Key promoter elements involved in transcriptional activation of the cancer-related gene coding for S100P calcium-binding protein

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Abstract. S100P gene encodes a calcium-binding protein expressed in different tumor tissues and is functionally implicated in malignant phenotype. Despite consistent relationship to cancer, regulation of S100P gene expression has remained unexplored. Here we determined the transcription start and defined the S100P core promoter. Using a series of the promoter constructs analyzed by dual luciferase reporter assay, we identified SMAD, STAT/CREB and SP/KLF binding sites as critical cis-elements required for S100P expression in cancer cells. We also demonstrated in EMSA that these elements bind nuclear factors, and showed their functional significance by promoter deletion analysis. This study represents the first coherent contribution to understanding of factors and pathways responsible for S100P gene activation in cancer.

Introduction

S100 proteins, belonging to a family of EF-hand calcium-binding proteins, act as intracellular and/or extracellular signaling molecules with regulatory functions in diverse cellular processes such as differentiation, proliferation and malignant transformation. Expression of S100 proteins has been associated with several human diseases including cardiomyopathy, diabetes, neurodegenerative disorders and cancer (1). S100P protein, originally isolated from placenta, is a small 11-kDa protein consisting of 95 amino acids (2,3). It forms calcium-dependent homodimers (4,5) and interacts with a cytoskeletal protein ezrin, which plays a role in cell adhesion and motility (6). S100P also binds to a CacyBP/SIP component of the ubiquitin pathway involved in degradation of the cell adhesion molecule ß-catenin (7). Moreover, S100P exists in a soluble form and functions as an extracellular RAGE receptor ligand modulating cell proliferation and survival via activation of MAP kinase pathway (8).

Based on molecular investigations, tissue expression studies and microarray analyses, S100P has been implicated in tumor development. Expression of S100P was detected in tumor cell lines and carcinomas derived from the breast, pancreas, prostate, colon, and other tumor types, and was associated with immortalized, malignant, hormone-independent and chemoresistant phenotype (9-17). However, only few reports describe regulation of S100P expression. In the prostate carcinoma cell lines and tissues, expression of S100P is controlled by androgens (18) and depends on hypomethylation of S100P gene (19,20). In the gastric cancer cells, S100P biosynthesis is induced by retinoic acid (21).

Despite increasing number of studies showing biological significance of S100P protein and its relationship to cancer, no promoter analysis of S100P gene has been published so far and data on its transcriptional control are missing. This fact motivated us to perform the present investigation and define critical regulatory elements involved in activation of S100P transcription.

Materials and methods

Cell culture. HeLa, SiHa and C33A cervical carcinoma cells, MFC-7 and BT-20 breast carcinoma cells were cultured under standard conditions in DMEM supplemented with 10% FCS, L-glutamine and 100 units/ml penicillin/streptomycin mixture (all from BioWhittaker, Verviers, Belgium) in humidified air containing 5% CO_2 at 37˚C.

5'-RACE mapping of transcription start site. Rapid amplification of the 5' S100P cDNA ends was performed using RACE kit (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations, using the gene-specific primers GSP1 S100P-A and GSP2 S100P-A (Table I) combined with the abridged anchor primers supplied with the kit. cDNA template was produced by reverse transcription

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Table I. Primers and oligonucleotides used in the RACE, PCR and EMSA experiments.

