

Clobetasol down-regulates SLPI expression in U937 monocytoid cells

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Abstract. In order to investigate how glucocorticoids affect the expression of secretory leukocyte peptidase inhibitor (SLPI), which is overexpressed in a variety of cancers, clobetasol was added to cell culture medium of U937 cells and the SLPI mRNA levels were examined. The *in vitro* effect of the treatment on SLPI expression was detected by reverse transcriptase-polymerase chain reaction. Clobetasol treatment of U937 cells induced an up- and down-regulation of SLPI expression in a dose-dependent manner. Western blotting confirmed the down-regulation of SLPI protein expression. We hypothesized a loop formation in the SLPI genome domain, in which the glucocorticoid receptor regulates bi-directional transcriptional activity.

Introduction

Secretory leukocyte peptidase inhibitor (SLPI) is a 12 kDa serine protease inhibitor, which is minimally produced by normal epithelial cells lining the respiratory, digestive, and reproductive tracts (1,2). SLPI is overexpressed in a variety of cancers, but its role in cancer is not well understood. SLPI genes are often up-regulated under tumorigenic conditions. Overexpression of this protein correlates with more-invasive forms of the cancer (3). The expression levels of SLPI gene have been associated to both disease-free and overall survival (4). In addition, gene-targeting experiments in mice have indicated that carcinogenesis is suppressed in the SLPI knockout mice (5). Accordingly, repression of SLPI expression may be expected to have interference effects on cancer progression. The SLPI gene is regulated by glucocorticoids, which play an essential role in embryonic and cancer development (6). Due to the wide spectrum of activity and pro-apoptotic

properties, glucocorticoids are one of the most commonly drugs used in hematological malignancies and are also used as co-treatment chemotherapy regimens in other types of cancers. Glucocorticoid receptor (GR) is a member of the nuclear hormone receptor family (7). Hormone activated GR binds to glucocorticoid responsive elements (GRE) near the target genes (8). However, glucocorticoids could play a complex role in cancer epidemiology, biology and treatment (9).

Materials and methods

Cell culture. The human cell lines U937 and K562 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Reagents preparation. Reagents used were dissolved in ethanol and subsequently diluted at a stock concentration of 10 mM. It was stored at -20°C until use. For the cell treatments, a range of 0.5-10.0 µl was added into 1 ml of cell culture medium.

Reverse transcriptase-polymerase chain reaction (RT-PCR). SLPI and GAPDH mRNAs were analyzed by semi-quantitative RT-PCR. Total-RNA was extracted by an RNA isolation kit (Takara, Japan). Total-RNA (2 µg) was reverse-transcribed using a Phusion RT-PCR kit (NEB) as described in the manufacturer's protocol. Cycle-based PCR was used to semi-quantitate the ubiquitin1 and presenilin1 gene level. GAPDH was also used as an internal loading control. All the samples were determined within 3 months after collection. Thermocycling was performed according to the manufacturer's instructions at 55°C annealing temperature in a final volume of 10 µl including TaqDNA polymerase.

Western blot analysis. Equal amounts of protein samples were used for Western blot analysis using anti-SLPI (Abnova) and anti-Erk2 (AnaSpec, Inc.) antibodies, and quantified by densitometry. All the Western blot analyses were repeated at least 3 times and the representative data are shown.

Results and Discussion

In order to investigate how glucocorticoids affect the expression of SLPI, clobetasol as well as dexamethasone were added into the cell culture medium of U937 or K562

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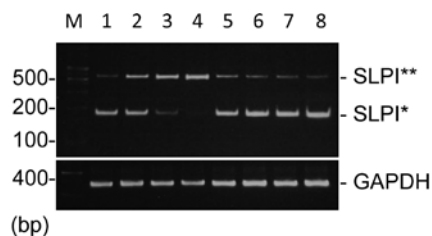


Figure 1. Semi-quantitative RT-PCR was performed using primers specific to SLPI on 100 ng total-RNA prepared from U937 cells treated without (lane 1, lane 5) or with clobetasol propionate (Sigma) at the final concentration 10^{-7} M (lane 2), 10^{-6} M (lane 3), 10^{-5} M (lane 4) or with dexamethasone (Sigma) at the final concentration 10^{-7} M (lane 6), 10^{-6} M (lane 7), 10^{-5} M (lane 8) for 24 h. Specific expression was determined in relation to the expression of the housekeeping gene GAPDH used as an internal loading control. At least 4 independent experiments were done, and a typical paired result is documented.

