Glucocorticoid Receptor-Mediated Transcriptional Activation of S100P Gene Coding for Cancer-Related Calcium-Binding Protein

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ABSTRACT

S100P is a member of the S100 family of calcium-binding proteins involved in calcium sensing and signal transduction. Its abnormal expression and biological activities are linked to tumor phenotype, namely to increased survival, proliferation, invasion and metastatic propensity of tumor cells. Association of S100P with outcome of tumor treatment and preliminary data from S100P promoter analysis prompted us to study regulation of S100P expression by glucocorticoids, which are implicated in tumor response to chemotherapy. We showed that dexamethasone (DX), a representative glucocorticoid, was capable to induce activity of S100P promoter by means of increased expression, nuclear translocation, and transactivation properties of the glucocorticoid receptor (GR). Moreover, DX treatment led to decreased phosphorylation of ERK1/2, reduced transcriptional activity of AP1, and modulated activity of some additional transcription factors. We identified a promoter region responsible for DX-mediated transactivation and proved GR binding to S100P promoter. We found that the effect of DX was enhanced by partial but not complete inhibition of the MAPK/ERK pathway, supporting an active crosstalk between GR and MAPK/ERK signal transduction in control of S100P expression. On the other hand, suppression of GR mRNA level by transient siRNA expression resulted in reduced S100P transcription. The role of GR activation in S100P regulation was supported by co-expression of GR with S100P in cells treated with DX. These data suggest that S100P is a direct transcriptional target of glucocorticoid-mediated signaling in tumor cells that is activated through the interplay of GR and MAPK pathways. J. Cell. Biochem. 112: 3373–3384, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: S100P PROMOTER; DEXAMETHASONE; GLUCOCORTICOID RECEPTOR; TRANSCRIPTION; MAPK PATHWAY; CANCER

S100P is a member of the S100 family of small (9–14 kDa) calcium-binding proteins implicated in calcium sensing and signal transduction. The S100 proteins usually exist as tight symmetric homo- or heterodimers that can bind calcium ions via two EF-hand motifs present in each monomer [Heizmann and Cox, 1998; Donato, 2003; Santamaria-Kisiel et al., 2006]. Upon binding of calcium, they recognize and selectively interact with specific target molecules and thereby modulate diverse biological processes including cell differentiation, proliferation, and malignant transformation [Heizmann et al., 2002; Santamaria-Kisiel et al., 2006]. Abnormal expression of S100 proteins has been associated with

several human diseases, such as cardiomyopathy, diabetes, neurodegenerative disorders, and cancer [Marenholz et al., 2004].

The S100P protein was originally isolated from placenta, but later it was shown to be over-expressed in various tumor cell lines and tissues and was, therefore, linked to cancer [Becker et al., 1992; Arumugam et al., 2005; Dowen and Crnogorac-Jurcevic, 2006; Wang et al., 2006; Surowiak et al., 2007; Parkkila et al., 2008]. Numerous studies, including microarray analyses, revealed relationships between elevated expression of S100P and different aspects of tumor phenotype, notably, increased survival, metastatic potential, and resistance to anticancer therapy. These observations were in

accord with functional studies demonstrating the capability of the S100P protein to transmit extracellular and intracellular signals.

The intracellular form of S100P apparently increases the metastatic potential of tumor cells and stimulates cell proliferation and survival by binding and activating ezrin [Koltzscher et al., 2003]. The secreted form of S100P was shown to exert a multitude of effects relevant to cancer biology, both in culture and in animal models, via binding to RAGE [Arumugam et al., 2004, 2005; Fuentes et al., 2007].

Despite the growing interest in S100P as a molecule functionally involved in tumor development, information on the regulation of its expression remains limited. Based on two recent reports, transcription of the S100P gene is activated by BMP4 (bone morphogenic protein) in a Smad-4 dependent manner and is also induced in response to NSAIDs via the ATF-4 transcription factor [Hamada et al., 2009; Namba et al., 2009]. Since both pathways participate in cancer-related phenomena, they might explain at least some of the observations of S100P over-expression in tumors.

We have previously defined a core promoter of the S100P gene and performed its detailed functional analysis, which revealed the importance of the STAT/CREB, SMAD, and SP/KLF binding sites in the transmission of trans-activating signals [Gibadulinova et al., 2008]. In the same study, we noted that the S100P upstream regulatory region contains several glucocorticoid receptor (GR)-binding sites. This region responds to the GR agonist hydrocortisone by increased transcriptional activation. S100P was also found among GR transcriptional targets in two additional studies [Wang et al., 2004; Kino et al., 2009] but no details at the promoter level have been provided.

Because of the biological significance of GR-mediated signaling and its relevance to the tumor phenotype, we decided to further investigate the role of glucocorticoids in the regulation of S100P expression. Here we describe the effects of dexamethasone (DX) on the transcriptional activation of S100P in the context of MAPK pathway modulation, and propose the mechanism of the crosstalk between GR and MAPK-mediated signaling acting on S100P promoter.

MATERIALS AND METHODS

CELL CULTURE
HeLa and C33a cervical carcinoma cells and MFC-7 breast carcinoma cells were cultured under standard conditions in Dulbeccós modified Eaglés medium (DMEM) without phenol red, supplemented with 10 or 5% fetal calf serum (FCS), L-glutamine, and 100 units/ml penicillin/streptomycin mixture (all from Lonza, Verviers, Belgium) in humidified air containing 5% CO_2 at 37°C.

CONSTRUCTION OF PROMOTER REPORTER PLASMIDS
Promoter fragments spanning the $-575/+58$, $-312/+58$, and $-236/+58$ (core) regions were amplified by PCR from HeLa genomic DNA using the following primers: -575: 5′-CTTCTGCCCGTTGATTTTCAGC-3′, -312: 5′GTCACCACTCACTCCACACACA-3′, and -236: 5′-CGGTACCTCAGTGATGGCGCCGAGACA-3′, respectively, each with $+58$: 5′-GAAGATCTGGTGCTAGATTCAGACCCAC-3′. Amplified fragments were digested and cloned

into pGL3-Basic vector (Promega, Madison, WI) upstream of the luciferase reporter gene. The $-663/+58$ fragment was recloned from the $-1,234/+58$ fragment [Gibadulinova et al., 2008], by digestion with *Bgl II* into the pGL3-Basic/*Bgl II* site.

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAY
The cells were plated onto 30-mm Petri dishes to reach approximately 60–80% monolayer density on the following day. Transfection was performed with 2 μg of the promoter-pGL3 plasmid and 100 ng of pRL-TK renilla vector (Promega) using Gene Porter 2 Transfection Reagent (Genlantis, San Diego, CA) according to the manufacturer's recommendations. Alternatively, promoter-pGL3 plasmid was co-transfected with 1 μg of plasmids coding for ERK1 and/or ERK2 dominant-negative mutants kindly provided by Dr. M.H. Cobb (Southwestern Medical Center, Dallas). After 24 h, the transfected cells were trypsinized and plated onto 24-well plates, allowed to attach for 20 h, then starved in serum-free medium for 24 h and treated in duplicates or triplicates by DX (10 μM) for an additional 20 h, with 45 min of pre-treatment with PD98059 (10 μM), LY294002 (10 μM), UO126 (5 μM), SB203580 (10 μM), and RU-486 (50 μM), (all from Sigma, St. Louis, MA). The cells were harvested for analysis at the end of the treatment period. Cytoplasmic extracts were analyzed for luciferase activity (Dual-Luciferase Reporter Assay System, Promega) using TD Luminometer 20/20 (Turner Design).