Purpose	Designation	Sequence (5'→3')
5' RACE	GSP1-A	ATCTGTGACATCTCCAGGGCATC
	GSP2-A	GAAACTCTTCCGTCCTGAGTTTA CCATGGGC
PCR of the promoter fragments	*Kpn*-prom-S100P-S (-236)	TCC*GGTACC*TCAGTGATGGCGCCGAGACA
	Bgl-prom-S100P-A (-14)	GA*AGATCT*GGTGCTTTATAAGGCAGCCTA
	Bgl-prom-S100P-A2 (+58)	GA*AGATCT*GGTGCTAGATTCAGACCCAC
	Kpn-prom-S100P-S (-1234)	TCC*GGTACC*TCTGAGTACCTCCGCTATAG
	Xho-prom-S100P-A2 (+58)	TCCG*CTCGAG*GTGCTAGATTCAGACCCACC
	del-prom-S100P-S (-124)	TGCATTTCATCAGAACTGAGCAC
RT PCR	S100P-S	AAGGGGGAGCTCAAGGTGCTGA
	S100P-A	ATCTGTGACATC TCCAGGGCATC
	h-ß-actin-S	CCAACCGCGAGAAGATGACC
	h-ß-actin-A	AGGATCTTCATGAGGTAGTCAGTC
EMSA	prom-SMAD	GATGGCG<u>CCGAGACA</u>CAGGT
	cons1-SMAD	GGAGGTATGCAGACAACGAGTCAG
	cons2-SMAD	AGCCAGACA
	prom-STAT/CREB	GGGAAAGG<u>TTCCAGAA</u>ACGTCAT
	cons-STAT1	ATTCCTGTAAG
	cons-STAT4	TTTCCCCCGAAA
	cons-CREB	GATTGCCTGACGTCAGACAGC
	prom-SP/KLF	AGGAGGAA<u>GGTGG</u>GTCTGAA
	cons-SP/KLF	GGGGGTGGG
Inverse	del-SMAD-A	CCATCACTGAGGTACCTATCG
PCR	del-SMAD-S	ACAGGTGAACACTGTAAAATGTGGATGC
	del-STAT/CREB-A	CCCCAGCCAAGGCCCAGG
	del-STAT/CREB-S	ATCACAACGATCCATTTCATCAG
	del-SP/KLF-A	TTCCTCCTGGGGGCTGGC
	del-SP/KLF-S	GTCTGAATCTAGCACCAGATC

Sequence motifs written in italic indicate restriction enzyme sites used for cloning of the amplified fragments, core sequences of the proposed transcription factor binding sites are underlined.

with Mo-MuLV reverse transcriptase (Finnzymes, OY, Finland) of RNA isolated from HeLa cells as described before (16). PCR consisted of initial denaturation step (2 min at 94˚C), 35 amplification cycles (45 sec at 94˚C, 40 sec at 60˚C and 40 sec at 72˚C) and the final extension (7 min at 72˚C). The 5' end of the RACE product was determined by sequencing.

Bioinformatic analysis. Potential cis-elements in S100P promoter were identified using different software including MatInspector (www.genomatics.de), Promoter Scan (bimas.dcrt.nih.gov/molbio/proscan), Promoter 2.0 (www.cbs.dtu.dk/services/promoter) and SoftBerry (www.softberry.com).

Construction of promoter reporter plasmids. Promoter fragments spanning the -236/-14, -236/+58 and -1234/+58 regions were amplified by PCR from HeLa genomic DNA using the primers listed in Table I. Amplified fragments were digested with *Kpn*I and *Bgl*II or *Xho*I and cloned into pGL3-Basic vector (Promega) upstream of the luciferase reporter gene. Deletion constructs were amplified by inverse PCR on the -236/+58 reporter plasmid using the listed primers (Table I) with the Phusion HF polymerase (Finnzymes) under the following conditions: denaturation for 5 min at 98˚C, 35 cycles for 40 sec at 98˚C, 40 sec at 58˚C, 80 sec at 72˚C and final elongation for 5 min at 72˚C.

Transient transfection and reporter gene assays. The cells were plated into 30-mm Petri dishes to reach approximately 60% monolayer density the next day. Transfection was performed with 2 μg of the fragment-pGL3 plasmid and 100 ng of pRL-TK renilla vector (Promega) using GenePorterII reagent (Genlantis, San Diego, CA) according to manufacturer's recommendations. Reporter gene expression was

assessed 48 h after transfection using a Dual-Luciferase Reporter Assay system (Promega). For the mitogenic or hormonal stimulation or inhibition, transfected cells were trypsinized and plated into 24-well plates, allowed to attach for 20 h, starved in serum-free medium for 24 h and treated in triplicates by different compounds [EGF (1 nM), hydrocortisone (1 μM), mithramycin A (250 nM), PD98059 (50 μM), LY294002 (50 μM), all from Sigma, St. Louis, MA] for additional 24 h.

