocampus, and the amygdala. Surprisingly, nuclear staining of PTPS was even more evident and prominent than GTPCH staining (Fig. 1). Few dendrites, no axons, and no terminal fields showed any contrast. Both anti-rat PTPS (SZ30) and anti-human PTPS (SZ28) affinity-purified immune sera were entirely inactivated by preincubation with rat and human PTPS or with the recombinant proteins (Fig. 1e). Preincubation of the antisera with maltose-binding protein did not modify the immunoreactivity observed using antiserum alone. No labeling was observed when pre-immune rabbit sera were used. In summary, strong nuclear and weak cytoplasmic staining for GTPCH and PTPS was observed in many neurons of

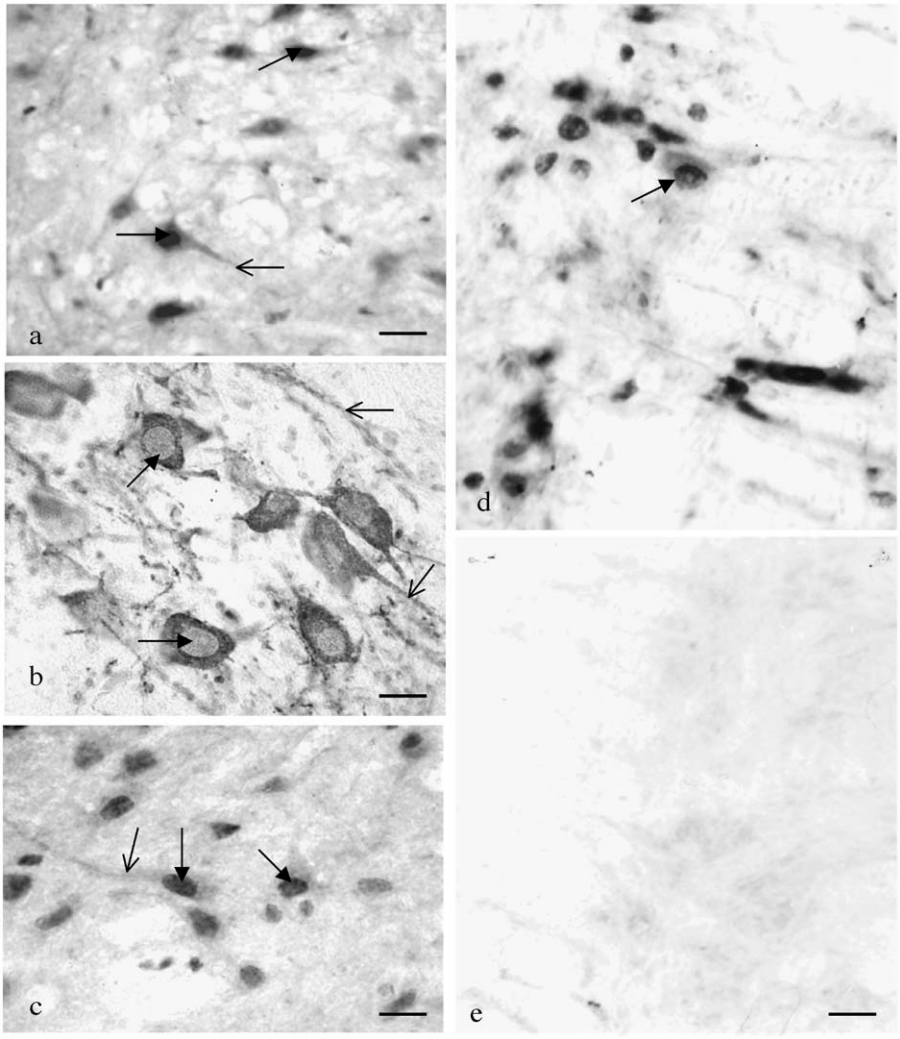

Fig. 1. Nuclear staining of GTPCH and PTPS in adult rat brain. Prominent nuclear distribution of GTPCH (a) and PTPS (c, d) immunoreactivities in rat brain as compared with prominent cytoplasmic distribution of TH (b). (a, b, c) Dopamine cells in group A10; (d, e) serotoninergic cells in the raphe obscurus; (e) section adjacent to d treated with inactivated immune serum. Nuclei are pointed by arrows and dendrites by open arrows. Scale bars: a, 27 μm; b, 15 μm; c, 14 μm; d, 30 μm; e, 30 μm.

the rat brain, which is in sharp contrast with TH intracellular distribution.

3.2. Nuclear localization of the PTPS-β-galactosidase fusion protein in PTS-transgenic mice

We generated a transgenic mouse with the endogenous *Pts* gene encoding the mouse PTPS replaced by an in-frame *Pts-LacZ* gene fusion. Whereas heterozygous $Pts^{+/-}$ mice exhibited normal development and behavior, homozygous $Pts^{-/-}$ knock-out mice developed in utero with no gross abnormalities but died within the first days after birth if not treated [11]. The *Pts-LacZ* gene fusion expressed a protein containing the N-terminal 35 amino acids for PTPS followed by the bacterial β-galactosidase. This fusion protein had enzymatic activity as determined by ONPG-assays in brain and liver (brain 1.4 mU/mg for $Pts^{+/-}$, and liver 2.2 mU/mg for $Pts^{+/-}$ or 3.5 mU/mg for $Pts^{-/-}$).

Upon analyzing cross sections of brain from newborn mice, we observed X-gal staining in either the nucleus or in the cytoplasm or both in heterozygous and knock-out animals, but no staining was observed in wild-type mice. Furthermore, there was no difference in intracellular distribution of a given cell type between heterozygous and knock-out newborn animals (not shown). In adult heterozygous mice, X-gal staining was observed in the nucleus of neurons located in prominent catecholaminergic cell groups such as substantia nigra (Fig. 2a) and locus coeruleus. Very many positive neuron nuclei were also observed in the ventral part of the hypothalamus, where they outdid the number of tyrosine hydroxylase-positive cells, and also in regions void of tyrosine hydroxylase cells such as in the hippocampus (Fig. 2b) and in the amygdala. In both heterozygous and knock-out newborn mice, many positive neurons were observed throughout the brain, particularly in the dorsal tegmentum differentiating field, in the hypothalamus, and in the ventral mesencephalon. Staining occurred prominently in the nucleus of the neurons even if they were still migrating and far from complete differentiation, but was both cytoplasmic and nuclear in the germinal periventricular layer (not shown).