Cignal™ Cell-based Multi-Pathway Activity Assay (SABioscience, Frederick, MD) was performed according to instructions of the manufacturer. Dual-luciferase results were calculated for each transfectant and analyzed by the Data Analysis Software (SABioscience). The change in the activity of each signaling pathway was determined by comparing the normalized luciferase activities of the reporter in treated versus untreated transfectants.

WESTERN BLOTTING
Cells grown in confluent monolayers in 60 mm diameter dishes were rinsed twice with cold PBS and solubilized in an ice-cold RIPA buffer (1% Triton X-100 and 1% deoxycholate in PBS) containing the commercial COMPLETE cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min on ice. The extracts were collected, cleared by centrifugation at 15,000 rpm for 10 min at 4°C and stored at −80°C. Protein concentrations of the extracts were quantified using the BCA protein assay reagent (Pierce, Rockford, IL). The extracts (50 μg/lane) were resolved in 12% SDS–PAGE and transferred to a PVDF membrane (Macherey-Nagel GmbH&Co.KG, Düren, Germany). After blocking in 5% non-fat dry milk with 0.2% Tween 20 in PBS, the membrane was probed with p44/42 and Phospho-p44/42 MAP kinase antibodies (New England Biolabs Inc., MA), S100P mouse monoclonal antibody 18-9 [Parkkila et al., 2008], or β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After that, it was washed and treated with secondary HRP-conjugated antibodies (Sevapharma, Prague, Czech Republic). Protein bands were visualized by enhanced chemiluminescence using the ECL kit (GE Healthcare Bio-Sciences Inc, Quebec, Canada).

WESTERN BLOTTING OF NUCLEAR AND CYTOPLASMIC EXTRACTS

Cells were cultured in 100 mm diameter dishes, and nuclear proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Rockford) according to the manufacturer's instructions. Protein concentration was quantified by the bicinchoninic acid (BCA) assay method (Pierce Rockford). Sixty micrograms of nuclear proteins and 120 µg of cytoplasmic proteins were separated by SDS–PAGE and transferred to a PVDF membrane (Macherey-Nagel). After blocking in 5% non-fat dry milk with 0.2% Tween 20 in PBS, the membrane was probed with the GR (H-300) antibody (Santa Cruz Biotechnology), and with the antibodies specific for tubulin (H-300) and TFIID (C-7) proteins representing cytoplasmic and nuclear fraction, respectively (Santa Cruz Biotechnology). After washing, the membranes were treated with secondary anti-rabbit/mouse HRP-conjugated swine antibody (Sevapharma). The protein bands were visualized by enhanced chemiluminescence using the ECL kit (GE Healthcare Bio-Sciences, Inc.).

REVERSE TRANSCRIPTION PCR (RT–PCR)

Total RNA was isolated from the cell monolayers using INSTAPURE solution (Eurogentech, Belgium) according to the protocol of the manufacturer. Reverse transcription was performed with M-MuLV reverse transcriptase (Finnzymes OY, Espoo, Finland) using random heptameric primers (400 ng/µl). The mixture of 3 µg of total RNA and random primers was heated for 10 min at 70°C, cooled quickly on ice, and supplemented with 0.5 mM dNTPs (Promega), M-MuLV reverse transcriptase buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol (Finnzymes OY). The mixture, in a final volume of 24 µl, was further supplemented with 200 U of reverse transcriptase M-MuLV, incubated for 1 h at 42°C, heated for 15 min at 70°C, and stored at −80°C until used. RT-PCR was performed with GoTaq Flexi DNA polymerase (Promega) in an automatic DNA thermal cycler (Eppendorf AG, Hamburg, Germany). Following an initial denaturation at 93°C for 3 min, the amplification program was set as follows: Denaturation at 93°C for 45 s, annealing at 65°C (S100P), 59°C (GR), and 57°C (β-actin) for 45 s, and extension at 72°C for 45 s for a total of 30 cycles, and finally, 5 min at 72°C. The resulting PCR fragments were separated in 1.3% agarose gels. The nucleotide sequences of the primers were as follows (s, sense; a, antisense): S100P-s (109–130) 5′-AAGGGG-GAGCTCAAGGTGCTGA-3′, S100P-a (330–308) 5′-ATCTGTGACATC TCCAGGGCATC-3′, GR-s (886–906) 5′-TCGACCAGTGTTCCAGA GAAC-3′, GR-a (1,558–1,579) 5′-TTTCGGAACCAAC GGGAATTG-3′, β-actin-s (414–433) 5′-CCAACCGCGAGAAGATGACC-3′, β-actin-a (649–629) 5′-GATCTTCATGAGGTAGTCAGT-3′.

REAL–TIME QUANTITATIVE PCR

Total RNAs obtained from HeLa and MCF-7 cell lines treated for 20 h with or without DX were reverse transcribed as described above. Amplification by RTQ-PCR was performed in StepOne^TM System Thermal Cycling Block (Applied Biosystems, Foster City, CA) for the transcripts of the S100P and GR genes. PCR reactions were carried out in quadruplicates in 20 µl using Power SYBRGreen PCR Master Mix (Applied Biosystems) for 10 min at 95°C for initial denaturation, followed by 30 cycles of 95°C for 15 s and 60°C for 1 min. Sample Ct values were normalized to those of β-actin cDNA. The relative expression was calculated by the ddCt method. Oligonucleids used for RTQ-PCR: S100P-s (109–130) 5′-AAGGGGGAGCTCAAG GTGCTGA-3′, S100P-a (330–308) 5′-ATCTGTGACATC TCCAGGG-CATC-3′, GR-s (886–906) 5′-TCGACCAGTGTTCCAGAGAAC-3′, GR-a (1,043–1,064) 5′-AAGGTGCTTTGGTCTGTGGTAT-3′, β-actin-s (414–433) 5′-CCAACCGCGAGAAGATGACC-3′, β-actin-a (649–629) 5′-GATCTTCATGAGGTAGTCAGT-3′.