Preparation of nuclear extracts and electromobility shift assay (EMSA). Nuclear extracts were prepared from HeLa cells grown to a confluent monolayer in a 10-cm dish. The cells were washed twice with ice-cold PBS, scraped, resuspended in 1 ml of buffer A (10 mM HEPES pH 7.9, and 1.5 mM $MgCl_2$) and kept on ice for 15 min. The nuclei were pelleted by centrifugation for 5 min at 4,000 g at 4˚C and resuspended in 200 μl of buffer B (30 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin and leupeptin, 500 mM KCl, 10% glycerol) and kept on ice for 15 min. The extract was cleared by centrifugation for 5 min at 15,000 g at 4˚C and the supernatant was stored in aliquots at -80˚C until use.

EMSA was performed by mixing 5 μg of the nuclear extract with 40 fmol of oligonucleotides (Table I), end-labeled with [γ-^{32}P]-ATP using T4-polynucleotid kinase, in the binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 2 μg polydI-dC) for 25 min at room temperature. One-hundred molar excess of the unlabelled oligonucleotides was added to the reaction in the case of competitive assay. DNA-protein complexes were separated on the 5% native gel in the presence of 1x Tris-glycine buffer (25 mM Tris-HCl pH 8.0, 190 mM glycine, 1 mM EDTA).

Results

Identification of the core promoter of S100P gene. As the first step towards characterization of S100P promoter, we decided to localize the transcription initiation site, because the information from three different entries in Genebank database (namely NM_005980, BC006819, X65614) was inconsistent. We therefore performed an amplification of the 5' S100P cDNA ends (RACE). Sequence analysis of the 5' RACE product revealed a major transcription initiation site corresponding to a guanosine nucleotide localized 58 nt upstream of the first ATG codon. This position was in full agreement with the transcription start site predicted by the SoftBerry Fprom promoter prediction program.

In order to identify the core promoter, we amplified a series of genomic fragments from the 5'-flanking region of S100P gene using the gene-specific primers listed in Table I and a genomic DNA isolated from HeLa cells as a template. The fragments were inserted into the pGL3-Basic reporter vector upstream of the firefly luciferase gene. Functional analysis of the resulting promoter constructs was done by transient transfections of cervical carcinoma HeLa cells that naturally express S100P mRNA (16) and thus apparently contain all relevant components of the transcriptional machinery needed for S100P gene transactivation.

