Differential expression of the stress-associated proteins FKBP51 and FKBP52 in human and New World primate cells.

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ABSTRACT

The proteins FKBP51 and FKBP52 are involved in regulation of the stress response due to their effects on the glucocorticoid receptor (GR) that binds cortisol. FKBP51 and FKBP52 may occupy the same site in the GR complex. When FKBP52 is included in the complex, cortisol responsiveness is enhanced. In contrast, the inclusion of FKBP51 inhibits cortisol responsiveness. Therefore, the relative expression of these proteins partly determines cellular responsiveness to cortisol. To begin to understand how these proteins are regulated, we have studied their gene expression in two cell lines, EBV-transformed squirrel monkey lymphoblasts (SML) and human lymphoblasts (HL), that exhibit different levels of the two proteins. The relative levels of FKBP51 and FKBP52 and their respective messenger RNAs were measured in SMLs and HLs. FKBP51 was 3.7-times higher in SMLs than in HLs, whereas FKBP52 in SMLs was 40% of that in HL. FKBP51 mRNA was 3.2-times higher in SML and FKBP52 mRNA was 53% of that in HL. Our results show that the changes in mRNA and protein levels correspond, suggesting that mechanisms controlling mRNA levels are important for determining the overall proteins levels in the two cell lines. Understanding regulation of the levels of FKBP51 and FKBP52 may be important for glucocorticoid signaling dysfunction.

INTRODUCTION

FKBP51 and FKBP52 are proteins associated with the glucocorticoid receptor (GR), a member of a family of steroid hormone receptors that proceed through an assembly pathway to form a mature receptor heterocomplex. This process involves a number of chaperone proteins, including Hsp90. The mature GR complex, that binds glucocorticoids with high affinity, contains the complex-stabilizing protein p23, Hsp90 and the FK506-binding protein FKBP52 (Nair et al. 1997; Riggs et al. 2003; Davies et al. 2005). However, FKBP52 is not the only protein that can incorporate into the GR complex. There are several proteins that occupy the same site in the GR complex through their association with Hsp90. These are FK506-binding FKBP51, cyclosporin A-binding cyclophilin 40, and the protein phosphatase 5 (PP5). A model has emerged for GR responsiveness in which incorporation of FKBP52 or FKBP51 into the GR heterocomplex has opposing effects on GR signaling. When FKBP52 is present in the GR heterocomplex, the GR exhibits high activity, whereas the presence of FKBP51 in the

heterocomplex confers low GR activity (Cheung and Smith 2000; Denny et al. 2000; Davies et al. 2005; Wochnik et al. 2005). Therefore, the *relative* expression of FKBP51 and FKBP52 would be expected to impact overall GR responsiveness.

To gain insight into the mechanisms controlling the levels of FKBP51 and FKBP52, cell lines that differentially express FKBP51 and FKBP52 were used for comparative studies. The cell lines chosen as models of differential expression of FKBP51 and FKBP52 were Epstein-Barr virus (EBV)-transformed human and squirrel monkey lymphoblasts (Reynolds et al. 1999). Squirrel monkeys are South American New World primates that exhibit reduced GR responsiveness and high levels of the GR-activating hormone cortisol relative to humans. New World primate glucocorticoid resistance has been associated with high levels of FKBP51 and low levels of FKBP52 (Scammell et al. 2001). The EBV-transformed squirrel monkey lymphoblasts (SML) were shown to exhibit low glucocorticoid hormone binding affinity relative to the EBV-transformed human lymphoblasts (HL). Furthermore, analysis of the glucocorticoid receptor (GR)-associated proteins, FKBP51 and FKBP52, by Western blot demonstrated that SML underexpress FKBP52 and overexpress FKBP51 relative to HL (Reynolds et al. 1999). Thus, the SML cell line is an in vitro model that recapitulates glucocorticoid resistance in New World primates. As a result of differences in the levels of FKBP51 and FKBP52, SML and HL also serve as useful models in which to study differential expression of FKBP51 and FKBP52.