CHROMATIN IMMUNOPRECIPITATION

HeLa cells were plated into 500 cm² plates at ∼90 density, left to attach overnight and incubated in the absence or presence of 10 µM DX for 4 h. The cells were fixed in 1% formaldehyde directly in medium at room temperature (∼21°C) for 10 min. Chromatin isolation, shearing to the size of ∼600–800 bp and its immunoprecipitation with rabbit polyclonal antibody against GRα (ab3580) Abcam, Cambridge, UK) were performed as described in [Lofstedt et al., 2004]. Anti-human IgG antibodies (rabbit polyclonal, Abcam, Cambridge, UK) were used as a negative control and for chromatin preclearing. Purified DNA was subjected to 33 cycles of PCR with primers flanking the putative GREs (−687s: 5′-ACTGTGTTTATG-GATAGTTTCCAC-3′, −500a: 5′-GCTTCCACGTCAATCTCGCTC-3′ for GRE −608/−592; −575s: 5′-CTTCTGCCCGTTGATTTTCAG-3′, −288a: 5′-GAGTGTGTGTGGAGTGAGTGG-3′ for GRE −468/−459) within the S100P promoter.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Cells grown on glass coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min and permeabilized with 0.2% Tween 20 in PBS. After blocking in 3% bovine serum albumin, the cells were incubated with the anti-S100P primary antibody (mouse monoclonal antibody 18-9) for 2 h at 37°C, and with the anti-GR primary antibody (H-300) (Santa Cruz Biotechnology) diluted 1:50 in PBS for 1 h at room temperature. Then the cells were incubated for 1 h at 37°C with secondary antibodies, namely Alexa Fluor 488-conjugated donkey anti-mouse IgG, and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, CA) diluted 1:1,000 in PBS. All experiments were also carried out in the absence of the primary, secondary, or both antibodies as negative control. Cell nuclei were stained with 10 µM Hoechst 33342 (Invitrogen) in PBS for 5 min. Finally, the cells were washed three times for 15 min in 0.2% Tween 20, mounted in Fluorescein-FragEL mounting media (Calbiochem, San Diego, CA), and analyzed using a confocal laser scanning microscope (LSM 510 Meta Microscope, Zeiss, objective 63×).

RNA INTERFERENCE

In order to suppress GR expression, the cells were transfected with 10 nM GR siRNA (h): sc-35505 (Santa Cruz Biotechnology) using the Gene Silencer siRNA Transfection Reagent (Genlantis) according to the manufactureŕs instructions. Ten nanomolar Silencer Negative Control siRNA (Applied Biosystems) was used as control. The RNA and proteins were isolated 48 h after transfection and analyzed by real-time quantitative PCR and Western blotting, as described above.

DEXAMETHASONE ACTIVATES THE S100P PROMOTER IN TUMOR CELLS

First, we wanted to confirm that the S100P promoter activation observed previously with hydrocortisone could be repeated with DX, another GR agonist frequently used as an anti-inflammatory drug in anticancer therapy. We therefore transiently transfected cervical carcinoma HeLa cells with a S100P promoter-luciferase construct covering the genomic region of −663/+58 nt (related to the S100P transcriptional initiation site). Transfected cells were subjected to treatment with 10 µM DX for 20 h and then analyzed by a dual luciferase assay, which showed about threefold increase in the promoter activity in response to DX when compared to non-treated
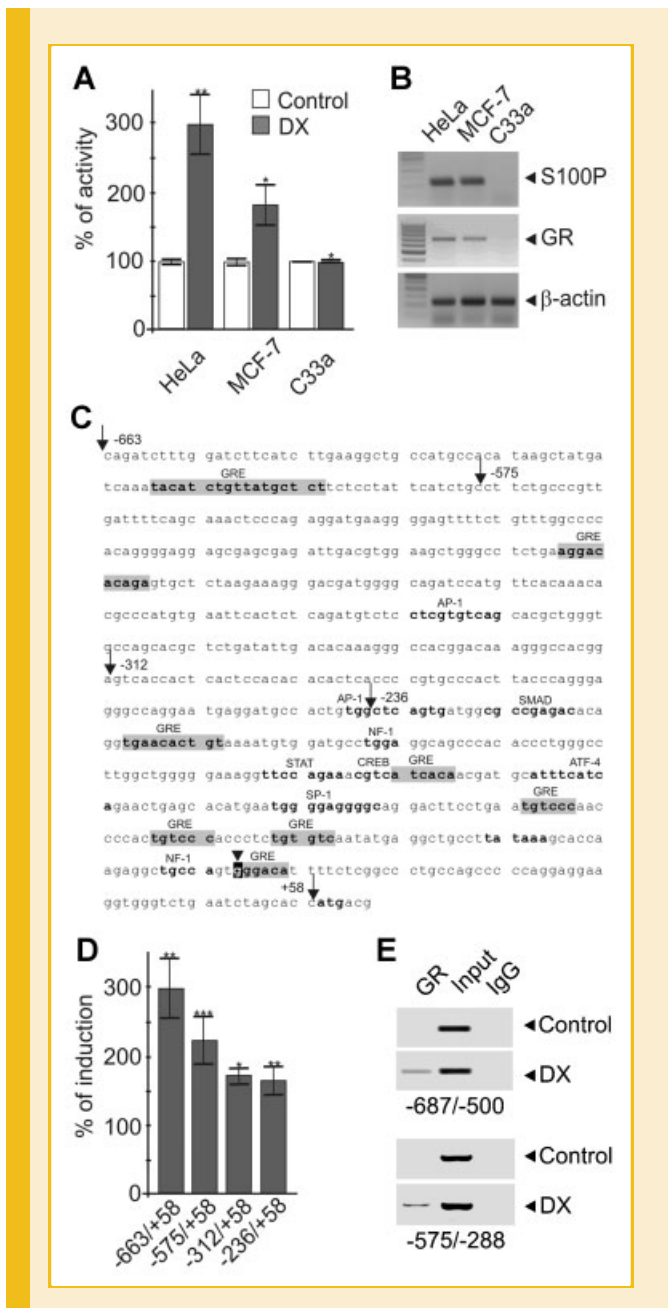
control (Fig. 1A). Similar promoter activation by DX was also observed in the MCF-7 cell line derived from primary breast carcinoma, but not in C33a cervical carcinoma cells (Fig. 1A), which do not express endogenous S100P, as demonstrated by RT-PCR (Fig. 1B). Since glucocorticoids usually exert their transactivating effects through direct binding of the activated and dimerized form of their receptor to the glucocorticoid responsive elements (GRE) in upstream genomic regions of target genes, we analyzed the promoter of the S100P gene for the presence of GREs. In silico analysis revealed potential GREs in the core promoter (−236/+58) region (one located just downstream of the transcription start). Two additional GREs were found in the longer promoter construct (Fig. 1C). Then we analyzed a series of 5′ truncated promoter constructs missing sequentially several GREs. As evident from Figure 1D, both GREs localized between the nucleotides −663 and −312 are responsible for the major part of the DX-mediated induction. This region was also found to bind GR in the CHIP assay (Fig. 1E). Thus, we can conclude that GREs at position −608/−592 and −468/−459 are instrumental for S100P transactivation by DX.