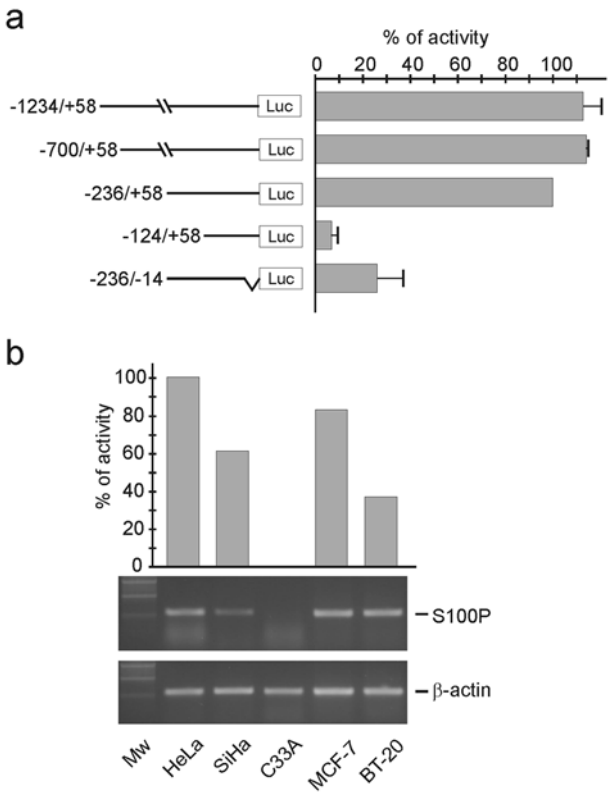

Figure 1. Basic promoter analysis and expression of S100P gene. (a) Transcriptional activities of the luciferase constructs containing truncated S100P promoter fragments. The numbers on the left indicate the position of terminal nucleotides of each genomic fragment with respect to transcription initiation site. Luciferase activities normalized to renilla are expressed in per cent relative to the core promoter construct (-236/+58) which was set to 100%, bars represent mean values with standard deviations. Each transfection experiment was repeated 3-5 times and examined in triplicates to yield reproducible data. (b) Luciferase activities of the core promoter in different cancer cell lines were assessed as described above. RT PCR analysis of S100P expression in the same cell lines is shown below the graph, C33A cell line was used as a negative control. PCR conditions were as follows: 95˚C for 3 min, 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 40 sec, and final extension 72˚C for 5 min. ß-actin was used as an internal standard.

The luciferase activity produced by different promoter constructs showed that the core promoter of S100P gene is located within the -236/+58 region (Fig. 1a). This region exhibited similar activity values as the larger -700/+58 and -1234/+58 constructs. Interestingly, removal of the terminal sequences from either side of this region resulted in significantly reduced activity, corresponding to less than 10% in the absence of -236/-125 fragment and to about 20% in the absence of -13/+58 region. This finding suggests that the terminal sequences of the core promoter contain critical regulatory elements whose cooperation is required for full transcriptional activation of the S100P gene.

Activity of the core promoter was also assessed in three additional human cancer cell lines, including SiHa cervical carcinoma cells, and MCF-7 and BT-20 breast carcinoma cells. Luciferase values measured in these cell lines were expressed as per cent of the activity obtained in HeLa cells (Fig. 1b). The core promoter displayed the highest activity in HeLa cells, slightly decreased activity in MCF-7, and SiHa cells and was considerably less active in BT-20 cells. RT PCR analysis of S100P gene expression showed that all these cell