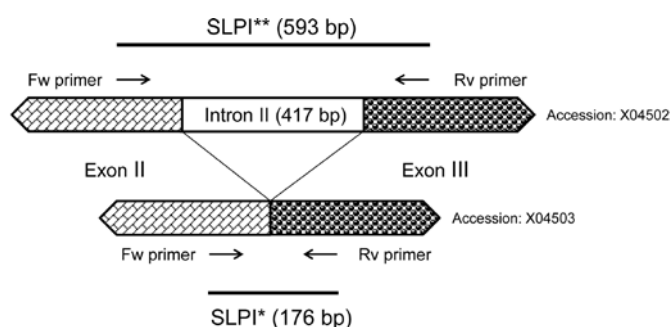


Figure 2. Schematic representation of the SLPI genome structure between exon 2 and exon 3, and the site for primers used in the RT-PCR experiment. The predicted PCR products (SLPI* and SLPI** correspond to short and long bands in Fig. 1, respectively) by sequencing are depicted.

cells and the levels of SLPI expression were examined. We employed RT-PCR analysis to quantify the expression level of the SLPI gene. Total-RNA was extracted 24 h after treatment for detection of the SLPI, and was reverse-transcribed. The levels of mRNA were determined by the conventional RT-PCR using primers specific to SLPI forward, 5'-CTGCCAGTGCCTTAGATACA-3' and reverse, 5'-CATCAAACATTGGCCATAAGT-3', (expected size, 176 bp); and GAPDH forward, 5'-TCCCATCACCATCTTCCA-3' and reverse, 5'-CATCACGCCACAGTTTCC-3', (expected size, 376 bp).

As shown in Fig. 1, there were 2 sizes of band for SLPI expression by the RT-PCR. After pre-treating the cells with a set of different concentrations of clobetasol, we found that expression levels of the short band (SLPI*) greatly decreased, whereas those of the long band (SLPI**) greatly increased, in a dose-dependent manner in U937 monocytoid cells. However, gene expression of the two SLPI bands was unaltered with increasing concentrations of dexamethasone compared with the untreated ethanol vehicle. A larger amount of dexamethasone (10^{-4} M) could reduce the expression levels of the SLPI* band and could increase SLPI** gene expression (data not shown), probably because of the efficacy difference of glucocorticoids. On the contrary, the expression of the housekeeping gene GAPDH was also unaltered. There was almost no difference in the results of gene expression between U937 and K562

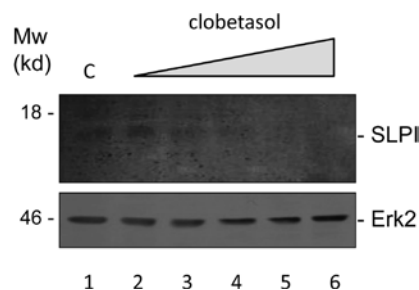


Figure 3. Dose-dependent repression of SLPI protein expression by clobetasol. U937 cells were treated without (lane 1) or with clobetasol propionate at a final concentration of 10^{-8} M (lane 2), 10^{-7} M (lane 3), 10^{-6} M (lane 4), 10^{-5} M (lane 5), 10^{-4} M (lane 6) for 48 h. The levels of SLPI protein were detected by Western blot analysis using anti-SLPI. Western blotting with anti-Erk2 antibody was also shown as equal levels of protein loading.

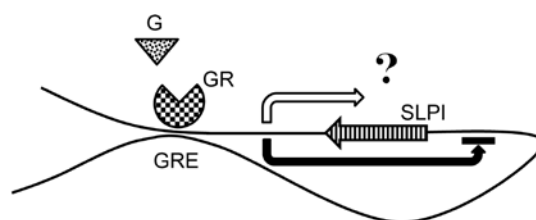


Figure 4. Schematic representation of a hypothetical loop formation in the SLPI domain. The glucocorticoid receptor regulates bi-directional transcriptional activity of both promoters by mechanisms related to the loop.

cells. To exclude the possibility of carry-over contamination, reactions containing all RT-PCR reagents including primers without sample RNA were performed as negative controls. No such contamination was detected (data not shown). Sequencing of the excised bands from the analysis gel revealed that the short SLPI band was the SLPI exon as expected, and that the long one included intron II in addition to the short one. The structures of the 2 bands are illustrated in Fig. 2. There was no band found with RT-PCR using the primers within either intron I or intron III of the SLPI genome even with similar clobetasol stimulation (data not shown).

To further confirm the expression status of SLPI, Western blotting methods with anti-SLPI and anti-Erk2 antibodies were performed to analyze the level of SLPI protein in U937 cells. As shown in Fig. 3, after pre-treating the cells with a set of different doses of clobetasol, we found that the SLPI protein expression was decreased with increasing concentrations of clobetasol. A final concentration 10^{-5} M of the clobetasol diminished the SLPI protein expression by more than 90% after 48 h stimulation. This protein expression profile approximately agreed with the result of the short band of RT-PCR shown in Fig. 1.

The kinetics of the GR-stimulated transcriptional output is very complex, and different cell types vary in their response to GR (10,11). It has been suggested that loop formation of GR responsive genome structure plays a role in transcriptional regulation (12). In the loop structure, specific long range interactions may play a role in GR function (12). It is plausible that the long size of RT-PCR product in Fig. 1 might be transcribed reversely from a site downstream the SLPI gene in the genomic loop (Fig. 4). Examinations of multiple sets of

GR-regulated genes will be required to explore the validity of the concept. There is accumulating evidence that a lack of SLPI in cancer cells suppresses tumor formation, and that SLPI is up-regulated in tumor formation (13,14). Here, we show that clobetasol is a potent transcriptional repressor for SLPI expression. The precise mechanism of transcriptional regulation of SLPI by clobetasol remains unclear. SLPI may be regulated by various factors. More studies including *in vivo* experiments need to be undertaken to elucidate the molecular mechanisms, and further analysis of SLPI function in relation to the tumor forming capacity may lead to the development of a novel treatment modality.

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