doi:10.4149/gpb_2012_036

Promoter-context as a determinant of glucocorticoid receptor-responsiveness to activation of p38 and JNK mitogen-activated protein (MAP) kinases

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Abstract. MAP kinases JNK and p38 play an important role in many immune and inflammatory processes, whereas glucocorticoids exert immunosuppressive and anti-inflammatory activities. We found previously that activation of p38 or JNK inhibits glucocorticoid receptor (GR)-mediated transcriptional activation of a mouse mammary tumor virus (MMTV) promoter-driven luciferase construct in HeLa cells. It appears that this effect is DNA regulatory element-specific, since p38 or JNK activation stimulates GR-dependent transcription from TAT3-ADH promoter-luciferase construct in the same cells. The apparent promoter-specificity of this action suggests that not all glucocorticoid-activated genes are negatively regulated by p38 or JNK. Using different MMTV/TAT3 chimeric reporters we demonstrate that the presence of other accessory binding sites of the MMTV construct contributes to the inhibitory effect of activated p38 or JNK on the MMTV-driven transcriptional activity; and diminishes, but does not reverse the stimulation observed using the TAT GREs from the TAT3-ADH promoter-luciferase construct. On the other hand, comparison of the effects of GRE sequences, either in isolation or in the context of the MMTV LTR accessory binding sites, demonstrates that, the responsiveness of the GR depends on the GRE sequence; indicating that, in addition to transcription factors bound nearby, interaction with the DNA itself modulates GR activity.

Key words: Promoter-context — Glucocorticoid receptor — p38 — JNK — Transcription

Abbreviations: ADH, alcohol dehydrogenase; AF, activation function; CBP, CREB-binding protein; CREB, cAMP response element-binding protein; DBD, DNA-binding domain; ER, estrogen receptor; ERE, estrogen response element; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; hsp90, heat shock protein 90; IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; LBD, ligand-binding domain; LTR, long terminal repeat; Luc, luciferase; MAP kinase, mitogen-activated protein kinase; MKK, MAP kinase kinase; MMTV, Moloney murine mammary tumor virus; MT-I, metallothionein-I; NF-1, nuclear factor-1; OCT, octamer transcription factor; SRC, steroid receptor coactivator; SUMO-1, small ubiquitin-related modifier-1; TAT, tyrosine aminotransferase; TNF, tumor necrosis factor.

Introduction

Glucocorticoids are in use more than 50 years for treatment of autoimmune and inflammatory diseases, including rheumatoid arthritis and asthma and for preventing transplant rejection; but surprisingly very little is known about the mechanism by which they exert their function. They are lipophilic steroid hormones derived from cholesterol and synthesized in adrenal cortex. Because of their lipophilic nature, glucocorticoids can pass through the plasma membrane of target cells and bind to the glucocorticoid receptor (GR) present in most mammalian cells (Beato and Klug 2000). The GR is structurally organized into functional domains. It harbors two activation domains, activation function (AF)-1

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in the N-terminal region of the receptor and AF-2 in the C-terminal region. The DNA-binding domain (DBD) is located between the N-terminal and the C-terminal regions, the latter of which has been shown to be organized into α-helices forming the ligand-binding domain (LBD). The unliganded GR exists in the cytoplasm as a large heteromeric complex that comprises hsp90 and other stabilizing proteins (Pratt and Toft 1997). Formation of the complex with hsp90 keeps the GR functionally inactive while retaining its high affinity for the ligand. Binding of the hormone causes dissociation of the hsp90/GR complex, enabling the receptor with the bound hormone to translocate to the nucleus and bind as a homodimer to the glucocorticoid response element (GRE) in promoter regions of target genes (Beato and Klug 2000). The transcriptional complex formed by the hormone-bound GR involves coactivators of the p160 family, which serve as docking platforms for other proteins engaged in transcriptional regulation, as CREB-binding protein (CBP) or its homologue p300 (Grenier et al. 2004).