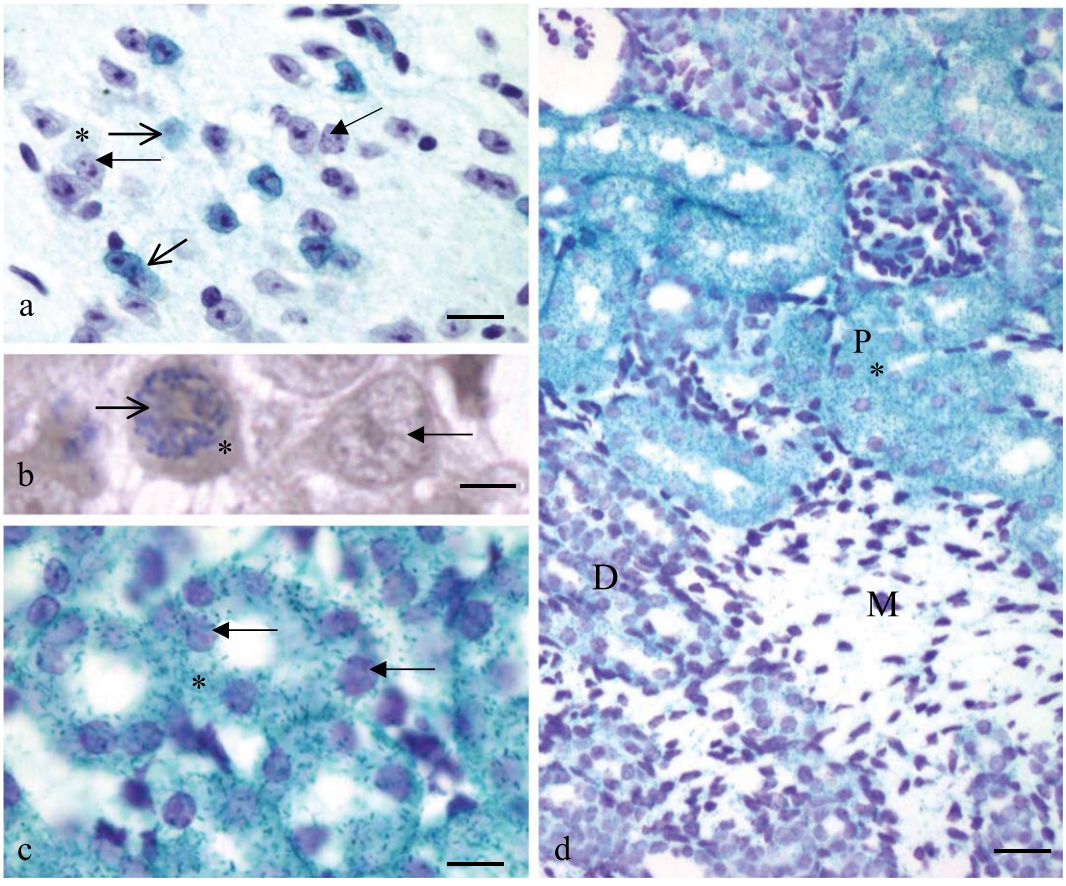

Fig. 2. X-gal staining in transgenic mice expressing the PTPS-β-galactosidase fusion protein. X-gal staining (blue-green) in transgenic mice expressing the PTPS-β-galactosidase fusion protein in adult $Pts^{+/-}$ heterozygous mice brain (a, b), and newborn $Pts^{-/-}$ homozygous knock-out mice kidney (c, d). Negative structures are stained violet by the cresyl violet (a, c, d,) or grey by hematoxylin (b). (a) Dopaminergic neurons in group A10; (b) neurons in the hippocampus; (c) kidney proximal convoluted tubes; (d) small magnification of the kidney showing different β-gal expression in proximal convoluted tubes (P), distal convoluted tubes (D) and medulla (M). Positive (open arrows) and negative (arrows) nuclei are present side by side in the brain cell groups (a, b) but all the kidney nuclei are negative. Cytoplasms are shown by stars. Scale bars: a, 12 μm; b, 8 μm; c, 15 μm; d, 30 μm.

To investigate an additional organ requiring BH_4 as cofactor for aromatic amino acid hydroxylase activity, we examined X-gal staining in the kidney of both newborn and adult heterozygous mice. β-Galactosidase expression was very weak in adults and strong in newborns. At both stages, all cells lining the proximal convoluted tube had a positive cytoplasm with a negative nucleus (Fig. 2c and d). However, while in the still undifferentiated kidney from newborn mice no staining was observed in the medulla (Fig. 2d), positive nuclei were evident in many cells of the thin limb of the Henle loops in adult mice.

Taken together, nuclear localization of the PTPS-β-galactosidase fusion protein in the transgenic mice corroborated our in situ observations with antibodies against the BH_4-synthesizing enzymes GTPCH and PTPS. Furthermore, it also demonstrated that the N-terminal 35 amino acids of PTPS contained a domain with the capacity for nuclear targeting, as the β-galactosidase fusion was targeted to the nucleus.