DEXAMETHASONE AND MAPK INHIBITOR PD98059 CROSSTALK IN S100P PROMOTER REGULATION

The transcriptional effects of glucocorticoids are often exhibited by interplay of the GR with components of the MAP kinase or PI3 kinase pathways [Takabe et al., 2008; Arancibia et al., 2009]. In order to find out whether these pathways participate in the GR-mediated regulation of the S100P gene, we analyzed the S100P

Fig. 1. Effect of dexamethasone (DX) on S100P promoter activity. A: Cervical carcinoma HeLa and C33a cells, and breast carcinoma MCF-7 cells were transfected with the S100P promoter–containing luciferase construct −663/+58 nt (related to the S100P transcriptional initiation site). Following treatment with 10 µM dexamethasone (DX) for 20 h, the promoter activity was measured and calculated as a ratio between luciferase and renilla values. The results of eight independent experiments were expressed as the mean percentage ± S.E.M. of activity of the control untreated cells, which was set to 100%. Statistical significance of the differences between the control and DX-treated cells was assessed using Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001). DX-mediated promoter activation occurred in HeLa and MCF-7 cells, but not in C33a cells. B: Relative levels of S100P and GR mRNA expressed under basal conditions in three cell models were evaluated by RT-PCR in parallel with β-actin mRNA that served as an internal standard. GR expression was not detectable in C33a cells. C: The nucleotide sequence of the 5′ genomic region of S100P gene shows highlighted GRE elements, AP-1 and ATF-4 binding sites that were predicted by different software programs, including MatInspector (www.genomatics.de), AliBaba2 (www.gene-regulation.com), and Promoter Scan (bimas.dcrt.nih.gov/molbio/proscan). Arrowhead indicates transcription initiation site and arrows depict the 5′ termini of the promoter regions used for analysis shown in the following part. D: HeLa cells were transfected with the 5′-truncated promoter constructs and treated with 10 µM DX. The results were expressed as the mean percentage ± S.E.M. of activity of the control untreated cells, which was set to 100%. Statistical significance of the differences between the control and DX-treated cells was assessed using Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001). Elimination of the −663/−312 region led to reduced DX-related promoter activation. E: Chromatin immunoprecipitation assay was done using HeLa cells treated with DX compared with untreated control. The cells were incubated with formaldehyde for crosslinking, DNA was extracted and immunoprecipitated with antibody specific for GR or for irrelevant IgG to visualize background signal. Recovered DNA was used in PCR with primers flanking the GRE regions to demonstrate that GR binds to GRE localized between −687 and −288 nucleotides.

promoter activity in HeLa cells treated with DX and with different kinase inhibitors. Using a single agent setting, the S100P promoter activity was significantly increased in cells treated with DX, as well as with PD98059, a class II inhibitor of MEK1 kinase, but not with U0126, a more potent class I MEK1/ERK inhibitor [Favata et al., 1998], p38 stress kinase inhibitor SB203580, or with the PI3 kinase inhibitor LY294002 (Fig. 2A). This indicates that the GR pathway and the MEK/ERK arm of the MAPK pathway each contribute individually to the stimulation of S100P expression. Upon simultaneous treatment with two compounds (DX and inhibitor), we observed additive effect only when DX was combined with PD98059, whereas the other combinations did not exceed the action

of DX alone. The inducing effect of DX and PD98059 was abolished by pre-incubating the cells with the GR antagonist RU38486/mifepristone (Fig. 2B), supporting the significant role of GR in S100P transactivation.

One of the mechanisms by which GR exerts transcriptional regulation includes reciprocal interference with activator protein 1 (AP-1), a transcription factor that transmits the MAPK cascade signals to its target genes and is itself a MAPK pathway as well as GR pathway target [Frost et al., 1994; Watts et al., 1998; Saklatvala, 2002]. The outcome of this antagonism is determined by equilibrium between these two pathways [Konig et al., 1992]. We, therefore, examined the response of the S100P promoter to increasing
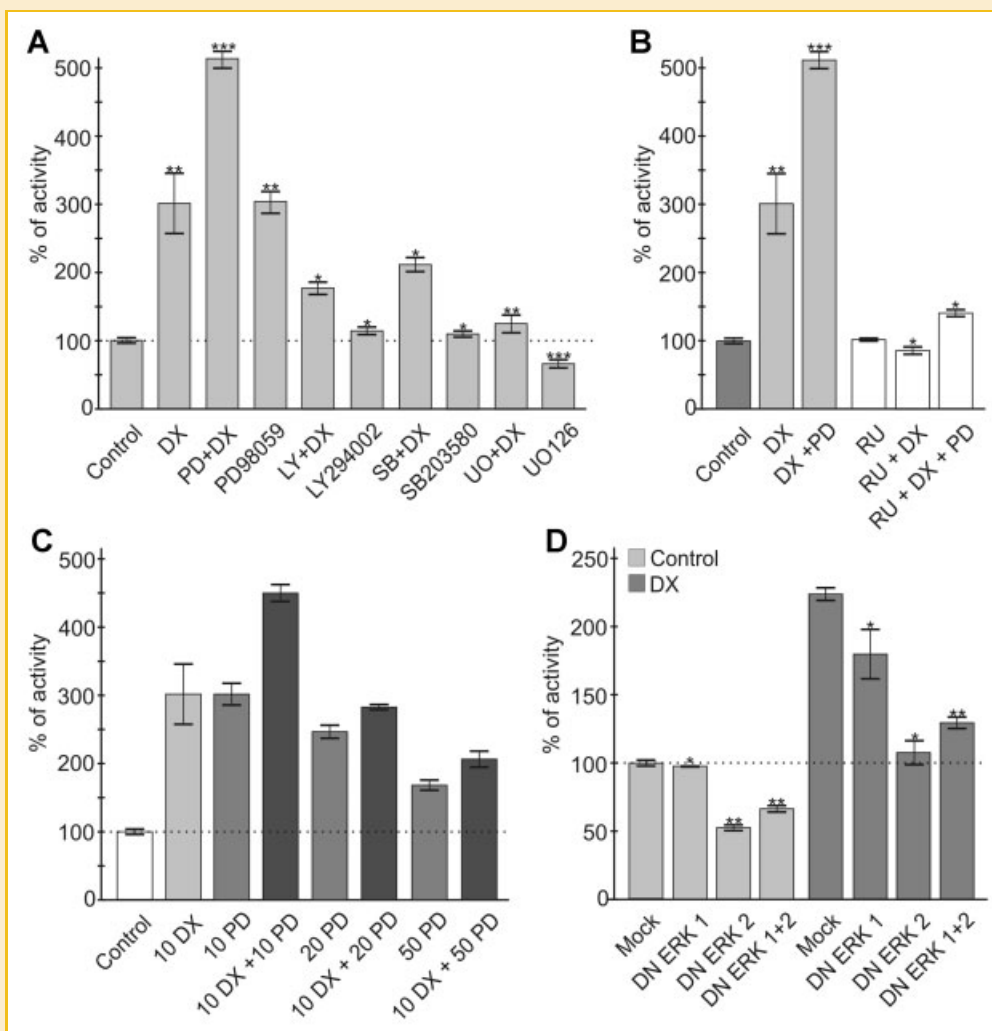

Fig. 2. Relationship between dexamethasone effect and MAPK inhibition. A: HeLa cells were transfected with S100P promoter construct −663/+58 nt, pre-treated with different kinase inhibitors for 45 min (including 10 μM PD98059 inhibitor of MEK1, 10 μM LY294002 inhibitor of PI3K, 10 μM SB203580 inhibitor of p38 MAPK, and 5 μM UO126 inhibitor of MEK1), and then co-treated with 10 μM DX for 20 h. An additive effect was observed only in combination of DX with PD98059. Statistical significance of the differences was assessed for single compounds versus control and for inhibitors combined wit DX versus DX alone (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). B: Pretreatment with 50 μM RU38486/mifepristone counteracted the enhancing effect of DX as well as DX + PD. Statistical significance of the differences was assessed for DX and DX + PD versus control and for RU + DX and RU + DX + PD versus RU-treated cells. C: Dose response of S100P promoter to PD98059 inhibitor shows decreasing promoter activation by increasing concentrations of the inhibitor alone or in combination with DX. Numbers below x-axis indicate compound concentration in μM. D: This decreasing tendency was also visible in both basal and DX-stimulated conditions upon co-transfection of S100P promoter with dominant-negative mutants of ERK1 and/or ERK2. In all variants of the experiment, the promoter activity was assessed as described in Fig. 1. Statistical significance of the differences was assessed for dominant negative ERK1 and/or ERK2 versus mock separately in co-transfected nontreated and DX-treated cells.