Natural promoters usually harbor recognition sequences for multiple transcription factors. Such a transcriptional control unit (composed of binding sites for several transcription factors) is called composite response element. The mouse mammary tumor virus (MMTV) promoter is one of the most studied regulatory regions containing a composite GRE (Payvar et al. 1983; Guido et al. 1996; Biola et al. 2001). Its transcriptional activation depends on the binding of the GR to a cluster of four GREs (two distal palindromic sites and two proximal hemipalindromes) located within the U3 region of the long terminal repeat (LTR). In addition, the promoter also contains binding sites for additional transcription factors, such as nuclear factor-1 (NF-1), octamer transcription factors-1 and -2 (OCT-1 and OCT-2), and perhaps for other unknown tissue-specific regulatory factors, controlling the expression of MMTV (Gouilleux et al. 1991; Tanaka et al. 1993; Biola et al. 2001). While NF-1 and OCT-1 are ubiquitous proteins, OCT-2 is expressed mainly in lymphoid cells (Prefontaine et al. 1998; Biola et al. 2001). Although the GREs are usually part of composite regulatory elements, certain glucocorticoid-inducible genes can be found to contain so-called simple GREs (Schoneveld et al. 2004). Among the glucocorticoid-inducible genes, the regulatory regions of which were found to contain simple GREs, is the gene for the gluconeogenic tyrosine hydroxylase, the serine/threonine protein kinase serum- and glucocorticoid-inducible kinase 1 (SGK1), and the β2-adrenergic receptor (Schoneveld et al. 2004).

JNK and p38 MAP kinases are proline-directed serine/threonine kinases activated in response to cellular stress (hyperosmotic shock, UV radiation, oxidative or chemical stress) and pro-inflammatory cytokines, such as TNF or IL-1 (Tibbles and Woodgett 1999; Ono and Han 2000). There are three principal MAP kinase families – the extracellular signal-regulated kinases (ERK), JNK and p38 – defined by their structural properties and unique phosphorylation sites (Widmann et al. 1999). Each of the MAP kinase subfamilies is activated by specific upstream MAP kinase kinases (MKK) that phosphorylate MAP kinases on a threonine and tyrosine residue separated by another intervening amino acid. Direct activators of JNK are MKK4 and MKK7 (Tournier et al. 1997; Wu et al. 1997), while activators of p38 are MKK3 and MKK6 (Raingeaud et al. 1996). JNK and p38 phosphorylate numerous downstream cellular effectors, including protein kinases such as MAPKAP-K2/3 (Clifton et al. 1996; Ni et al. 1998), Mnk1/2 (Cohen 1997), PRAK (also known as MAPKAP-K5) (New et al. 1998), and transcription factors including Elk-1, c-Jun, ATF-2, MEF2C and CHOP (New and Han 1998; Tibbles and Woodgett 1999; Yang et al. 1999; Ono and Han 2000). The GR-mediated transcriptional activation is also modulated both positively and negatively by phosphorylation (Bodwell et al. 1998; Ismaili 2004).

Earlier, we demonstrated that activation of MAP kinases inhibited GR-mediated transcriptional activation of the MMTV promoter-driven luciferase construct (MMTV-Luc) in HeLa cells (Rogatsky et al. 1998; Szatmary et al. 2004). While JNK inhibits GR transcriptional activation by direct receptor phosphorylation (Rogatsky et al. 1998), ERK and p38 do so indirectly (Rogatsky et al. 1998; Szatmary et al. 2004). In the present study we demonstrate that the effect of p38 and JNK is DNA regulatory element-specific and that the responsiveness to the MAP kinase activation is more complex then expected. The presence of other accessory binding sites of the MMTV construct contributes to the inhibitory effect of activated p38 or JNK on the MMTV-driven transcriptional activity; moreover, the responsiveness of the GR depends on the GRE sequence, as well. The apparent GRE-specificity of this action suggests that not all glucocorticoid-activated genes may be negatively regulated by p38 or JNK MAP kinases. It also indicates that protein-protein and DNA-protein interactions all influence GR activity.

