Analytical Biochemistry 412 (2011) 96–101

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Quantitative and selective polymerase chain reaction analysis of highly similar human alpha-class glutathione transferases

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ARTICLE INFO

Article history:
Received 6 December 2010
Received in revised form 11 January 2011
Accepted 19 January 2011
Available online 24 January 2011

Keywords:
Quantitative PCR
Primer design
Alpha-class glutathione transferase expression
Human steroidogenic cells

ABSTRACT

Alpha-class glutathione transferases (GSTs) found expressed in human tissues constitute a family of four homologous enzymes with contrasting enzyme activities. In particular, GST A3-3 has been shown to contribute to the biosynthesis of steroid hormones in human cells and is selectively expressed in steroidogenic tissues. The more ubiquitous GST A1-1, GST A2-2, and GST A4-4 appear to be primarily involved in detoxification processes and are expressed at higher levels than GST A3-3. We are interested in studying the cell and tissue expression of the GST A3-3 gene, yet the existence of highly expressed sequence-similar homologs and of several splice variants is a serious challenge for the specific detection of unique transcript species. We found that published polymerase chain reaction (PCR) primers for GST A3-3 lack the specificity required for reliable quantitative analysis. Therefore, we designed quantitative PCR (qPCR) primers with greatly increased discrimination power for the human *GSTA3* full-length transcript. The improved primers allow accurate discrimination between GST A3-3 and the other alpha-class GSTs and so are of great value to studies of the expression of the *GSTA3* gene. The novel primers were used to quantify *GSTA3* transcripts in human embryonic liver and steroidogenic cell lines.

Glutathione transferases (GSTs)[1] play important roles in the biotransformation of xenobiotics as well as in the detoxification of genotoxic substances that arise from normal constituents of living organisms [1,2]. GSTs catalyze reactions with a myriad of electrophilic compounds, and the individual GSTs have partly overlapping activity profiles. Therefore, single gene deletions generally do not have a high penetrance resulting in a distinctive phenotype. Nevertheless, the frequently occurring null alleles of human *GSTM1* and *GSTT1* have been associated with different forms of cancer [3]. In addition, certain GSTs are involved in the metabolism of endogenous substances, including steroids, and the expression of many human GSTs is subject to regulation by xenobiotics such as electrophilic compounds [4]. Therefore, measurement of the expression of individual GSTs is important to untangle the role of individual enzymes in chemoprotection and drug resistance as well as in the maintenance of normal cellular functions.

The human enzyme GST A3-3 is an efficient steroid isomerase, catalyzing the migration of a double bond from the Δ^5 position to the Δ^4 position in Δ^5-androsten-3,17-dione and Δ^5-pregnen-3,20-dione [5]. GST A3-3 is expressed in steroid-producing organs, and we have shown that it contributes to steroidogenesis, thereby complementing the catalytic activity of the 3β-hydroxysteroid dehydrogenase [6]. The closest human homolog of GST A3-3 is GST A1-1, which shares 85% of amino acid sequence similarity with GST A3-3. GST A1-1 displays noteworthy steroid isomerase activity, although its catalytic efficiency is approximately 20-fold lower than that of GST A3-3. These homologous enzymes are encoded on chromosome 6 in a gene cluster that includes three additional alpha-class GST genes: *GSTA2*, *GSTA4*, and *GSTA5* [7]. The enzymes expressed from the latter genes have not been found to have any significant steroid isomerase activity. GST A2-2, like GST A1-1, is expressed at high levels in liver and kidney, whereas GST A4-4 is ubiquitously occurring. No transcript or protein product of the *GSTA5* gene has so far been experimentally identified in human tissues or cells, even though transcription and translation have been demonstrated in an in vitro system [8]. The differential tissue distribution of the various alpha-class GSTs and the divergence in their activity profiles with alternative substrates obviously influence physiological functions and toxicological processes in different tissues and cells. Therefore, quantification of the enzymes is of significance for elucidation of their biological roles. However, in view of the sequence similarities among the enzymes, unambiguous analysis of the individual proteins is a difficult task. The GST

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[1] *Abbreviations used:* GST, glutathione transferase; PCR, polymerase chain reaction; qPCR, quantitative PCR; UTR, untranslated region; mRNA, messenger RNA; TBS–Tween, Tris-buffered saline–Tween 20; D-MEM, Dulbecco's modified Eagle's medium; F12, Ham's F-12; FBS, fetal bovine serum; cDNA, complementary DNA.

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doi:10.1016/j.ab.2011.01.024

A4-4 sequence is markedly divergent from the other GST sequences and can be clearly distinguished, but the other sequences are more challenging. For example, the sequences of GST A1-1 and GST A2-2 proteins are 95% identical. The nucleotide sequences of the enzymes are also highly similar, but oligonucleotide primers have nevertheless been reported to distinguish the sequences in various polymerase chain reaction (PCR) analyses [7–9].

In investigations of the role of human GST A3-3 in steroid hormone biosynthesis, we have attempted to quantify expression levels by use of antibodies as well as by quantitative PCR (qPCR). However, neither immunoassays with available antibodies nor PCR with the published oligonucleotide primers [9] achieved the desired specificity for GST A3-3. In the current article, we demonstrate how the use of oligonucleotide primers targeting untranslated regions (UTRs) of the *GSTA3* nucleotide sequence allows efficient distinction from other alpha-class GST sequences. The primers also distinguish between the full-length message and alternatively spliced messenger RNA (mRNA). The novel primers are shown to be suited for quantitative analysis of *GSTA3* expression in samples of steroidogenic cells that display high or low levels of the enzyme.