3.3. Sub-cellular localization of PTPS in in vitro cultured cells

The prominent nuclear distribution of GTPCH and PTPS in rodent neurons, including in regions important for dopamine and catecholamine synthesis, was unexpected and prompted us to further examine this phenomenon with in vitro cultivated cells. This included the human neuroblastoma cell line SK-N-BE and the monkey kidney cell line COS-1. Only the latter were transfected with plasmid DNA expressing the human PTPS (plasmid pHSY2013 [16]), as untransfected COS-1 cells have very low PTPS activity (≤ 0.3 μU/mg protein). Immunofluorescent staining with the SZ28 anti-human PTPS antibody resulted in a picture similar to that found for most PTPS+ cells in brain: SK-N-BE cells, which have relatively high amounts of intrinsic PTPS activity (~ 18 μU/mg total protein), showed predominant nuclear staining but also some immunoreactivity in the cytoplasm. Normal COS-1 cells showed no PTPS

Name / mutation (plasmid)	Construct	Activity (%) in COS-1 cells	Localization in COS-1 cytoplasmic	nuclear
Flag-GTPCH / wt (pHGCH10)	8 aa — 250 aa	100%	+	+
Flag-PTPS / wt (pHSY45)	8 aa — Ⓟ — 145 aa	100%	+	+
PTPS-Flag / wt (pHSY46)	Ⓟ — 145 aa — 8 aa	45%	+	+
Flag-PTPS / C10A (pHSY59)	8 aa — * — Ⓟ — 145 aa	50%	+	+
Flag-PTPS / R16C (pHSY57)	8 aa — * — 145 aa	11%	+	+
Flag-PTPS / R25Q (pHSY60)	8 aa — Ⓟ — * — 145 aa	4%	+	+
Flag-PTPS / K143A (pHSY67)	8 aa — Ⓟ — 145 aa — *	65%	+	+
Flag-PTPS / Δ1-11 (pHSY62)	8 aa — Δ — Ⓟ — 134 aa	<3%	+	+
Flag-PTPS / Δ143-145 (pHSY68)	8 aa — Ⓟ — 142 aa — Δ	n.d.	+	(-)
Flag-PTPS / Δ119-145 (pHSY69)	8 aa — Ⓟ — 118 aa — Δ	n.d.	+	(-)
NLS-Flag-PTPS / wt (pHSY80)	16 aa — Ⓟ — 145 aa	149%	+	+
Flag-SR / wt (pHSR6)	8 aa — 261 aa	100%	+	+

Fig. 3. Sub-cellular localization of the different GTPCH, PTPS, and SR Flag fusion-constructs transfected into COS-1 cells. The position of the Flag-tag with an extension of eight amino acids is given as a black bar. For GTPCH and SR only wild-type (wt) sequences with Flag-fusions were tested, whereas for PTPS, besides an N-terminal und C-terminal Flag-fusion with the wild-type enzyme, several mutations and deletions containing an N-terminal Flag were included. The circled 'P' in the PTPS constructs mark the location of phosphoserine 19, and the asterisk (*) indicates the site of single amino acid alterations. Note that the Flag-PTPS/R16C is a phosphorylation-deficient mutant. The extensions of the deletions (△) in PTPS are indicated by the dashed line. The NLS-Flag-PTPS expressed from pHSY80 contains besides the N-terminal Flag sequence an eight-amino-acid nuclear localization signal (NLS). Sub-cellular localization of Flag proteins was verified by confocal microscopy and Western blot analysis. Due to the strongly reduced expression or instability of the C-terminal Flag-PTPS deletions, Δ143–145 and Δ119–145, only the cytoplasmic PTPS was visible by immunostaining; the nuclear absence is thus set in parentheses. COS-1 cells were transiently transfected with the different constructs. For normalization, plasmid pSVβgal was co-transfected and the extracts were assayed for β-galactosidase activity (100% of enzyme activity = x μU/mg protein). n.d., not detectable.

immunoreactivity, but exhibited almost exclusively nuclear staining when transfected with a human PTPS-expressing plasmid (data not shown). These studies substantiated our previous observations of strong but not necessarily exclusive nuclear distribution of PTPS.

3.4. Intracellular distribution of GTPCH, PTPS, and SR in COS-1 cells

Next, we were interested in examining in more detail the intracellular distribution of all three BH_4-biosynthetic enzymes. As an in vitro model, we chose COS-1 cells, which exhibit SR activity (~ 1.2 mU/mg) but do not synthesize BH_4 because they have no detectable GTPCH and very low PTPS activity (see above). Furthermore, as we had no specific antibodies against all three enzymes suitable for in situ cell staining, we engineered pcDNA3 vectors for transient cell transfection that expressed Flag-tagged fusion proteins (for details on the constructs see Fig. 3 and Materials and methods). Localization of the BH_4 enzymes was individually monitored by indirect immunofluorescence with antibodies against the Flag-epitope in cells expressing Flag-GTPCH, Flag-PTPS, or Flag-SR. A representative image for each enzyme distribution in fixed COS-1 cells stained with anti-Flag antibody is shown in Fig. 4. All three proteins were detected in the nucleus and in the cytoplasm, although Flag-GTPCH and Flag-SR were only weakly present in the nucleus. While most of the Flag-PTPS immunofluorescence staining was in the cytoplasm at the vicinity of the nucleus, Flag-PTPS appeared to be irregularly located in the nucleus, more at the periphery and not at all in the nucleoli, exactly as it was in the X-gal staining.

In order to compare the distribution of the three proteins, we fractionated COS-1 cells after transfection and prepared soluble cytoplasmic and nuclear extracts. The extent of cross-contamination was monitored by measuring the LDH activity as a marker enzyme for the cytoplasmic fraction, and the SV40 large T-antigen as a marker protein for the nuclear fraction. Contamination of LDH in the nuclear fraction was 1% (maximally 3%), whereas the cytoplasmatic extract exhibited >97% of activity. The presence of SV40 large T-antigen was monitored by Western blot analysis. Only the nuclear fraction contained immunoreactive material, while nothing was detectable in the cytoplasmatic preparation. The same result was observed when p53 antibodies were tested instead of antibodies against SV40 large T-antigen (not shown). Fig. 5 depicts Western blot analyses of COS-1 cells that were transfected with Flag-GTPCH, Flag-PTPS, or Flag-SR, fractionated into cytoplasmatic and nuclear extracts, and probed with the anti-Flag antibody. As shown by immunofluorescence, both fractions contained all three enzymes, with most of the protein located to the cytoplasm.

