Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

Functional characterization of the human translocator protein (18 kDa) gene promoter in human breast cancer cell lines

Amani Batarseh [a,1], Keith D. Barlow [b,2], Daniel B. Martinez-Arguelles [a], Vassilios Papadopoulos [a,b,c,d,*]

[a] The Research Institute of the McGill University Health Centre and the Department of Medicine, McGill University, Montreal, Quebec, Canada H3G 1A4
[b] Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington DC, 20057 USA
[c] Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1A4
[d] Department of Pharmacology & Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1A4

ARTICLE INFO

ABSTRACT

The translocator protein (18 kDa; TSPO) is a mitochondrial drug- and cholesterol-binding protein that has been implicated in several processes, including steroidogenesis, cell proliferation, and apoptosis. Expression of the human *TSPO* gene is elevated in several cancers. To understand the molecular mechanisms that regulate TSPO expression in human breast cancer cells, the *TSPO* promoter was identified, cloned, and functionally characterized in poor-in-TSPO hormone-dependent, non-aggressive MCF-7 cells and rich-in-TSPO hormone-independent, aggressive, and metastatic MDA-MB-231 breast cancer cells. RNA ligase-mediated 5′-rapid amplification of cDNA ends analysis indicated transcription initiated at multiple sites downstream of a GC-rich promoter that lacks functional TATA and CCAAT boxes. Deletion analysis indicated that the region from −121 to +66, which contains five putative regulatory sites known as GC boxes, was sufficient to induce reporter activity up to 24-fold in MCF-7 and nearly 120-fold in MDA-MB-231 cells. Electrophoretic mobility shift and chromatin immunoprecipitation assays indicated that Sp1, Sp3 and Sp4 bind to these GC boxes *in vitro* and to the endogenous *TSPO* promoter. Silencing of Sp1, Sp3 and Sp4 gene expression reduced TSPO levels. In addition, *TSPO* expression was epigenetically regulated at one or more of the identified GC boxes. Disruption of the sequence downstream of the main start site of *TSPO* differentially regulated *TSPO* promoter activity in MCF-7 and MDA-MB-231 cells, indicating that essential elements contribute to its differential expression in these cells. Taken together, these experiments constitute the first in-depth functional analysis of the human *TSPO* gene promoter and its transcriptional regulation.

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1. Introduction

The translocator protein (18 kDa) (TSPO), previously known as the peripheral-type benzodiazepine receptor, is a widely expressed 18-kDa mitochondrial protein that is biochemically and pharmacologically distinct from the central benzodiazepine receptor binding sites that allosterically regulate the GABA$_A$ receptor [1–3]. Subcellular fractionation and radioligand binding studies have shown that TSPO is predominantly localized to the mitochondria [4] and is concentrated at contact sites between the outer and inner mitochondrial membranes [5]. TSPO associates with the voltage dependent anion channel and adenine nucleotide translocator [6–8], which together contribute to the formation of the mitochondrial permeability transition pore [9]. The functional role of TSPO has been best described in steroidogenic cells, in which it acts as a high affinity cholesterol-binding protein that participates in the intra-mitochondrial transport of cholesterol, the rate-determining step in the synthesis of steroids [10]. Additional functions have been suggested by the pharmacological effects of high affinity TSPO ligands, which have been shown to modulate mitochondrial respiration, cell proliferation, apoptosis, and differentiation, although the mechanisms underlying these effects are poorly understood.

The levels of TSPO expression vary according to tissue and cell type and can be altered pathologically. In normal tissues, high levels of TSPO expression are observed in the adrenal cortex, steroidogenic cells of the gonads, and clusters of differentiated cells within glandular epithelia. Increased expression of the human *TSPO* gene has been described in several cancers, including high-grade glioblastomas, prostate, ovarian, colon, and breast carcinomas [11]. In breast cancer cell lines and clinical specimens, expression of *TSPO* mRNA and

radioligand binding and/or immunoreactivity increases in a manner that correlates positively with invasiveness and/or malignancy [12–14]. The mechanism by which TSPO gene expression is increased physiologically in specific cell types and pathologically in tumors is poorly understood. We have previously reported the presence of TSPO gene amplification in aggressive metastatic breast cancer cells and biopsies [11,15]. However, TSPO gene amplification does not appear to be sufficient to account for the levels of increased expression of TSPO in aggressive human breast cancer cells without contributions from additional mechanisms of aberrant gene expression. Thus, we investigated the mechanism(s) regulating transcription of the TSPO gene in MCF-7 and MDA-MB-231 cells, which express relatively low and high levels, respectively, of TSPO mRNA and protein [14].