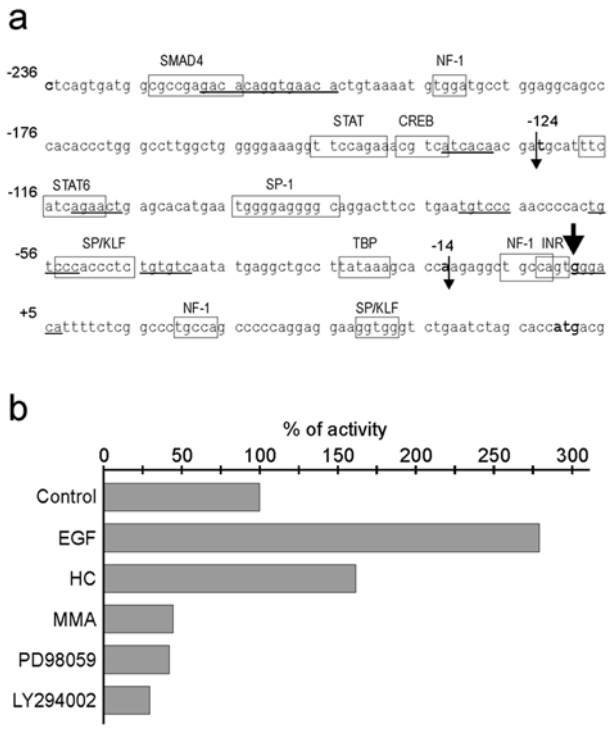

Figure 2. Sequence of the S100P core promoter and effects of different compounds on its activity in HeLa cells. (a) Nucleotide sequence of the -236/+58 region with potential binding sites for transcription factors enclosed in the boxes. Thick arrow indicates position of the transcription start site determined by RACE, thin arrows show the ends of terminal truncations. Underlined sequences correspond to GRE motifs. (b) Transcriptional activity of the S100P core promoter in response to epidermal growth factor (EGF), hydrocortisone (HC), mithramycin A (MMA) and inhibitors of signal transduction PD98058 and LY294002. Luciferase activities normalized to renilla are expressed in per cent relative to the activity measured in untreated HeLa cells (control) that was set to 100%.

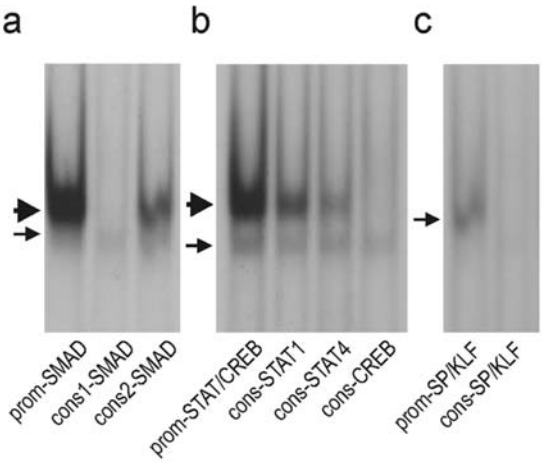

Figure 3. Electromobility shift assay of the putative binding sites for SMAD, STAT/CREB and SP/KLF transcription factors. EMSA was performed with the nuclear extracts from HeLa cells using the oligonucleotides derived from the predicted transcription factor-binding regions of the promoter. (a-c) DNA-protein complexes formed at putative SMAD-binding region a), STAT/CREB-binding region (b) and SP/KLF-binding region (c) in the absence and presence of the competitor oligonucleotides are depicted by arrows.

lines exhibit the endogenous S100P transcript. On this basis, further promoter experiments were done in HeLa cells that naturally express S100P gene and show the best transcriptional activation.

Prediction of potential cis-regulatory elements. Computer analysis of the core promoter sequence with different software predicted several cis-acting elements potentially contributing to regulation of S100P gene transcription. Fig. 2a shows selected promoter elements of the highest predictive significance. For example, Inr initiator binding site was predicted at position -1/-4 just in front of the transcription initiation site (TIS) and TATA box was found at -20/-25 position within the expected distance from TIS, supporting the view that the localization of TIS by 5' RACE was correct. In addition, the region upstream of TATA box was proposed to contain the binding sites for SMAD, overlapping STAT/CREB and SP-1 transcription factors, as well as several scattered GREs (glucocorticoid response elements). On the other hand, the sequence downstream of TIS was predicted to bind NF-1 and SP/KLF (Krüppel-like) transcription factors.

Some of the transcription factors potentially binding to S100P promoter are known to respond to signal transduction pathways activated by mitogenic or hormonal stimulation (22-24). These pathways involve signal transmission through MAPK or PI3K (phosphatidyl inositol 3-kinase) and thus can be blocked by specific inhibitors. Therefore, we analyzed the S100P promoter activity in HeLa cells transiently co-transfected with the core promoter luciferase construct and renilla plasmid. As expected, S100P activity was induced >2-fold by epidermal growth factor and 1.5-fold by hydrocortisone, whereas mithramycin A, PD98059 and LY294002, inhibitors of SP-1, MAPK and PI3K, respectively, reduced the promoter activity to less than a half (Fig. 2b). These data indicated that the modulation of S100P promoter activity is consistent with the predicted set of the promoter elements and that at least some of them can transmit the signaling to transcriptional regulation of the S100P gene.