Materials and Methods

Cell culture

HeLa human cervical carcinoma cells (ATCC, CCL2) and mouse 3T3 fibroblast cells (obtained from Dr. David E. Levy, NYU School of Medicine) were maintained in Dulbecco's modified Eagle's medium (D-MEM) with 2 mM L-glutamine (GIBCO/BRL), supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), and a mixture of 100 units/ml of penicillin and 100 μg/ml of streptomycin (Gemini Bio-Products). Hormone treatments were done in DMEM/10% charcoal-treated FBS containing 100 nM dexamethasone (Dex).

Reagents

Dexamethasone was purchased from Sigma, SB203580 and SP600125 were from Tocris Cookson Inc. Dexamethasone was dissolved in ethanol, whereas SB203580 and SP600125 were dissolved in dimethylsulfoxide (DMSO). The rabbit polyclonal anti-p38 (C-20) and anti-JNK1,3 (C-17) sera were purchased from Santa Cruz Biotechnology. The rabbit polyclonal anti-phospho-p38 (recognizing p38 dually phosphorylated on Thr180 and Tyr182) and monoclonal anti-phospho-JNK antibodies (recognizing JNKs dually phosphorylated on Thr183 and Tyr185) were obtained from New England Biolabs. The rabbit polyclonal antibody against LexA DBD was a gift from E. Golemis.

Plasmids

Full-length human MKK6b(E) and MKK7(D) sequences cloned into the pcDNA3 mammalian expression vector were gifts from Dr. Jiahuai Han (Scripps Research Institute, La Jolla, CA, USA), and were described previously (Huang et al. 1997; Yang et al. 1998; Szatmary et al. 2004). Briefly, the MKK6b(E) and MKK7(D) constructs encode constitutively active kinases containing mutations of the critical Ser and Thr residues in their activation loops to Asp or Glu, respectively. To asses the endogenous GR transactivation activity: MMTV-Luc, TAT3-Luc, TAT1-Luc, MT-Luc and TM-Luc reporter constructs were used. The MMTV-Luc reporter construct comprises a composite GRE from mouse mammary tumor virus LTR upstream of the luciferase gene in the pGL3-Basic vector. The TAT1-Luc contains a single copy of tyrosine aminotransferase (TAT) GRE2, whereas the TAT3-Luc is made up of three copies of this distal TAT GRE (Jantzen et al. 1987; Schoneveld et al. 2004), linked to the luciferase gene in the pΔODLO vector. The chimeric reporters, MT-Luc and TM-Luc, were gifts from Dr. Jeffrey N. Miner (Ligand Pharmaceuticals), and were described elsewhere (Guido et al. 1996). The pCMV NLxC construct consists of the full-length N- and C-termini of rat GR with a heterologous LexA DBD. The LexA-responsive luciferase reporter pΔ4x-LALO containing four LexA operators upstream of a minimal *Drosophila* ADH promoter linked to luciferase gene was used to measure transcriptional activity of the pCMV NLxC construct. The pcDNA3 vector (gift from Dr. David Wallach, Weizmann Institute) was used to equalize the total amount of transfected DNA.

Transient transfection and luciferase assay

Transient transfections and luciferase assays were performed in HeLa cells as described previously (Szatmary et al. 2004). Briefly, one day before transfection the cells were plated on 35-mm dishes in DMEM/10% charcoal-treated FBS. The following day, the subconfluent cells were transfected for 4 h with the indicated plasmids using Lipofectamine Plus Reagent (Life Technologies, Inc.). Twenty-four hours after the transfection the cells were treated with dexamethasone or left untreated (i.e., treated with an ethanol vehicle). Following the treatments, transfected HeLa cells were lysed in 150 µl 1 × Reporter Lysis Buffer (Promega). 50 µl aliquots of lysates were assayed for luciferase activity following addition to a 300 µl reaction volume containing 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml BSA, and 1 mM DTT. Luciferase activity was measured with Lumat LB9507 Luminometer (EG&G Berthold) using 1 mM D-luciferin (Pharmingen) as a substrate, and normalized to total protein concentration as determined by the Bio-Rad DC Protein Assay (modified Lowry method).