To begin to understand how the FKBP51 and FKBP52 genes (*FKBP5* and *FKBP4,* respectively) are expressed, protein and mRNA levels in SML and HL were determined. Protein levels were evaluated by Western blot to confirm differential expression of FKBP51 and FKBP52 in the two cell lines. Next, steady state mRNA levels were determined by qPCR. The results show that differences in mRNA levels were consistent with the observed differences in protein levels in the two cell lines. These data provide a foundation on which researchers may continue to explore the regulation of FKBP51 and FKBP52 gene expression.

MATERIALS AND METHODS

Materials

Culture medium, penicillin-G, streptomycin and defined fetal bovine serum (FBS) were obtained from MidSci (St. Louis, MO). Nitrocellulose, precast 7.5% polyacrylamide gels, anti-mouse IgG secondary antibody and the Clarity Western ECL Substrate immunodetection kit were from Bio-Rad Laboratories (Hercules, CA). Generation of monoclonal antibodies to FKBP51 and FKBP52 was described earlier (Nair et al. 1997). Monoclonal antibody to beta actin was purchased from Santa Cruz Biotechnology, Inc. (Dallas TX). RIPA buffer and protease inhibitor were from Cell Signaling Technology, Inc. (Danvers, MA).

Cell culture

Squirrel monkey and human lymphoblasts were described previously (Reynolds et al. 1999). Squirrel monkey lymphoblasts may be obtained from American Type Cell Collection (Rockville, MD), ATCC number CRL-2311. Both cell lines were grown in

suspension cultures in RPMI 1640 supplemented with 10% FBS, 50 U/mL penicillin G and 0.05 mg/mL streptomycin, at 37° C in a humidified atmosphere of 5% CO_2-95% air.

Comparison of Protein Levels: Western Blot

Whole cell extracts were prepared in RIPA buffer containing protease inhibitors, then dissolved in 2X concentrated sample buffer. Total protein (10 µg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. The blots were incubated in PBS (pH 7.4), containing 0.1% Tween-20 and 5% nonfat milk (blocking buffer), for 1 h at room temperature. Incubation with primary antibodies was carried out at 4° C in blocking buffer overnight. After washing, blots were incubated with secondary antibody for 1 h at room temperature and developed using the Clarity Western ECL Substrate (Bio-Rad Laboratories) Western Blotting Immunodetection System. Western blots were quantified by densitometry and normalized against beta actin, the loading control. Results were expressed as protein levels relative to HL.

Comparison of mRNA levels: qPCR

Cell collection, RNA Extraction and cDNA Synthesis

Cells were collected by centrifugation at 500 g for 5 min, washed with PBS and placed immediately on dry ice for shipment to ARQ Genetics (Bastrop, TX). Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Sciences, Germantown, MD) according to the manufacturer's instructions with optional DNase treatment. Subsequently, 1 µg total RNA was used as template to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Primer Design

Primers for all SYBR assays were designed using Primer 3 (Ye et al. 2012) and alignment tools available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). Squirrel monkey and human FKBP51 and FKBP52 mRNA sequences were compared using data from GenBank at the National Center for Biotechnology Information, NCBI, (FKBP51 mRNA accession numbers NM_001145775.2, NM_001145777.1, NM_0011457776.1, NM_004117.3 and NM_001280014.1; FKBP52 mRNA accession numbers NM_002014.3 and XM_010330055.1). Regions of identical sequences were selected for analysis and primers were designed to target multiple regions of each transcript. Four regions of FKBP51 mRNA were targeted using the primers FKBP51.1-FKBP51.4 in Table 1. Two regions were suitable for targeting the FKBP52 mRNA in squirrel monkey and human using the primers FKBP52.1-FKBP52.2 in Table 1. Melting curve analysis was performed to ensure single-product amplification for all primer pairs. Sequences for all primers are listed in Table 1.

Real Time PCR Analysis

Real time PCR was performed on the ABI 7900HT Fast Real Time PCR System (Applied Biosystems) using assays specific for each gene of interest. For SYBR assays, each reaction well contained 5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems), cDNA equivalent to 20 ng of total RNA and 400 nM each of forward and reverse amplification primers in a reaction volume of 10 µL. Cycling conditions were as

follows: 95° C for 10 minutes for polymerase activation, followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute. Data analysis was performed using Sequence Detection System software from Applied Biosystems, version 2.4. The experimental Ct (cycle threshold) was calibrated against an endogenous control, 18s RNA or beta actin RNA. Relative gene expression levels were calculated by the ddCt method (Pfaffl 2001).