inhibition of the MAPK pathway by different doses of the MEK1 inhibitor PD98059, alone and in combination with DX. We could see that elevated concentrations of PD98059 as a single agent led to a reduced activation of the S100P promoter construct that contains both AP-1 and GR-binding sites (Fig. 2C). Moreover, increasing PD98059 concentrations diminished the additive effect of DX (Fig. 2C), supporting the view that GR and MAPK pathways cross-talk in regulation of the S100P gene expression, and that only a partial MAPK pathway inhibition allows for increased S100P transactivation by GR. This observation was corroborated by co-expression of dominant-negative mutants of ERK1 and/or ERK2 that reduced DX-stimulated promoter activity (Fig. 2D) and thus behaved similarly as U0126 inhibitor or as PD98059 inhibitor at higher concentrations. This was in agreement with the data from literature showing that expression of kinase inactive ERK1 and/or ERK2 mutants completely inhibited AP-1 transctivation [Frost et al., 1994; Watts et al., 1998].

UP-REGULATION OF S100P IS ASSOCIATED WITH DECREASED PHOSPHORYLATION OF ERK, NUCLEAR TRANSLOCATION OF GR, AND ITS ELEVATED TRANSCRIPTION

It has been described that stimulation of GR with DX can lead to induction of MAPK phosphatase MKP-1, which, in turn, depho-sphorylates ERK1/2, a substrate of MEK1 [Kassel et al., 2001; Wu et al., 2005]. This results in inactivation of signal transduction through the MAPK pathway and decreased activation of AP-1 transcription factor. On the other hand, MKP-1 is induced in response to increased activation of the AP-1 transcription factor [Casals-Casas et al., 2009]. This pendulum effect offers a feedback mechanism maintaining a balance between the GR and MAPK pathways. We wanted to learn whether this mechanism contributes to the transcriptional control of the S100P gene. For this reason, we analyzed the phosphorylation status of ERK1/2 in HeLa cells treated with DX and MEK1 inhibitors. Figure 3A shows that the treatment of cells with DX and DX + PD98059 for 30 min led to inhibition of ERK1/2 phosphorylation. However, after 24 h only a slightly decreased level of the phosphorylated ERKs was detectable in the DX-treated cells, whereas the decrease was more apparent upon co-treatment with DX and PD, and complete inhibition was observed with U0126 inhibitor. Interestingly, the longer treatment with DX and/or PD led to a significantly increased level of S100P, probably as a consequence of reduced MAPK signaling, which potentiated GR transactivation.

Activation of GR by DX is followed by its translocation to the nucleus [Kumar and Thompson, 2005; Takabe et al., 2008]. This was also confirmed here by immunodetection of GR in the cytosolic
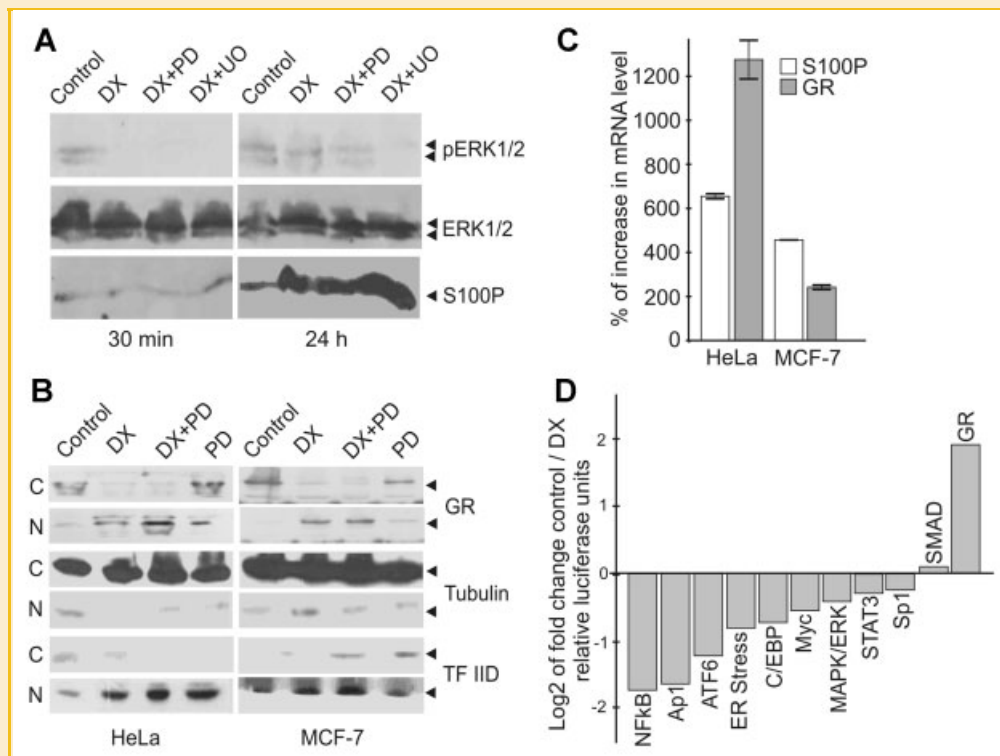

Fig. 3. Cross-talk between the GR and MAPK pathways. A: Western blotting analysis of the phosphorylation status of ERK1/2 and expression level of S100P in HeLa cells treated with 10 μM DX, 10 μM PD98059, and 5 μM U0126. The data clearly show partial inhibition of ERK1/2 by PD98059 and their full inhibition by U0126. B: Detection of GR in cytosolic and/or nuclear fractions of HeLa and MCF-7 cells reveals its translocation in response to treatment with 10 μM DX, 10 μM PD98059, or their combination. Tubulin and TFIID subunit were used as controls for cytoplasmic and nuclear localization, respectively. C: Real-time PCR analysis of GR and S100P mRNAs in DX-treated cells. The graph shows % of increase in mRNA levels normalized to β-actin RNA. D: Cignal cell-based dual luciferase reporter assay of HeLa cells treated with 10 mM DX versus non-treated control cells revealed alterations of diverse signal transduction pathways leading to changes in transactivation capacity of various transcription factors, including GR.

versus nuclear fractions of the control HeLa and MCF-7 cells compared to cells treated with DX and MAPK inhibitors. In accordance with S100P promoter activation, nuclear translocation was observed in HeLa cells in response to DX and was even more pronounced when DX was combined with PD98059 (Fig. 3B). GR also partially translocated to the nucleus upon treatment with PD98059 in the absence of DX, in agreement with the data from literature showing that inhibition of the MAPK pathway causes activation and nuclear translocation of GR [Onda et al., 2006].