A semi-quantitative analysis by Western-blotting serial dilutions of these extracts and comparing intensities of the immunoreactive bands is shown for these enzymes in Fig. 6. We found approximately 10-fold more GTPCH and PTPS per total protein in the cytoplasmatic fraction compared to the nuclear fraction, while for SR the ratio of cytoplasm to nucleus was approximately 100:1. From the known total amount of protein per fraction and the estimated ratio of immunoreactivity in Fig. 6, we determined the total amount of enzyme in the fractions. For example, the total protein per dish in Flag-GTPCH-transfected COS-1 cells was between 4.4 and 4.9 mg for the cytoplasm, and between 2.6 and 2.9 mg for the nucleus. Thus, the total soluble protein content in the cytoplasm of these cultured COS-1 cells was ~ 2-fold higher in comparison with the total nuclear content, resulting in a ratio of 10- to 20-fold more GTPCH in the cytoplasm. In

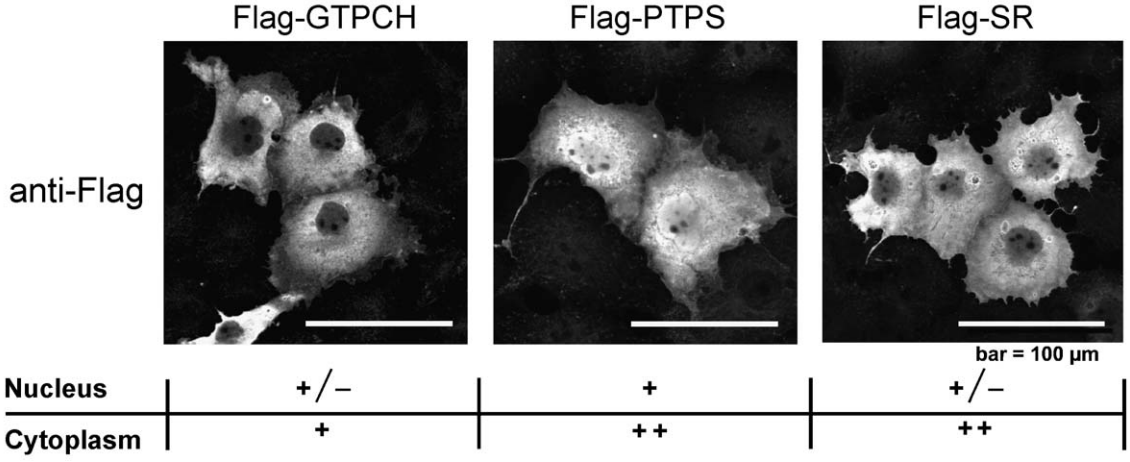

Fig. 4. Distribution of the Flag fusion proteins GTPCH, PTPS, and SR in COS-1 cells by confocal immunofluorescence. COS-1 cells were individually transfected with plasmids pHGC10, pHSY45, and pHSR6, expressing Flag-GTPCH, Flag-PTPS, and Flag-SR, respectively. Typical examples of captured immunofluorescence of fixed COS-1 cells incubated with the anti-Flag antibody are shown.

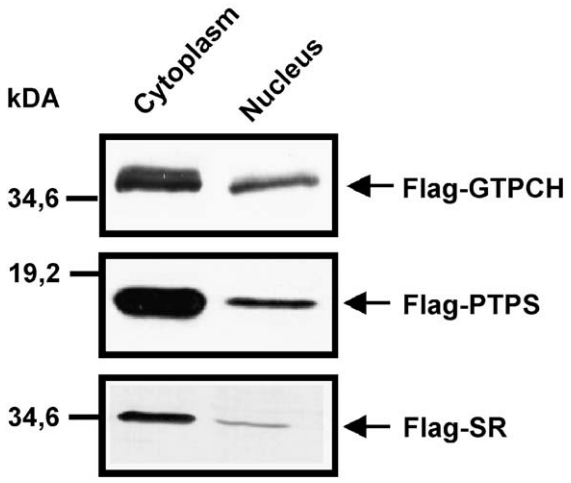

Fig. 5. Western blot analysis of sub-cellular fractions of transfected COS-1 cells expressing Flag-GTPCH, Flag-PTPS, and Flag-SR confirms the nuclear localization of BH_4 biosynthetic enzymes. Immunoblotting of transfected COS-1 cells shown in Fig. 4 was performed after separation into cytoplasmic and nuclear fractions. Cells were harvested 48 h post transfection and fractionated as described under Section 2. Soluble protein fractions were separated on SDS-PAGE and probed with anti-Flag monoclonal antibodies. Sixty-microgram protein of each fraction was loaded for Flag-GTPCH, and 40 μg for Flag-PTPS. For Flag-SR 0.5-μg protein from the cytoplasmic fraction and 80-μg protein from the nuclear fraction were loaded.

percentage, 5–10% of total GTPCH was found in the nuclear fraction, and 90–95% in the cytoplasm. Similarly, about 5–10% of total PTPS was in the nucleus, and only ~ 1% of total SR in the nuclear fraction.

3.5. GTPCH but not PTPS is an active enzyme in the nuclear fraction

The cytoplasmatic and nuclear fractions from the transfected COS-1 cells were tested for corresponding enzymatic activities (see Fig. 7). For Flag-GTPCH we observed in the nucleus an activity of approximately 10% of that in the cytoplasm, which was in agreement with the detected amount from the semi-quantitative Western analysis above. For Flag-PTPS, we observed activity in the cytoplasm but found no activity in the nucleus, although we had expected one tenth of the activity in this fraction. In a further experiment, we aimed at enhancing nuclear localization of PTPS in COS-1 cells by transient expression of a Flag-PTPS containing a classical N-terminal eight amino acid nuclear localization signal (NLS; amino acid sequence DPKKKRKV in construct pHSY80 in Fig. 3) [23]. However, we did not find more NLS-Flag-PTPS immunoreactivity in the nucleus compared to wild-type Flag-PTPS. Regarding Flag-SR, we estimated from Fig. 6