Data Analysis

For Western blot analysis, protein levels were quantified by densitometry and normalized to beta-actin as a loading control. Each experiment was repeated at least two times. Statistical analysis was performed on normalized data using a t-test with $p < 0.05$ considered as statistically different. Data was then represented as the average fold change relative to HL protein levels.

For real time PCR, relative levels of SML and HL amplicons were calculated by the ddCt method (Pfaffl 2001) for each mRNA region targeted by the primers in Table 1. Each experiment was performed at least two times. Data from all FKBP51 or FKBP52 primer pairs in a single analysis were statistically analyzed using a t-test with $p < 0.05$ considered as statistically different. The data was represented as the average fold change relative to HL mRNA levels.

RESULTS

Comparison of Protein Levels: Western Blot

Western Blot was performed on SML and HL to confirm differential expression of FKBP51 and FKBP52 in the cell lines used for our experiments (Figures 1a and 1b). For comparison of protein levels, data were graphically represented as protein levels relative to HL protein levels. Our results show that SML express 3.7-times greater FKBP51 protein relative to HL (3.7 ± 0.75, Figure 1c). As reported previously, squirrel monkey FKBP51 exhibits greater electrophoretic mobility than human FKBP51 (Reynolds et al. 1999; Scammell et al. 2001). In contrast to FKBP51 protein, the levels of FKBP52 were 40% of that in HL (0.4 ± 0.02, Figure 1d). These data confirm differential expression of FKBP51 and FKBP52 in SML and HL cell lines, as previously reported in the literature (Reynolds et al. 1999).

Comparison of mRNA Levels: qPCR

qPCR was used to determine the relative amount of FKBP51 and FKBP52 mRNA in each cell line. Because the cells were from distinct species, the primers for qPCR were designed to target exon sequences that are conserved in squirrel monkeys and humans. For comparison of mRNA levels, data were graphically represented as mRNA levels relative to HL mRNA levels. The comparison of mRNA levels for each cell line showed FKBP51 mRNA was 3.2-times higher in SML than in HL (3.2 ± 0.19, Figure 2a). FKBP52 mRNA in SML was 53% of HL (0.5 ± 0.08, Figure 2b). These results are consistent with the relative differences in FKBP51 and FKBP52 protein observed in SML and HL. Furthermore, the results suggest that differences in mRNA levels may contribute to the differences in relative FKBP51 and FKBP52 protein levels in these cells.

DISCUSSION

A number of mechanisms may contribute to the regulation of gene expression. These include promoter activity, nuclear processing and transport of mRNA, mRNA stability, translational regulation, protein stability and epigenetic mechanisms such as gene silencing. As a result, changes in protein may be a reflection of changes occurring by any or all of these mechanisms. To date, the underlying mechanisms by which cellular FKBP51 and FKBP52 protein levels are regulated are not well understood. Our results show that, in SML and HL cell lines, the relative levels of FKBP51 and FKBP52 mRNA and protein are consistent. These data provide evidence that regulation of FKBP51 and FKBP52 protein levels occur, in part, at the level of mRNA.

There are at least three mechanisms that may explain the observed differences in squirrel monkey and human FKBP51 and FKBP52 mRNA levels: (1) interspecies differences in enhancer or promoter regions of the FKBP51 and FKBP52 genes, *FKBP5* and *FKBP4* respectively, (2) differences in microRNA activity in cells, and (3) single nucleotide polymorphisms. We previously isolated and sequenced a 2000 bp segment immediately upstream of squirrel monkey *FKBP5* (Accession number JX503530). Comparison of this segment with the corresponding region of the human transcript variant 1 promoter reveals the presence of two Alu elements in the human *FKBP5* promoter sequence that are absent in the squirrel monkey promoter sequence (unpublished data). Similarly, the human *FKBP4* 5'-flanking region contains multiple Alu-like sequences that are absent in the squirrel monkey sequence. The presence of Alu insertions may affect the activity of regulatory sequences (Schmitz 2012). Therefore, such insertions may impact the relative levels of mRNA present in cells from different species.