Interestingly, DX-induced transcription of the S100P gene was accompanied by increased transcription of GR mRNA, as assessed by real-time PCR (Fig. 3C). This was confirmed by a cell-based multi-pathway reporter assay, which showed that the treatment with DX led to increased GR-mediated transactivation capacity. In accord with the above mentioned balance mechanism, it also led to decreased transactivation capacity of AP-1 transcription factor (Fig. 3D). Noteworthy, DX treatment also affected additional pathways and transcriptional factors potentially implicated in S100P regulation and/or crosstalk with GR, including NFκB, ATF6, ER stress, ERK, SP1, etc. (Fig. 3D).

These results suggest that DX displays simultaneous effects on the levels, translocation, and activation of GR, leading to induction of S100P expression, and that it operates in context of changed transactivation capacity of various transcription factors that might contribute to S100P induction.

Nuclear translocation of GR was also clearly visible in an immunofluorescence image acquired by confocal microscopy (Fig. 4A). Closer insight into the temporal relationship between nuclear translocation of GR and transcriptional activation of S100P revealed that GR could be detected in the nucleus as early as 15 min following DX stimulation, whereas GR was mostly retained in the cytoplasm in the non-stimulated cells (Fig. 4B). GR then persisted in the nucleus of DX-treated cells for up to 6 h (or even longer, for 24 h, as shown in Fig. 3). Interestingly, S100P promoter activation, evaluation of which includes both luciferase transcription and translation, could be detected after 4 h of DX treatment (Fig. 4C), suggesting that GR translocation is a prerequisite for S100P transcriptional up-regulation.

S100P IS CO-EXPRESSED WITH GR

Direct implication of GR involvement in induction of S100P prompted us to examine possible co-expression of these two proteins. For this purpose, we performed dual immunofluorescence staining of control HeLa cells versus HeLa cells treated with DX or with DX and PD98059, using the specific antibodies against both proteins. Cell nuclei were labeled with Hoechst 33342 dye to visualize all cells in the analyzed monolayer. Co-expression of S100P and GR could be seen in cell subpopulations in all samples (Fig. 5). However, it was more prominent in the cells treated with DX, both alone and combined with PD98059. In addition, the control cells showed nuclear as well as cytoplasmic staining of GR, whereas DX-treated cells displayed exclusive nuclear signal for GR, in accordance with DX-stimulated translocation demonstrated by immunoblotting. It is noteworthy that S100P was visible in the cytoplasm as well as in the nucleus of the control cells and partially also of the DX-treated cells, whereas it was mostly nuclear in the
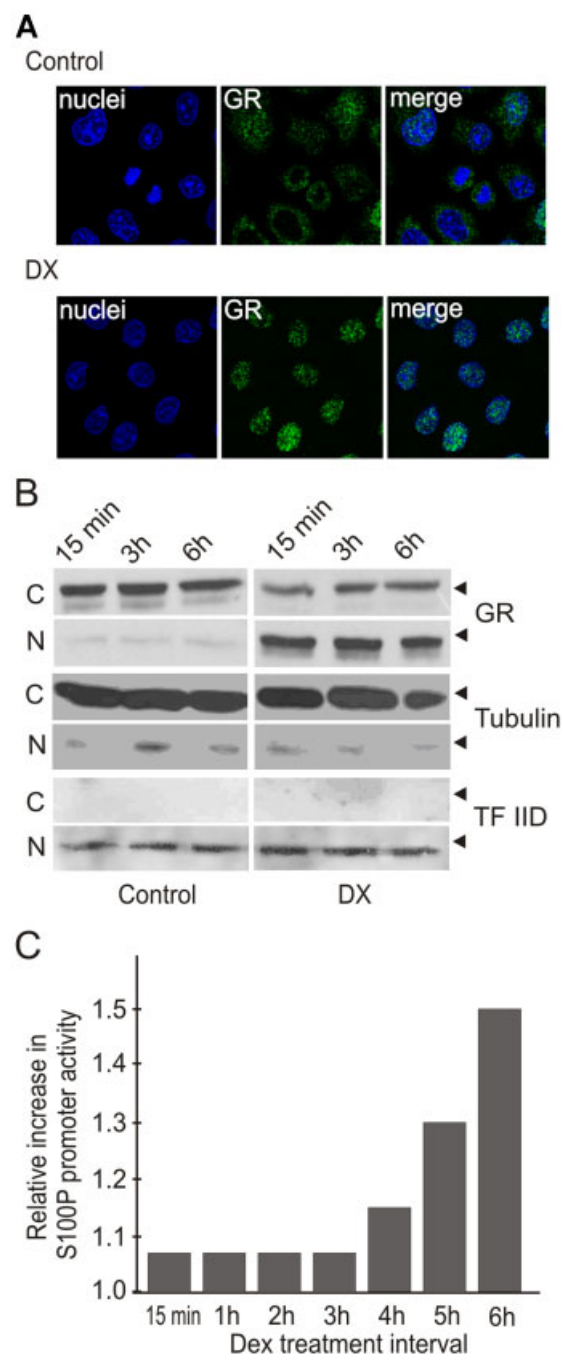