Western blotting

To make protein extracts, the cells were washed twice with ice-cold PBS and lysed directly on the dishes in 150 µl lysis buffer (1% NP-40, 50mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 mM PPi, 10 mM NaVan, 1 mM PMSF, 100 mM NaF; pH 7.5). The lysates were collected and centrifuged at 10 000 × *g* for 20 min at 4°C. Protein concentrations were adjusted in all samples with the lysis buffer, and the whole cell extracts were boiled with an equal volume of 2 × SDS sample buffer. For Western blotting, protein extracts were fractionated on 8–10% SDS/polyacrylamide gel (depending on the molecular weight of the protein), transferred to Immobilon paper (Millipore), and probed with anti-LexA DBD, anti-phospho-p38 (after stripping: anti-p38), anti-phospho-JNK (after stripping: anti-JNK1,3), respectively. The blots were developed with horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG (pp38; JNK1,3, or GR containing the LexA DBD), HRP-conjugated anti-mouse IgG (pJNK), or HRP-coupled protein A (p38) and enhanced chemiluminescence (Kirkegaard and Perry Laboratories).

Northern blotting

Mouse 3T3 fibroblast cells were treated with the indicated agents or left untreated (i.e., treated with the corresponding vehicle); then RNA was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. 10 µg of RNA was then electrophoretically separated after denaturation at 65°C using 1% agarose-formaldehyde gel, and transferred onto nylon membranes (Nytran, Schleicher & Schuell) by UV cross-linking. The membranes were then prehybridized for 30 min. at 68°C with QuikHyb (Stratagene). Murine glucocorticoid-induced leucine zipper (GILZ) (GenBank: AI 326808) and metallothionein I (MT-I) (Genbank: AI 427514) probes were prepared by cutting the pT7T3D-PAC cloning vec-

tors, containing coding sequences from the respective cDNA, with EcoRI and NotI. The control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) plasmid was cut with PstI. The cDNA probes were isolated after fractionation by electrophoresis using the QIAquick Gel Extraction Kit (Qiagen), and labeled with [α-^{32}P]dCTP using the Rediprime II Random Prime Labeling System (Amersham Biosciences). The labeled probes were then purified using G-25 sephadex columns and boiled with salmon sperm DNA. After a snap cool they were added to the QuikHyb solution and hybridized with the RNA attached to the membranes for 2 hours at 68°C. After washing, autoradiography was performed.

Results

Effect of p38 or JNK activation on GR-mediated transcriptional activation is promoter-context dependent

We have shown previously that activation of MAP kinases p38 (Szatmary et al. 2004), JNK (Rogatsky et al. 1998; Szatmary et al. 2004), or ERK (Rogatsky et al. 1998) inhibits GR-mediated transcriptional activation of the mouse mammary tumor virus promoter-driven luciferase construct (MMTV-Luc) in HeLa cells. Similarly, treatment with TNF (Szatmary et al. 2004) or IL-1 (Wang et al. 2004), pro-inflammatory cytokines capable to stimulate p38 and JNK, were found to have similar, although less pronounced effect on the same promoter construct.

The MMTV-Luc is the most extensively used GR-responsive reporter construct containing a so-called composite glucocorticoid response element (Fig. 1A). Similar composite response elements, composed of binding sites for several other transcription factors, can be found in regulatory regions of the majority of glucocorticoid-regulated genes. However, the regulatory regions of some glucocorticoid-inducible genes were found to contain so-called simple GREs (Schoneveld et al. 2004). The TAT3-Luc reporter construct exemplifies such a simple GR-responsive promoter, and contains three copies of the distal GRE from a tyrosine aminotransferase regulatory region (Jantzen et al. 1987; Schoneveld et al. 2004) (Fig. 1A). To further characterize the effects of p38 and JNK on GR-dependent transactivation, we compared their effects within these two different promoter-contexts.