MicroRNAs (miRs) miR-511, miR-100, miR-15a and miR-29c have been identified as regulators of FKBP51 (Bhushan and Kandpal 2011; Li et al. 2013; Volk et al. 2016; Zheng et al. 2016) or FKBP52 levels (Joshi et al. 2016). A comparison of the 3' untranslated region sequences (3' UTRs) in human and squirrel monkey *FKBP5* revealed that sequences potentially targeted by miR-511, miR-100 and miR-15a (AAAAGA, UACGGGU and UGCUGCU, respectively) were identical in human and squirrel monkey DNA. In addition, the target sequence for miR-29c, GGUGCUA, was identical in human and squirrel monkey *FKBP4*. A comparison of miR-511, miR-100, miR15a and miR-29c sequences in human and squirrel monkey DNA revealed no differences in the regions that align with the target sequences (above) in *FKBP4* and *FKBP5* 3' UTRs. These data suggest that these miRs may interact with and regulate *both* human and squirrel monkey FKBP51 and FKBP52 mRNAs.

A number of single nucleotide polymorphisms (SNPs) have been identified in human *FKBP5*. *FKBP5* SNPs have been associated with altered stress responses (Ising et al. 2008; Bortsov et al. 2013), altered susceptibility to mental disorders (Binder et al. 2004; Koenen et al. 2005; Klengel et al. 2013), and development of or recovery from substance abuse disorders (Levran et al. 2014). Such influences are hypothesized to relate to the effect of FKBP51 on cortisol responsiveness. Currently, how these SNPs affect *FKBP5* expression levels is not fully understood.

The present study has shown that FKBP51 and FKBP52 protein levels are consistent with mRNA expression levels in two cell types that exhibit differential expression of the two proteins. These data contribute to our understanding of the regulation of these important antagonistic glucocorticoid signaling proteins by suggesting that mechanisms controlling mRNA levels are significant contributors to overall protein levels. As a consequence of differential expression of FKBP51 and FKBP52 in New World primates and humans, cell lines derived from squirrel monkeys and humans may provide a useful model for the discovery of important regulatory mechanisms of FKBP51 and FKBP52 gene expression.

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TABLES

Table 1. Primer sequences used for qPCR of FKBP51 and FKBP52 mRNA.

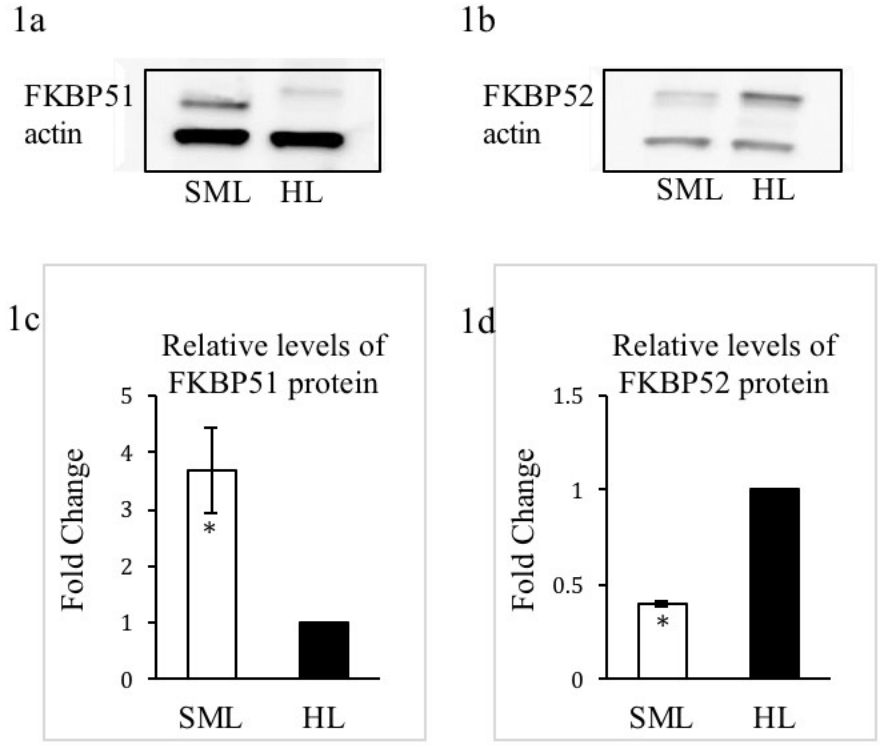
Primer Name	Exon	Forward or Reverse	Sequence
FKBP51.1F	6	F	5’ATGGGACATTGGGGTGGCTA3’
FKBP51.1R	6	R	5’TGCATATTCTGGTTTGCACAGT3’
FKBP51.2F	5	F	5’CATCAAGGCATGGGACATTGG3’
FKBP51.2R	5	R	5’GCATATTCTGGTTTGCACAGT3’
FKBP15.3F	8	F	5’AGATGTGGCATTCACTGTGGG3’
FKBP51.3R	8	R	5’CTCCAGAGCTTTGTCAATTCCA3’
FKBP51.4F	8	F	5’GAGATGTGGCATTCACTGTGG3’
FKBP51.4R	8	R	5’TCTCCAGAGCTTTGTCAATTCC3’
FKBP52.1F	6	F	5’GGTTGCACTGGAAGGGTACT3’
FKBP52.1R	6	R	5’TGGCCCTCTCCAGACCATAA3’
FKBP52.2F	3	F	5'CAGTCTGGATCGCAAGGACA3'
FKBP52.2R	4	R	5'TATGGCAATGTCCCAAGCCT3'