Fig. 4. Nuclear translocation of GR and activation of S100P promoter. A: Immunofluorescence detection of GR in DX-treated and untreated cells. Image acquired by confocal microscopy at original magnification 200×. B: Western blotting analysis of cytoplasmic versus nuclear GR in HeLa cells treated with 10 μM DX for different time intervals shows GR translocation to the nucleus after 15 min of stimulation. Tubulin and TFIID were used a controls for subcellular localization. C: Time-dependent activation of S100P promoter in DX-treated HeLa cells transiently transfected with S100P promoter construct −663/+58 nt. The graph shows relative increase of the promoter activity compared to non-treated control cells.
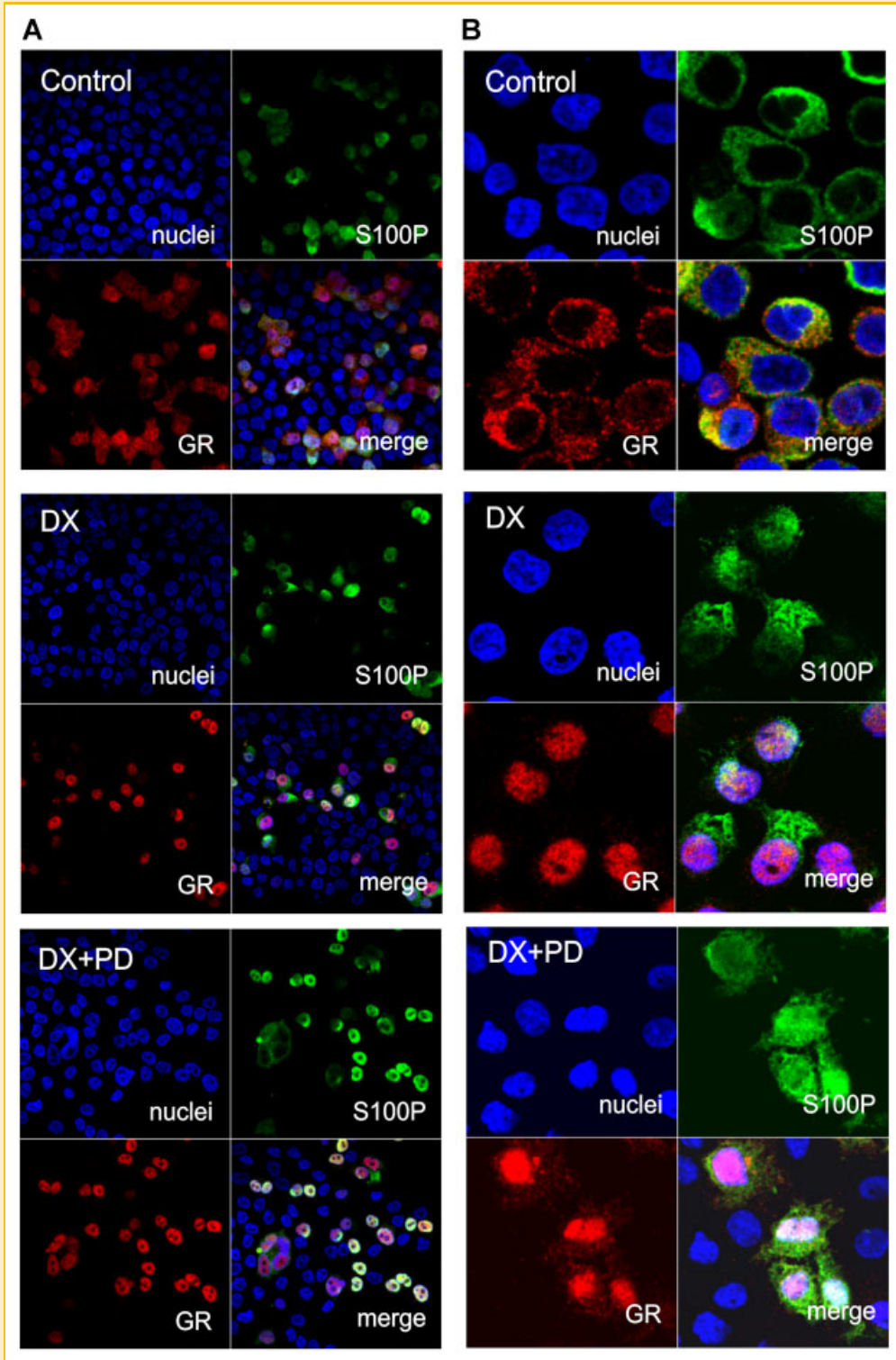
Fig. 5. Co-expression of S100P and GR. Immunofluorescent double-staining of control HeLa cells and the cells treated with 10 μM DX or DX and 10 μM PD98059 was done using anti-S100P primary antibody 18-9 with Alexa Fluor 488 anti-mouse IgG (green) and anti-GR primary antibody H-300 with Alexa Fluor 594 anti-rabbit IgG (red). Nuclei were stained with Hoechst 33342. The staining signals were analyzed by confocal microscopy at original magnifications 50× (A) and 200× (B), and show considerable overlap that was most prominent in DX and PD98059-treated cells.

cells treated simultaneously with DX and PD98059. This finding indicates possible relationship between the S100P expression and the presence and/or activation of GR.

SiRNA SUPPRESSION OF GR REDUCES S100P LEVELS

To prove the above conclusion, we also accomplished a transient siRNA transfection to specifically suppress GR expression. In both cell models, namely in HeLa and in MCF-7 (not treated by DX), transient siRNA transfection resulted in decreased GR protein levels compared to sham-transfected controls (Fig. 6A). Real-time PCR analysis of transfected HeLa cells showed that siRNA expression almost completely suppressed the GR mRNA levels and also significantly reduced S100P transcription (Fig. 6B). On the other hand, GR siRNA transfection of MCF-7 cells only partially suppressed GR expression. S100P mRNA levels were reduced to a similar extent, thus perfectly reflecting the decline in GR mRNA (Fig. 6C). This data further supported the fact that S100P expression is related to GR expression even in the absence of DX stimulation.

DISCUSSION

Glucocorticoids have been widely used as components of chemotherapy regimens for treatment of leukemias, lymphomas, and myelomas because of their potent pro-apoptotic and anti-
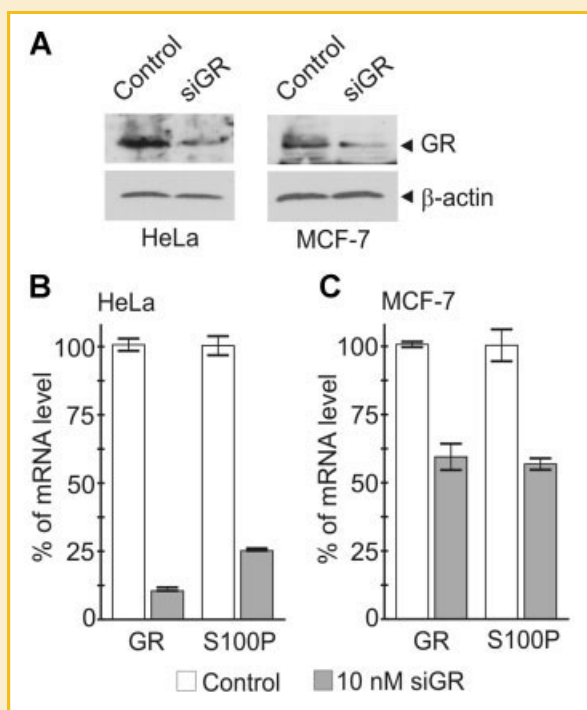

Fig. 6. Suppression of GR and S100P by GR-specific siRNA. A: Western blotting analysis of both HeLa and MCF-7 cells transiently transfected with GR-specific siRNA and in parallel with the non-relevant negative control siRNA. GR was visualized using anti-GR antibody H300. B, C: Real-time PCR analysis of S100P and GR mRNA levels. The graph shows decrease in mRNA levels normalized to β-actin RNA.

inflammatory properties and capability to reduce nausea and acute toxicity in normal tissues. However, in non-hematological tumors, glucocorticoids display diverse and even contradictive effects on response to chemotherapy and are highly suspected of inducing resistance and increasing frequency of metastases [Herr and Pfitzenmaier, 2006; Mattern et al., 2007; Moutsatsou and Papavassiliou, 2008].

Glucocorticoids act by means of cytosolic GR activation. Activated GR undergoes nuclear translocation and mediates the transcriptional regulation of numerous genes known to play key roles in cell and tissue functions, including growth, apoptosis, differentiation, metastasis, and survival. While their beneficial anti-inflammatory effects appear to occur mainly via gene repression, the undesired side effects, including chemoresistance, seem to involve predominantly the activation of gene transcription [Necela and Cidlowski, 2004]. An additional mode of action has been proposed that includes membrane-associated GR, and although several published studies have explored this GR variant, there is still some controversy as to its surface versus inner plasma membrane localization (related to caveolin-1) and its significance outside of alveolar and endothelial cell types. This mGR has been proposed to mediate rapid non-genomic glucocorticoid action [Spies et al., 2006; Matthews et al., 2008].

In accordance with the former mechanism, glucocorticoids were shown to induce transcription of the S100P gene, which has been associated with decreased sensitivity to a range of anticancer drugs in different tumor types [Bertram et al., 1998; Arumugam et al., 2005; Jiang et al., 2005; Surowiak et al., 2007; Basu et al., 2008; Song et al., 2009]. Although initial information on S100P up-regulation by glucocorticoids came from earlier studies [Wang et al., 2006; Gibadulinova et al., 2008; Kino et al., 2009], the context of their action in the S100P promoter remained unresolved.