Materials and methods

Dot-blot immunoblotting

Human GSTs were available as recombinant proteins [10]. The GSTs were blotted onto Hybond ECL membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was blocked with 5% milk and Tris-buffered saline–0.1% Tween 20 (TBS–Tween). The polyclonal primary antibodies against human GST A3-3 (Atlas Antibodies) were diluted 1:1000 in TBS–Tween and incubated with the membrane overnight at 4 °C before the addition of the secondary antibodies (goat anti-rabbit diluted conjugated with horseradish peroxidase from Bio-Rad) diluted 1:3000 in TBS–Tween. Following incubation for 3 h at room temperature, detection was performed with the Amersham ECL blotting system (GE Healthcare, Uppsala, Sweden).

Cell cultures and RNA preparations

All reagents were obtained from Gibco unless otherwise indicated. Human adrenal H295R cells (obtained from William E. Rainey, Medical College of Georgia, USA) were cultured in Dulbecco's modified Eagle's medium and nutrient mixture Ham's F-12 (D-MEM/F12). The medium contained 5% Nu-Serum (BD Biosciences), 10 μg/ml streptomycin, 10 U/ml penicillin, and 1 μg/ml gentamicin. Human placental JEG-3 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in MEM containing 10% fetal bovine serum (FBS), 10 μg/ml streptomycin, 10 U/ml penicillin, and 0.25 μg/ml fungizone. The cells were cultured under a humidified atmosphere containing 5% CO_2.

Total RNA from the different cell lines was extracted with RNeasy (Qiagen), the concentration and purity (A_{260}/A_{280}) were measured with a NanoDrop 1000 (Thermo Scientific), and the samples were stored at −80 °C. Total RNA from fetal liver was obtained from Biocat (Heidelberg, Germany).

Construction of plasmid stocks

Plasmid stocks with target sequence inserts were constructed by amplifying the GST-specific sequences from clones provided by DNA2.0 (Menlo Park, CA, USA) or available in our laboratory [10,11] using PCR and subsequent cloning into the pGEM-T Easy vector (Promega). Gene-specific products were amplified using primers designed for qPCR. GenBank Accession No. and primer sequences for GST A1-1, GST A2-2, GST A3-3 and β-actin are given in Table 1. For alignments, GST A4-4 and GST A5-5 complementary DNA (cDNA) sequences were also included based on the GenBank Accession No. NM_001512.3 and NM_153699.1, respectively.

Reverse transcription and real-time qPCR

One microgram of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Real-time qPCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a MiniOpticon Real-Time PCR Detection System (Bio-Rad). The reaction conditions were as follows: 3 min at 95 °C followed by 39 cycles of 1 s at 95 °C, 30 s at 57 °C, and 20 s at 72 °C. PCR products were subjected to melting curve analysis, according to the manufacturer's instructions, to confirm the presence of a single amplicon. Triplicates were used throughout the experiments. Quantitation of GST A3-3 transcript was performed using the $2^{-\Delta\Delta C_t}$ method [12], and β-actin was chosen as a reference gene.

Table 1
Primer sequences for cloning and qPCR analysis.

Gene (and GenBank accession number)	Name	Orientation	Sequence (5'→3')	Positions
Primers for cloning of full-length cDNAs				
GSTA1	P1[a]	Forward	attgagaggaacaaagagcttata	1–24
GSTA2	P1[a]	Forward	attgagaggaacaaagagcttata	39–62
GSTA1	P2[a]	Reverse	atagtttccttttttactgaatttttaatt	1048–1019
GSTA2	P3[a]	Reverse	gaataggagttgtattatttaattagcatataatt	969–935
GSTA3	P4[a]	Forward	ctgagcggagaccggctag	29–47
GSTA3	P5[a]	Reverse	aagttagcaaataggagtttttattattta	907–878
Primers for qPCR				
GSTA1 (NM_145740.3)	P6[b]	Forward	gactccagtcttatctccagcttcc	624–648
	P7[b]	Reverse	tgcttcttctaaagatttctcatccat	760–735
GSTA2 (NM_000846.4)	P8[b]	Forward	tggaagagcttgactctagccttatt	614–639
	P9[b]	Reverse	ggctgccaggctgtagaaac	721–702
GSTA3 (NM_000847.4)	P10[b]	Forward	ctctactatgtggaagagcttgactcca	510–537
	P11[b]	Reverse	tgcatctgcgggaggctt	750–733
GSTA3 (NM_000847.4)	P12[a]	Forward	caaagaaaccaagagactgt	56–75
	P13[a]	Reverse	ccaaatcttctgcagatcc	103–85
ACTB (β-actin) (NM_001615.3)	P14[c]	Forward	aagagctatgagctgcctga	837–856
	P15[c]	Reverse	tacggatgtcaacgtcacac	996–976

[a] From this study.
[b] From Ref. [9].
[c] Kindly provided by Johan Heldin (Department of Medical Biochemistry and Microbiology (IMBIM), Uppsala University).