FIGURE LEGENDS

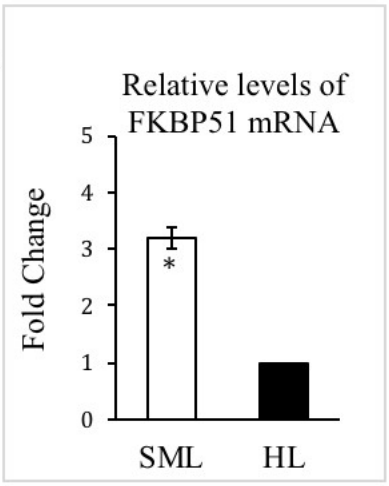
Figure 1. FKBP51 and FKBP52 proteins are expressed differently in SML and HL. Whole cell extracts were separated by SDS-PAGE and analyzed by Western Blot for FKBP51 (1a) or FKBP52 (1b) and beta actin as a loading control. Protein levels were quantified by densitometry and represented graphically as fold change relative to HL protein levels (Figures 1c and 1d). Bar represents standard error, n=2. *, protein levels considered statistically different.

Figure 2. The relative levels of FKBP51 and FKBP52 mRNA differ in SML and HL. Total RNA was extracted and used as template for qPCR. Primers shown in Table 1 were designed to target specific exons that contain conserved sequences in humans and squirrel monkeys. Relative levels of mRNA were calculated by the ddCt method for FKBP51 (2a) or FKBP52 (2b) and represented graphically as fold change relative to HL mRNA levels. Bar represents standard error, n=2 for FKBP5 and n=3 for FKBP4. *, mRNA levels considered statistically different.

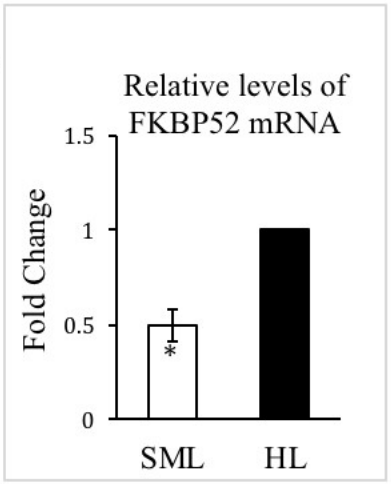
FIGURES



2a


Relative levels of
FKBP51 mRNA
Fold Change
5
4
3
2
1
0
*
SML
HL

2b


Relative levels of
FKBP52 mRNA
Fold Change
1.5
1
0.5
0
*
SML
HL