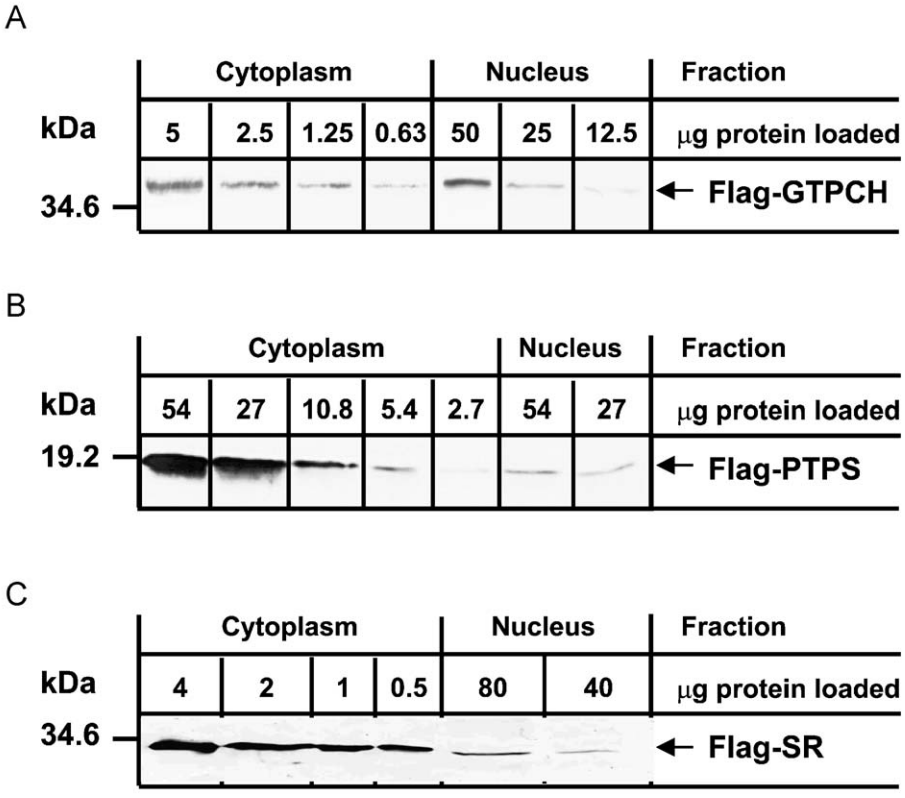

Fig. 6. Semi-quantitative determination of cytoplasmic and nuclear concentration of Flag-GTPCH (A), Flag-PTPS (B), and Flag-SR (C) from fractionated COS-1 cells. Immunoblotting of the transfected COS-1 cells is as shown in Fig. 5. Different amounts of total protein fractions as indicated were separated on an SDS-PAGE, blotted, and probed with anti-Flag monoclonal antibodies.

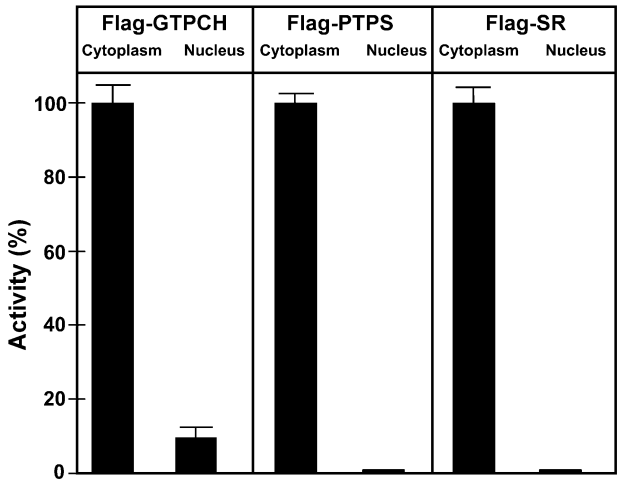

Fig. 7. The Flag-GTPCH is an active enzyme in the nucleus. Sub-cellular fractions from transfected COS-1 cells as shown in Fig. 6 were assayed for specific enzyme activity. In the figure, relative activities are given in % of cytoplasmic fractions.

that only 1% or less of the protein was in the nuclear fraction. From this we could not expect to see any SR activity in the nucleus, as an activity of $\leq 1\%$ in the cytoplasm is below our threshold value for detection. In conclusion, although all three BH_4-biosynthetic proteins were localized to the nucleus *and* to the cytoplasm, only GTPCH activity was detectable in the soluble nuclear fraction. While for SR it was not clear whether the small amount of nuclear protein had any activity, the PTPS present in the nuclear fraction had clearly no activity.

3.6. Sub-cellular localization of different PTPS alleles

To delineate a domain or define residues in PTPS responsible for signaling nuclear import (or export), we used COS-1 cell transfection followed by immunolocalization and cell fractionation, as described, and looked for potential cytoplasmic (or nuclear) accumulation of mutant enzymes (Fig. 3). From investigations with patients diagnosed with BH_4 deficiency due to mutations in PTPS, we already had a collection of mutant alleles some of which we characterized in more detail by expression studies in COS-1 cells [16,19,24–26]. The mutant PTPS tested included the PTPS-R25Q allele from a central type of BH_4 deficiency with an Arg to Glu exchange, and the PTPS-R16C allele detected in peripheral BH_4 deficiency which is a phosphorylation-deficient mutant. Additionally tested alleles that were not isolated from patients had either an exchange of the N-terminal Cys (PTPS-C10A), or an exchange of Arg to Ala at position 143 (PTPS-K143A). The N-terminal Cys at position 10 of the human PTPS is a putative palmitoylation site, a reversible post-translational type of acylation often involved in translocation and/or enzyme regulation [27,28]. Furthermore, we expressed several mutant Flag-PTPS with amino acid deletions, one at the N terminus (PTPS-Δ1-11) and two at the C terminus (PTPS-Δ143-145 and PTPS-Δ119-145). PTPS-Δ1-11, PTPS-Δ143-145, and PTPS-Δ119-145 included loss of 2, 1, or 4 Arg residues, respectively. The N terminus contains in addition a putative myristoylation site at the Gly of position 6 in human wild-type PTPS, which can function as an anchor for membrane binding or attachment [29].