In the present study, we aimed to functionally characterize the human TSPO promoter and to investigate its transcriptional regulation in breast cancer cells, as well as the similarities in its structure and regulation to that of the mouse promoter. As an initial step in the analysis of transcriptional regulation, the promoter directing expression of the human TSPO gene in breast cancer cell lines was identified, cloned, sequenced, and functionally characterized to determine the minimal sequence necessary to support basal levels of promoter activity. Specific substitution mutations were then introduced into the proximal region identified as necessary for maintaining near-maximal promoter activity, in order to define important regulatory elements. Based on the functional analysis of the TSPO promoter, potential protein/DNA interactions were investigated using electrophoretic mobility shift assay (EMSA) and supershift analyses. In silico analysis of the cloned human TSPO promoter sequence revealed high GC content in the proximal region of the promoter, whereas further analysis showed that the TSPO gene is situated within a CpG island. A methylation inhibitor (5-Azacytidine) and deacetylation inhibitor (TSA) were used to reveal the involvement of epigenetic mechanisms, such as methylation and acetylation, in the regulation of TSPO gene expression. As a result, this work constitutes the first functional description of the promoter of the human TSPO gene and compares and contrasts its regulation in two breast cancer cell lines that can be distinguished on the basis of hormonal dependence, chemotactic and chemoinvasive potential, and differential expression of several markers of malignancy, including differential expression of the TSPO gene.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 and MCF-7 human breast cancer cell lines were obtained from the Cell Culture Core Facility of Lombardi Comprehensive Cancer Center (LCCC) and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. (GIBCO, Burlington, ON). Human mammary epithelial cells (HMECs) from Cambrex, Inc. (East Rutherford NJ) were maintained in Mammary Epithelial Growth Medium (MEGM; Clonetics-Lonza, Walkersville, MD). Drosophila SL2 cells (D.Mel.2) were purchased from Invitrogen (Carlsbad, CA) and maintained as previously described [16]. Both MCF-7 and MDA-MB-231 cell lines were subjected to DNA fingerprinting and tested for mycoplasma contamination as part of routine maintenance by LCCC.

2.2. RNA ligase-mediated 5′-rapid amplification of cDNA ends (RLM-5′RACE)

Total RNA was isolated using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen). RLM-5′RACE was performed using the GeneRacer Kit, according to the manufacturer's instructions (Invitrogen). 5′RACE-ready cDNA libraries were prepared by reverse transcription using SuperScript II RT and total RNA isolated from HMEC, MCF-7, and MDA-MB-231 cells. Commercial RACE-ready cDNA libraries prepared from normal human testes and kidney were purchased from Invitrogen. Nested PCR was performed using a commercial nested 5′ primer in combination with a reverse gene-specific primer complementary to either TSPO exon 3 or exon 4. Amplified PCR products were gel-purified and subcloned into pCR4-TOPO (Invitrogen) for sequencing. Primers used in 5′RACE are listed in Supplemental Table S1.

2.3. Plasmid construction

The putative promoter sequence was amplified by PCR using the GC-Rich PCR System (Roche Molecular Biochemicals, Indianapolis, IN) and BAC clone dJ526I14 (Wellcome Trust Sanger Institute, Cambridge, UK; GenBank accession number z82214) as template. Amplified TSPO promoter fragments were directionally cloned into the promoter-less pGL3-Basic reporter vector (Promega, Madison, WI) using Mlu I and Bgl II restriction sites incorporated into the forward and reverse PCR primers, respectively. Promoter sequences were verified using Taq FS Dye Terminator Cycle Sequencing performed by the LCCC DNA Sequencing Core Facility. Primers used in the construction of deletion and substitution mutants are listed in Supplemental Table S1. Substitution mutants were prepared by PCR using either a splicing by overlap extension or conventional ligation protocol to join promoter fragments containing 6-bp substitutions, creating Xba I, Pst I, or Nhe I restriction sites in place of the wild-type sequence. All potential substitution mutants were evaluated by a search of the MatInspector v2.2 database [17] to ensure that the mutated sequence did not create a known consensus DNA binding motif. In the splicing by overlap extension protocol, two sets of primer pairs were used in the initial PCR to generate products that overlapped at the position where the mutation was to be inserted. In the second PCR, a mutated double-stranded TSPO promoter fragment was amplified from the overlapping primary PCR products using flanking primers hybridizing to positions −121 and +66 of the TSPO promoter.

2.4. Transient transfections and luciferase reporter assays

All cells were transfected in 12-well plates using Lipofectamine Plus reagent (Invitrogen). Each transfection consisted of 150 fmol of the specific TSPO promoter-pGL3Basic reporter construct, 10 ng of pRLTK (Promega), and the necessary amount of pUC19 plasmid to bring the total amount of plasmid DNA per transfection to 845 ng. In epigenetic analyses, MDA-MB-231 and MCF-7 cells were treated with 50 nM Trichostatin A (TSA) or 5 μM 5-Azacytidine (AZA) 24 h after transfection with the F11 -121/+66 promoter construct and lysates were harvested 24 h later. The luciferase activities of all lysates were measured using the Dual Luciferase Reporter Assay System (Promega) and a Wallac Victor2 equipped with two reagent injectors (Perkin Elmer, Waltham, MA).

2.5. Quantitative real time (QRT)-PCR

MDA-MB-231 and MCF-7 cells were seeded in 6-well plates and cell lysates were collected 24 h later. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed using the TaqMan reverse transcription reagents kit (Roche) in a 20-μl final volume, according to manufacturer's instructions. The resulting cDNA was diluted to 60-μl final volume with RNase free water and used as a template for real-time PCR using TSPO specific primers and SYBR GREEN dye. Amplification of 18S ribosomal RNA served as a control.

2.6. Immunoblotting

MDA-MB-231 and MCF-7 cells were seeded at 150,000 cells per well in 6-well plates for 24 h. In the epigenetic analyses, cells were treated with either TSA or 5-AZA for 24 h before harvesting and extracting protein. Cells were lysed in 1X cold RIPA buffer (Cell