Here we demonstrate that a representative GR agonist DX binds to S100P promoter, induces its transactivation and increases S100P mRNA levels, and that this effect is accompanied by the translocation of cytosolic GR to the nuclei of cells expressing S100P. Conversely, both the addition of a GR antagonist RU38486 and siRNA suppression of GR mRNA levels in the absence of DX result in decreased S100P transcription. This data proves that GR is directly implicated in the regulation of S100P gene expression.

On the other hand, mGR operates via activation of PKB/Akt and Src-related pathways, but has negligible effect on transcriptional regulation and is not inhibited by RU38486 [Spies et al., 2006; Sun et al., 2006; Matthews et al., 2008]. This is in contrast to our finding that glucocorticoids considerably transactivate S100P gene expression, suggesting that our observation is predominantly related to GR-mediated genomic effects.

Furthermore, we show that GR-mediated induction of S100P can be increased by inhibition of the ERK1/2 arm of the MAPK pathway. This is in agreement with the well-established concept of interference between GR and MAPK signaling. Activation of GR usually leads to suppression of the MAPK-regulated genes (as also shown in this study) and vice versa. The need for a certain balance between the GR and MAPK signaling status is clearly apparent also in the case of the S100P promoter, which is optimally activated only when the MAPK pathway is partially inhibited by the MEK1

inhibitor PD98059. Under this condition, GR translocates to the nucleus, suggesting that it is activated. However, DX-induced, GR-mediated activation of the S100P promoter is decreasing with increasing concentrations of PD98059 and cannot be achieved with
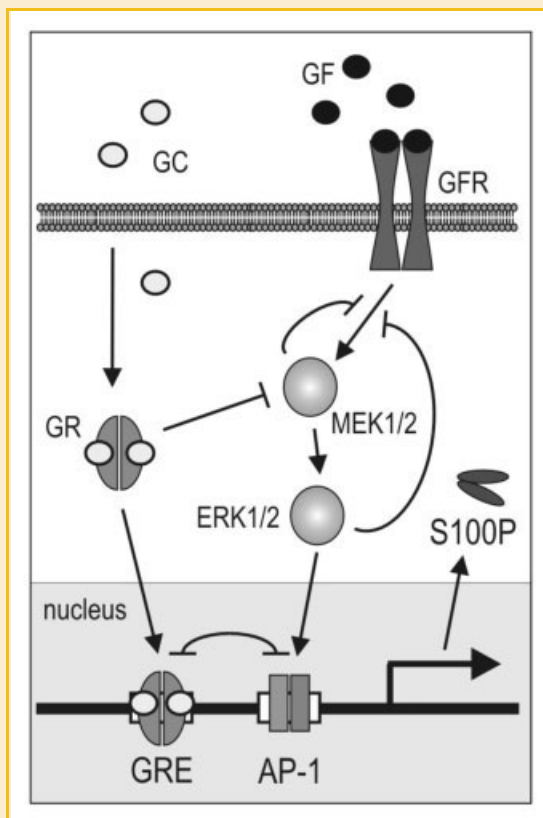

Fig. 7. A model of the interplay between GR and MAPK pathway in S100P regulation based on experimental data from this work as well as from the literature. The left side shows activation of the glucocorticoid receptor (GR) by dexamethasone as a representative glucocorticoid (GC). GCs activate GR which is translocated to the nucleus and binds to GRE element(s) in the S100P promoter [our data, Kumar and Thompson, 2005]. The right side shows activation of the MAPK pathway via growth factor receptors (GFR) transmitting signaling to MEKs and downstream to ERKs leading to transcription, activation and binding of AP-1 to its binding site. In basal situation in absence of GC, MAPK/ERK/AP-1 arm is activated by serum growth factors (GF) and drives S100P trascription [in cooperation with other transcription factors described earlier, Gibadulinova et al., 2008; Hamada et al., 2009; Namba et al., 2009]. Upon stimulation with GC, the GR arm is activated, contributes to S100P induction, but at the same cross-talks with AP-1 through mutual trans-repression [our study and Saklatvala, 2002]. When MAPK/ERK pathway is partially inhibited in concurrent activation of GR, AP-1 level is decreased and is not able to repress GR, but is sufficient to support basal transcription. However, upon full inhibition of MAPK/ERK pathway, AP-1 expression/activation is very low and basal transcription as well as DX stimulation of S100P promoter is reduced [our study]. The GR–MAPK crosstalk also involves feedback loops directed to upstream control points (e.g., MEKs/ERKs signal back to their upstream kinases), which might be released by inhibitors [Dougherty et al., 2005; Mirzoeva et al., 2009] however, this only illustrates the complex nature of this regulatory network and goes beyond this study. The symbols indicate induction (↑), inhibition or transrepression (⊥) and start of transcription (→).

more potent MEK1 inhibitor U0126, which completely blocks phosphorylation of the MEK1 substrates ERK1/2. Thus, full inhibition of the MAPK pathway is not permissive for the transcription of the S100P gene. This is understandable considering that the S100P promoter is responsive to EGF (known mitogenic activator of MAPK signaling), as shown earlier [Gibadulinova et al., 2008], and its activity is diminished upon expression of dominant-negative mutants of ERK1 and ERK2. So it appears that S100P promoter activation can be driven by both GR and MAPK signaling, and that the outcome of their cross-talk depends on the actual situation that favors either the former or the latter path of signal transmission as proposed in the model presented on Figure 7.

The principal involvement of GR (cross-talking with MAPK) in regulation of S100P is exemplified by their co-expression in the same subpopulation of HeLa cells grown in monolayer. At present, it is difficult to say whether a similar co-expression pattern exists in vivo in tumor tissues. Interestingly, abnormal GR expression has been detected in various neoplasms, including carcinoma of the breast, cervix, lungs, gastrointestinal tract, etc., but its contribution to the clinical outcome has not been fully clarified [Lu et al., 2006; Lien et al., 2008; Belova et al., 2009]. S100P is expressed in similar tumor types, and its clinical value is also controversial [Dowen and Crnogorac-Jurcevic, 2006; Surowiak et al., 2007; Parkkila et al., 2008].

Interestingly, S100P transcription is also activated in steroid-independent manner through ER stress-related ATF4 transcription factor binding to the core promoter of S100P [Namba et al., 2009]. It has been proposed that in this case the up-regulation of S100P might represent protective cellular mechanism responsible for reduction of therapeutic efficacy of NSAIDs. Since glucocorticoids repress both ATF4 [Adams, 2007] and ER stress response (see Fig. 3D), we can anticipate that steroid-dependent and independent pathways might operate on S100P promoter in mutually exclusive or counter-balanced manner. Nevertheless, experimental evidence for this assumption remains to be acquired.

In conclusion, potential GR-S100P relationship and its interplay with other pathways definitely deserve further investigation, as it can bring important insights into cancer biology. In light of our results, as well as available data on functional aspects of S100P, it is conceivable that the effects of glucocorticoids on cancer response to chemotherapy is accomplished at least partially via GR-induced expression of S100P. From this point of view, it would be interesting to look particularly at the correlation between GR-S100P co-expression and tumor phenotype, since it might provide useful predictive information and potentially result in better treatment planning.

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