Relative enzyme activities of expressed Flag-PTPS alleles in whole COS-1 cells extracts are shown in Fig. 3. A fusion of the Flag-tag at the C terminus resulted in a reduction of roughly 50% of the activity seen from that at the N terminus, which was set at 100%. The reduction of PTPS activity with a C-terminal Flag-tag, in comparison with the N-terminal Flag-tag, was most likely due to lower protein stability, as a Western blot with comparable amounts of total protein loaded showed about a 10-fold reduction in immunostaining for the C-terminal fusion (not shown). All single amino acid mutations resulted in a reduction of activity between 30% and 96%. The N-terminal deletion had very low activity (2–5% of wild-type), whereas both C-terminal deletions were completely inactive. When the transfected COS-1 cells were examined by confocal microscopy, we saw no difference between wild-type and mutants, i.e. all PTPS enzymes were located also in the nucleus independent of the reduction of activity in whole cell extracts. The only cases that did not show any nuclear staining were the C-terminal deletions PTPS-Δ143-145 and PTPS-Δ119-145, which had no activity and were most likely expressed at levels too low for detection (not shown). Taken together, from these results we could not conclude whether the N- or the C-terminal ends contain a signal for nuclear targeting. Furthermore, none of the single amino acid exchanges resulted in an alteration of nuclear localization, indicating that single residues may not be a signal for nuclear import.

4. Discussion

The main conclusions to be drawn from the experiments presented here are that the BH_4 biosynthetic enzymes GTPCH, PTPS, and SR have a nuclear localization in specific cell types or tissues, and that for PTPS the N-terminal domain is responsible for nuclear targeting. However, with these unprecedented observations we are left with unanswered questions, including mainly a functional or physiological explanation for nuclear localization. Three independent approaches, immunohistochemistry of GTPCH and PTPS with rat tissues, X-gal staining with the *Pts-LacZ* knock-in mouse, and in vitro cell culturing experiments including biochemical analysis of fractionated cells and indirect immunofluorescence microscopy with GTPCH, PTPS, and SR, corroborate the observed nuclear localization. We are thus convinced that an experimental artifact for the

nuclear localization of the BH_4 biosynthetic enzymes can be ruled out. The advantage of Flag epitope-tagging is that the addition of the unique eight amino acids spanning sequence (DYKDDDDK) is unlikely to interfere with the distribution, function, or activity of a protein. In our hands, the activity in whole extracts of COS-1 cells was well preserved upon expression of the N-terminally Flag-tagged BH_4-biosynthetic enzymes, and we did not observe any difference in distribution in these cells at least between native PTPS or Flag-PTPS detected with the corresponding antibodies.

Several details of the localization studies performed with PTPS immunoreaction and X-gal staining should be stressed. PTPS immunoreactivity and X-gal staining are prominently nuclear in the neurons but cytoplasmic in other cells, for example the kidney convoluted tubules. In general, X-gal staining is present in neurons of some brain regions in an amount that is higher than that of catecholaminergic cells, and is unrelated to catecholamine or serotonin synthesis. PTPS is also present in many differentiating structures of the fetal brain. This certainly argues in favor of a nuclear function unrelated to its known metabolic activity. Such an observation has been reported for PCD/DCoH, the dehydratase involved in BH_4-recycling, which shows also a prominent nuclear distribution [30,31]. Furthermore, the nuclear dimerization function of the dimeric DCoH does not require the tetrameric structure that is a prerequisite for the cytosolic dehydrates activity [32,33]. Interestingly, BH_4 was reported to up-regulate brain tyrosine hydroxylase several-fold upon intraperitoneal injection in a PTPS knock-out mouse [34]; however, it has to be clarified whether this is due to a direct nuclear response of the BH_4 cofactor.

The *Pts-LacZ* gene fusion expressing in transgenic mice a functional β-galactosidase with specific nuclear localization delineates the functional unit for nuclear targeting to the 35-N-terminal amino acids of PTPS. Since nuclear X-gal staining in neurons is present in both heterozygous and homozygous transgenic mice, the 35 amino acids are thus sufficient to target the protein to the nucleus even in the absence of full-length PTPS. The N-terminal domain includes a known phosphorylation site, and putative recognition motifs for acylation like palmitoylation and myristoylation. Besides the fact that we have not been able to demonstrate such N-terminal acylation for PTPS (unpublished observation), there is no indication from our transfection experiments with PTPS mutants that these single amino acid sites are individually involved in nuclear localization. Since the N terminus of PTPS acts as an import signal, as has been demonstrated for the β-galactosidase, one might speculate that a carrier that recognizes the N-terminal amino acids is responsible for nuclear transfer. The imported PTPS might be a nonenzymatically active and a non-hexameric protein, as only the native, homohexameric form gives rise to an active enzyme [35]. Although the size of the monomeric PTPS with $\sim$ 16 kDa is small enough to allow passive passage through a nuclear pore complex [36], passive diffusion is unlikely not only due to the demonstrated import capacity of the N terminus of PTPS, but also due to its highly cell-specific nuclear presence.

In another series of experiments, we sought to test for potential alteration of nuclear PTPS localization and/or activity upon induction of GTPCH expression by cytokine stimulation of human dermal fibroblasts. Whereas induction of PTPS (and GTPCH) expression in COS-1 cells by such a cytokine treatment is not possible, addition of cytokine to various human cells including fibroblasts leads also to an increase of PTPS activity by a factor of 1.5–3 [37–39]. Unfortunately, the detection level for PTPS immunoreactivity and enzymatic activity was not sensitive enough to observe nuclear PTPS in fractionated fibroblasts. Furthermore, attempts to quantify of biopterin and its metabolites in the two sub-cellular fractions from GTPCH- and/or PTPS-transfected COS-1 cells were unsuccessful. This was probably due to the low molecular weight of pterin metabolites that are lost during washing procedures for fractionation. In addition, pterins can probably freely travel between cytoplasm and nucleus through the nuclear pore complexes.