Electromobility shift assay of the predicted binding sites. As shown above, both terminal regions of the core promoter were required for the full promoter activation and their removal led to significant decrease of the luciferase values. The 5' region (-236/-125) was predicted to contain SMAD4 and STAT/CREB binding sites, and the 3' region (-13/+58) appeared to include a recognition element for SP/KLF transcription factors. In order to see, whether these binding sites are occupied by nuclear proteins, we carried out an electromobility shift assay (EMSA) using oligonucleotides derived from corresponding promoter fragments alone or in competition with excess of consensus binding motifs (Table I).

First we used an oligonucleotide corresponding to -230/-211 region that was proposed to contain the binding site for SMAD transcription factor (Prom-SMAD), which produced one dominant band and one less shifted weak band (Fig. 3a). The dominant band was completely lost in competitive assay with non-labeled 24-nt SMAD-binding consensus (cons1-SMAD), whereas only part of it was competed by a shorter 9-nt consensus probe (cons2-SMAD) supporting the view that the promoter fragment can bind a SMAD-related transcription factor and that the flanking sequences contribute to this interaction. In the second analysis, we used a probe

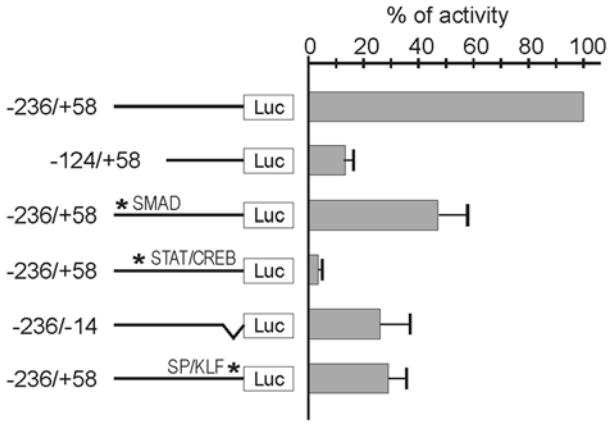

Figure 4. Transcriptional activities of S100P promoter constructs with internal deletions in putative regulatory elements. Reporter constructs with internal deletions were produced by inverse PCR and transfected to HeLa cells. Luciferase activities normalized to renilla are expressed in per cent relative to the core promoter construct (-236/+58) which was set to 100%, bars represent mean values with standard deviations. Each transfection experiment was repeated 3-5 times and examined in triplicates to yield reproducible data.

corresponding to -155/-133 region presumably containing the overlapping binding sites for STAT and CREB transcription factors. The EMSA profile showed one weak band that was independent of the addition of non-labeled competitor oligonucleotides and one very strong band, which was partially competed by two 11-nt and 12-nt consensus motifs corresponding to STAT1 and STAT4 binding sites, respectively (Fig. 3b). A complete loss of this strong band was observed with a non-labeled CREB consensus probe suggesting either preferential/stronger binding of CREB over STAT to this promoter region or reflecting a bigger longer size of the CREB-related competitor. The third oligonucleotide represented the +30/+49 region located downstream of the transcription initiation site within the leader sequence. This probe, predicted to contain CACC-element for SP/KLF transcription factors, produced only a weak single band that was entirely competed by a non-labeled 12-nt consensus motif indicating that also this region has the capacity to bind a transcription factor (Fig. 3c).

Functional analysis of cis-regulatory elements using deletion constructs. In order to demonstrate a functional relevance of the regulatory elements located within the analyzed core promoter regions, we produced promoter constructs with internal deletions of the binding sites for SMAD, overlapping STAT/CREB, and for SP/KLF transcription factors. These were generated by inverse PCR using the primers related to sequences flanking the intended deletion (Table I) and the -236/+58 promoter construct as a template. All deletion constructs were transiently transfected to HeLa cells as described above and luciferase activities were measured 48 h later. As shown on Fig. 4, the lowest activity (<5% of the intact promoter activity) was obtained with the STAT/CREB deletion construct (-153/-137) suggesting that this region is the most critical for activation of S100P transcription. Less prominent reduction (46%) was obtained with the SMAD deletion construct (-225/-217). Analysis of an additional construct with the deletion of SP/KLF element (+38/+42), revealed that the promoter activity decreased to about 25% of the intact core promoter. Comparable activity value was obtained in HeLa cells in the absence of the entire -13/ +58 region (Fig. 4). Taking these data together, we can propose that the S100P promoter activation is principally mediated by three regulatory elements: STAT/CREB-binding region, SMAD-binding site and SP/KLF element.