HeLa cells were transiently transfected with MMTV-Luc or TAT3-Luc reporter constructs, the MAP kinase activator MKK6b(E) to activate p38 or MKK7(D) to activate JNK, respectively; along with appropriate controls, as indicated (Fig. 1B). Dexamethasone treatment for 16 h resulted in an about 6-fold higher stimulation of TAT3-Luc reporter activity containing three TAT GREs (Fig. 3B), as compared with the activity of MMTV-Luc with four MMTV GREs and additional binding sites (Fig. 2B); indicating, that the TAT GREs are stronger cis-acting regulatory elements than the MMTV GREs. For this reason, the data were normalized within each group (defined by the reporter construct) separately. Activation of p38 or JNK by its specific upstream activator, MKK6b(E) and MKK7(D), respectively, inhibited GR-induced activation of the MMTV promoter-driven luciferase construct, as we have reported previously (Szatmary et al. 2004). Surprisingly, using the TAT3-Luc reporter construct, no inhibition of the hormone-induced TAT3 promoter activity was observed in the presence of activated p38 or JNK. Rather, activation of p38 by MMK6b(E) transfection (Fig. 1C, top panel) enhanced dexamethasone-induced luciferase activity by approximately 2-fold, whereas MKK7-mediated JNK activation (Fig. 1C, panel 3) by ~3-fold (Fig. 1B). Transfection of MKK6b(E) or MKK7(D) did not affect the GR expression pattern, which was uniform throughout all of the experiments (Szatmary et al. 2004). The selective activation of endogenous p38 and JNK is evidenced by phosphorylation of the appropriate target protein (Fig. 1C, panels 1 and 3, respectively), without a change in the respective overall protein levels (Fig. 1C, panels 2 and 4, respectively).

Similarly, whereas TNF treatment reduced GR-mediated transcriptional activation using MMTV-Luc (Szatmary et al. 2004), it had no effect on it in the context of the TAT3 promoter (data not shown). These results suggest that the effect of MAP kinases and the pro-inflammatory cytokine TNF (probably IL-1, as well) on glucocorticoid-stimulated genes is promoter-context dependent. To address the question whether such a context-effect reflects the contribution of other transcriptional regulators bound nearby, or rather indicate a direct effect of the DNA sequence instead, we performed the following experiments.

Deletion of the additional accessory binding sites from the MMTV construct diminishes GR-dependent transcriptional activation and its inhibition by p38

The cells were co-transfected with MKK6b(E) along with MMTV-Luc or a recombinant MT-Luc reporter construct, and treated for 16 h with dexamethasone, as indicated. The recombinant MT-Luc construct contains MMTV GREs without additional accessory binding sites, upstream of the luciferase gene and a minimal ADH promoter (Guido et al. 1996). There are reports that interactions between the GR and transcription factors bound nearby to the accessory binding sites, as NF-1 and OCT-1/-2, result in transcriptional synergism, which is a prerequisite for an efficient responsiveness to the hormone-activated GR (Biola et al. 2001). Indeed, deletion of the accessory binding sites from the MMTV construct (Fig. 2A) resulted in a significant decrease (~50%) of glucocorticoid stimulation (Fig. 2B);