Nuclear localization can also be a result of an only partially efficient nuclear export signal (NES) that is responsible for cytoplasmic localization of a protein. Although we had no indication for such a NES in PTPS, we tested for nuclear export by the application of leptomycin B as a covalent inhibitor of the corresponding nuclear export receptor exportin 1 [36,40]. As controls, we used transient transfections of COS-1 cells expressing either the green fluorescent protein alone or fused to the heat stress transcription factor HsfA2. HsfA2 contains an NES and its export can be specifically inhibited in mammalian cells in presence of leptomycin B [41]. However, inhibition of exportin 1 by leptomycin B treatment had no effect on the nuclear localization of Flag-PTPS in transfected COS-1 cells (not shown).

The unprecedented nuclear localization of all BH_4-biosynthetic enzymes reminds of an interesting observation published on mutations in the *Drosophila melanogaster Punch* locus, which encodes GTPCH [42]. There it was demonstrated that nuclear GTPCH, and indirectly SR, in the pre-cellular blastoderm are directly involved in nuclear divisions, and it was postulated that GTPCH has a role in chromosome separation. Whether this early developmental function in the fruit fly has any relevance to our observation of nuclear localization of BH_4-biosynthetic enzymes in specific tissues of cells in mammalians remains to be clarified.

Acknowledgements

We thank Drs. M. Gütlich and H. Ichinose for providing cDNA clones, F. Neuheiser for technical assistance with DNA cloning, M. Killen for help with the preparation of this

manuscript, the animal laboratory facilities under the guidance of Drs. B. Ledermann and K. Bürki for their collaborative efforts, and Prof. C.W. Heizmann for continuous support of this work. We would like to specially acknowledge the 'Jubiläumsstiftung' of the 'Rentenanstalt'/ Swiss Life Insurance Company, and the Swiss National Science Foundation (Grants nos. 31-64154.00 and 31-66953.01 to BT and NB), and the Belgian Founds de la Recherche Scientifique Medicale (Grant no. 3.4411.88 to AR) for financial support of our research.

References

[1] S.S. Gross, C.L. Jones, Y. Hattori, C.S. Raman, in: L.J. Ignarro (Ed.), Nitric Oxide: Biology and Pathobiology, Academic Press, San Diego, 2000, pp. 167–185.
[2] S. Kaufman, New tetrahydrobiopterin-dependent systems, Annu. Rev. Nutr. 13 (1993) 261–286.
[3] H. Taguchi, W.L. Armarego, Glyceryl-ether monooxygenase [EC 1.14.16.5]. A microsomal enzyme of ether lipid metabolism, Med. Res. Rev. 18 (1998) 43–89.
[4] N. Blau, B. Thöny, R.G.H. Cotton, K. Hyland, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed., McGraw-Hill, New York, 2001, pp. 1725–1776.
[5] G. Zorzi, B. Thöny, N. Blau, Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency, J. Neurochem. 80 (2002) 362–364.
[6] B. Thöny, G. Auerbach, N. Blau, Tetrahydrobiopterin biosynthesis, regeneration, and functions, Biochem. J. 347 (2000) 1–16.
[7] N. Blau, L. Bonafe, B. Thöny, Tetrahydrobiopterin deficiencies without hyperphenylalaninemia: diagnosis and genetics of dopa-responsive dystonia and sepiapterin reductase deficiency, Mol. Genet. Metab. 74 (2001) 172–185.
[8] J.C. Jensenius, I. Andersen, J. Hau, M. Crone, C. Koch, Eggs: conveniently packaged antibodies. Methods for purification of yolk IgG, J. Immunol. Methods 46 (1981) 63–68.
[9] D. Dassesse, B. Hemmens, L. Cuvelier, A. Resibois, GTP cyclohydrolase I-like immunoreactivity in rat brain, Brain Res. 777 (1997) 187–201.
[10] S.M. Hsu, E. Soban, Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry, J. Histochem. Cytochem. 30 (1982) 1079–1082.
[11] L. Elzaouk, W. Leimbacher, M. Turri, B. Ledermann, K. Burki, N. Blau, B. Thöny, Dwarfism and low insulin-like growth factor-1 due to dopamine depletion in Pts −/− mice rescued by feeding neurotransmitter precursors and H4-biopterin, J. Biol. Chem. 278 (2003) 28303–28311.
[12] A. Togari, H. Ichinose, S. Matsumoto, K. Fujita, T. Nagatsu, Multiple mRNA forms of human GTP cyclohydrolase I, Biochem. Biophys. Res. Commun. 187 (1992) 359–365.
[13] M. Gütlich, E. Jaeger, K.P. Rucknagel, T. Werner, W. Rodl, I. Ziegler, A. Bacher, Human GTP cyclohydrolase I: only one out of three cDNA isoforms gives rise to the active enzyme, Biochem. J. 302 (1994) 215–221.
[14] B. Thöny, W. Leimbacher, D. Bürgisser, C.W. Heizmann, Human 6-pyruvoyl-tetrahydropterin synthase: cDNA cloning and heterologous expression of the recombinant enzyme, Biochem. Biophys. Res. Commun. 189 (1992) 1437–1443.
[15] H. Ichinose, S. Katoh, T. Sueoka, K. Titani, K. Fujita, T. Nagatsu, Cloning and sequencing of cDNA encoding human sepiapterin reductase—an enzyme involved in tetrahydrobiopterin biosynthesis, Biochem. Biophys. Res. Commun. 179 (1991) 183–189.
[16] T. Oppliger, B. Thöny, H. Nar, D. Bürgisser, R. Huber, C.W. Heizmann, N. Blau, Structural and functional consequences of mutations in 6-pyruvoyl-tetrahydropterin synthase causing hyperphenylalaninemia in humans—phosphorylation is a requirement for in vivo activity, J. Biol. Chem. 270 (1995) 29498–29506.
[17] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[18] H. Ichinose, T. Ohye, E. Takahashi, N. Seki, T. Hori, M. Segawa, Y. Nomura, K. Endo, H. Tanaka, S. Tsuji, et al., Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene, Nat. Genet. 8 (1994) 236–242.
[19] B. Thony, W. Leimbacher, N. Blau, A. Harvie, C.W. Heizmann, Hyperphenylalaninemia due to defects in tetrahydrobiopterin metabolism: molecular characterization of mutations in 6-pyruvoyl-tetrahydropterin synthase, Am. J. Hum. Genet. 54 (1994) 782–792.
[20] J. Ferre, E.W. Naylor, Sepiapterin reductase in human amniotic and skin fibroblasts, chorionic villi, and various blood fractions, Clin. Chim. Acta 174 (1988) 271–282.
[21] A. Niederwieser, W. Staudenmann, E. Wetzel, High-performance liquid chromatography with column switching for the analysis of biogenic amine metabolites and pterins, J. Chromatogr. 290 (1984) 237–246.
[22] E.R. Werner, G. Werner-Felmayer, H. Wachter, High-performance liquid chromatography methods for the quantification of tetrahydrobiopterin biosynthetic enzymes, J. Chromatogr. 684 (1996) 51–58.
[23] D. Kalderon, B.L. Roberts, W.D. Richardson, A.E. Smith, A short amino acid sequence able to specify nuclear location, Cell 39 (1984) 499–509.
[24] T. Oppliger, B. Thöny, C. Kluge, A. Matasovic, C.W. Heizmann, A. Ponzone, M. Spada, N. Blau, Identification of mutations causing 6-pyruvoyl-tetrahydropterin synthase deficiency in four Italian families, Human Mutat. 10 (1997) 25–35.
[25] T. Scherer-Oppliger, A. Matasovic, S. Laufs, H.L. Levy, E.J. Quackenbush, N. Blau, B. Thöny, Dominant negative allele (N47D) in a compound heterozygote for a variant of 6-pyruvoyltetrahydropterin synthase deficiency causing transient hyperphenylalaninemia, Human Mutat. 13 (1999) 286–289.
[26] N. Blau, T. Scherer-Oppliger, A. Baumer, M. Riegel, A. Matasovic, A. Schinzel, J. Jaeken, B. Thony, Isolated central form of tetrahydrobiopterin deficiency associated with hemizygosity on chromosome 11q and a mutant allele of PTPS, Human Mutat. 16 (2000) 54–60.
[27] M.D. Resh, Fyn, a Src family tyrosine kinase, Int. J. Biochem. Cell Biol. 30 (1998) 1159–1162.
[28] R.B. Rucker, C. McGee, Chemical modifications of proteins in vivo: selected examples important to cellular regulation, J. Nutr. 123 (1993) 977–990.
[29] D.A. Towler, J.I. Gordon, S.P. Adams, L. Glaser, The biology and enzymology of eukaryotic protein acylation, Ann. Rev. Biochem. 57 (1988) 69–99.
[30] A. Resibois, L. Cuvelier, M. Svoboda, C.W. Heizmann, B. Thony, Immunohistochemical localisation of pterin-4alpha-carbinolamine dehydratase in rat peripheral organs, Histochem. Cell Biol. 111 (1999) 381–390.
[31] V. Depaepe, L. Cuvelier, B. Thony, A. Resibois, Pterin-4alpha-carbinolamine dehydratase in rat brain: I. Patterns of co-localization with tyrosine hydroxylase, Brain Res. Mol. Brain Res. 75 (2000) 76–88.
[32] J.L. Kim, S.K. Burley, PCD/DCoH: more than a second molecular saddle, Curr. Biol. 3 (1995) 531–534.
[33] D. Suck, R. Ficner, Structure and function of PCD/DCoH, an enzyme with regulatory properties, FEBS Lett. 389 (1996) 35–39.
[34] C. Sumi-Ichinose, F. Urano, R. Kuroda, T. Ohye, M. Kojima, M. Tazawa, H. Shiraishi, Y. Hagino, T. Nagatsu, T. Nomura, H. Ichinose, Catecholamines and serotonin are differently regulated by tetrahydrobiopterin. A study from 6-pyruvoyltetrahydropterin synthase knockout mice, J. Biol. Chem. 276 (2001) 41150–41160.
[35] H. Nar, R. Huber, C.W. Heizmann, B. Thöny, D. Bürgisser, Three-dimensional structure of 6-pyruvoyl tetrahydropterin synthase, an en-