Discussion

Expression of the S100P gene, originally identified in placenta, has been associated with cancer in a number of immunohistochemical and microarray studies of human tissues. These consistently showed that S100P transcription correlates with various features of malignant phenotype including hormone independence and resistance to chemotherapy (9-15,17). Two independent studies also demonstrated that ectopic expression of S100P was accompanied by acquisition of tumorigenic potential *in vivo* (16,17). In spite of this consistent link to cancer, regulation of S100P expression has remained unexplored, although some clues could be deduced from data showing the role of hormones and growth factors that influence the level of S100P in certain types of tissues.

Herein we provide the first information relevant for understanding the S100P gene regulation in cancer cells. We determined the position of transcription initiation site and identified the genomic region that encompasses the core promoter. We also identified cis-regulatory elements that play a critical role in transcriptional activation of S100P gene and bind nuclear proteins. Our data clearly suggest the functional implication of the STAT/CREB, SMAD and SP/KLF binding sites in the control of S100P expression.

Involvement of these regulatory elements is quite compatible with the cancer-related expression pattern of S100P gene, because the signal transduction pathways that converge on these types of transcription factors are frequently activated in different tumors (22-27).

The STAT proteins mediate transcriptional responses to many cytokines and growth factors (including EGF). These stimuli induce the formation of dimers composed of different combinations of six isoforms. STATs, particularly the isoforms 1, 3 and 5, operate in a wide variety of tumors where they up-regulate genes involved in control of cell proliferation and survival and thereby contribute to tumor progression (22).

The cAMP response element-binding protein CREB responds to various stimuli such as growth factors and stress signals that elevate intracellular cAMP or Ca^{2+} levels. CREB supports proliferation, survival and malignant transformation via transcriptional regulation of several thousand genes including proto-oncogenes, cytokines and cell cycle components, and is frequently constitutively active in tumor cells (23).

SMAD proteins, existing in eight isoforms, are intracellular transducers of TGF-ß signaling and their malfunctions are implicated in cancer and other serious human diseases (24). SMADs seem to be unable to directly recruit the basal transcriptional machinery to responsive promoters and instead regulate transcription through chromatin remodeling (25). This fact could explain why deletion of SMAD-binding motif

did not have such a dramatic effect on the activity of S100P promoter when compared to deletion of STAT/CREB region. SMADs can also cooperate with other transcription factors including AP-1, CREB/ATF3 and SP-1 (26). Binding sites for these transcription factors are present also in the core promoter and thus it is quite conceivable that such cooperation could occur also in the context of S100P regulation.

SP/KLF family contains at least 20 members (including SP-1) that have different transcriptional properties. They are involved in many growth-related signal transduction pathways, interact with oncoproteins and tumor suppressors, and can be oncogenic (27). Furthermore, these types of transcription factors can modulate each other's activities and cross-talk with glucocorticoid receptors (28,29). Indeed, GREs (not investigated in detail in this study) can also play a role in regulation of S100P gene because the core promoter contains several GRE motifs (some of them overlap with the herein identified key elements) and responds to hydrocortisone.

Due to complex relationships among these transcription factors, better understanding of the molecular anatomy of S100P promoter and related upstream pathways will require identification of STAT, SMAD and SP/KLF isoforms participating in regulation of S100P gene expression, because various isoforms differ by target selectivity and specific roles in responses to various stimuli. This study provides a solid basis enabling the achievement of this goal.

Acknowledgements

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