zyme involved in tetrahydrobiopterin biosynthesis, EMBO J. 13 (1994) 1255–1262.
[36] D. Gorlich, U. Kutay, Transport between the cell nucleus and the cytoplasm, Annu. Rev. Cell. Dev. Biol. 15 (1999) 607–660.
[37] I. Ziegler, K. Schott, M. Lubbert, F. Herrmann, U. Schwulera, A. Bacher, Control of tetrahydrobiopterin synthesis in T lymphocytes by synergistic action of interferon-gamma and interleukin-2, J. Biol. Chem. 265 (1990) 17026–17030.
[38] P. Linscheid, A. Schaffner, N. Blau, G. Schoedon, Regulation of 6-pyruvoyltetrahydropterin synthase activity and messenger RNA abundance in human vascular endothelial cells, Circulation 98 (1998) 1703–1706.
[39] K.L. Leitner, M. Meyer, W. Leimbacher, A. Peterbauer, S. Hofer, C. Heufler, A. Muller, R. Heller, E.R. Werner, B. Thony, G. Werner-Felmayer, The low tetrahydrobiopterin biosynthetic capacity of human monocytes is caused by exon skipping in 6-pyruvoyl tetrahydropterin synthase, Biochem. J. 373 (2003) 681–688.
[40] N. Kudo, B. Wolff, T. Sekimoto, E.P. Schreiner, Y. Yoneda, M. Yanagida, S. Horinouchi, M. Yoshida, Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1, Exp. Cell Res. 242 (1998) 540–547.
[41] D. Heerklotz, P. Doring, F. Bonzelius, S. Winkelhaus, L. Nover, The balance of nuclear import and export determines the intracellular distribution and function of tomato heat stress transcription factor HsfA2, Mol. Cell. Biol. 21 (2001) 1759–1768.
[42] X. Chen, E.R. Reynolds, G. Ranganayakulu, J.M. O'Donnell, A maternal product of the Punch locus of Drosophila melanogaster is required for precellular blastoderm nuclear divisions, J. Cell Sci. 107 (Pt. 12) (1994) 3501–3513.