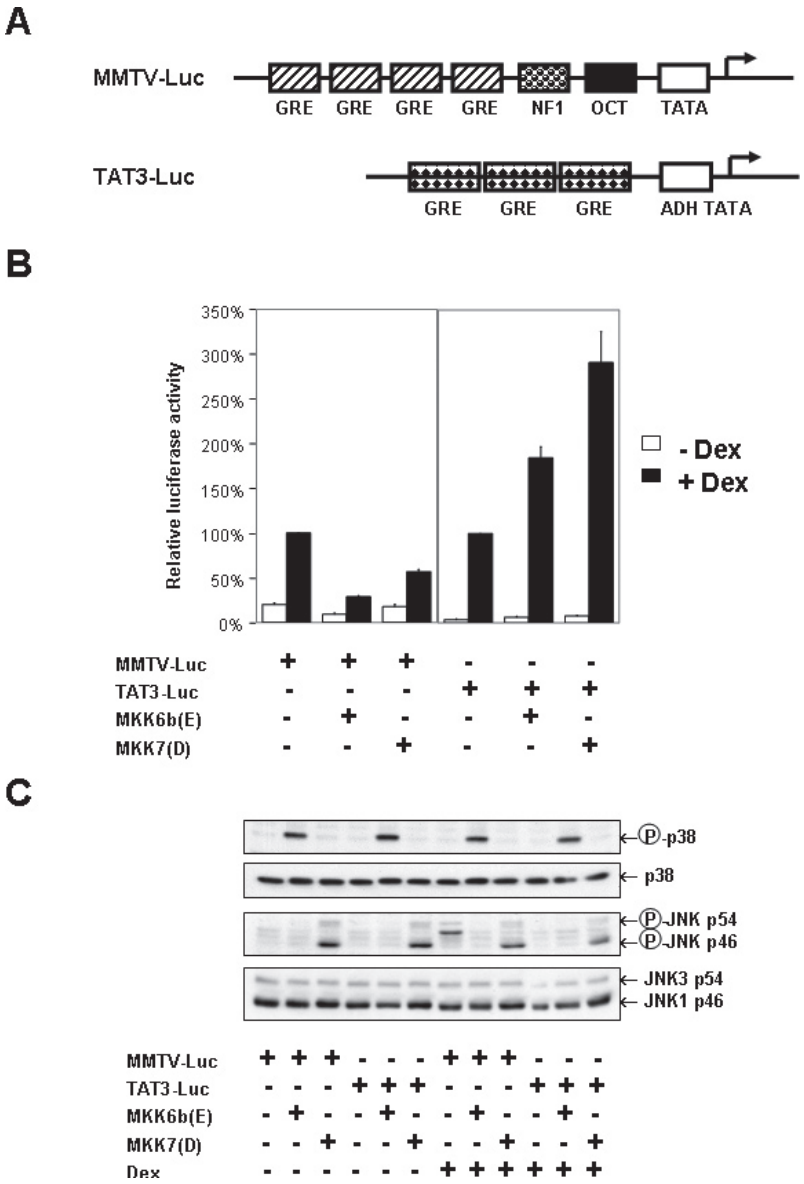

Figure 1. The effect of p38 or JNK activation on GR-dependent transcriptional activation is promoter-context dependent. **A.** Schematic representation of the composite MMTV-Luc reporter construct with accessory binding sites (for the simplicity, only the NF-1 site and one of the two OCT sites are shown; according to Guido et al. (1996)) and the simple TAT3-Luc reporter construct. **B.** Sub-confluent HeLa cells grown in 35-mm dishes were transfected with 0.1 μg of a pMMTV-Luc or pTAT3-Luc reporter construct, along with 1.35 μg of either MKK6b(E) or MKK7(D), as indicated. Empty pcDNA3 vector was used to equalize the amount of DNA in all transfection experiments. Twenty-four hours after transfection, the cells were treated for 16 h with 100 nM dexamethasone (+Dex) or control diluent (-Dex) prior to harvest. Luciferase activities were determined and normalized to total protein concentration as described under "Materials and Methods". Luciferase activities induced by dexamethasone alone, in the absence of MKK6b(E) and MJKK7(D) cotransfection, were set as 100 %. Results represent means ± S.E. from three independent transfections, each performed in duplicate. **C.** Whole cell lysates were prepared from a parallel set of transfected cells. The lysates were immunoblotted with an antibody against phosphorylated p38 and total p38 (top panels), as well as with anti-phospho-JNK (pJNK p54 and pJNK p46) and total JNK (JNK1 p46 and JNK3 p54) (bottom panels), to demonstrate comparable activation of endogenous p38 or JNK by MKK6b(E) or MKK7(D) transfection, and as loading controls, respectively. The circled letter P indicates phosphorylation.

which was, however, sufficient enough to accomplish the study of p38 and JNK inhibition. Transfection of the MKK6b(E) construct resulted in a specific activation of p38 (Fig. 2C, top panel) and inhibition of GR transcriptional activation in the context of MMTV GREs (Fig. 2B, left-hand bars). In the context of the same GREs, but without the accessory binding sites, activation of p38 failed to inhibit GR-mediated transcriptional activation (Fig. 2B, right-hand bars). The observed lack of inhibition was not due to reduced activity of p38 as shown by its selective phosphorylation without affecting the overall protein level (Fig. 2C). The same experiment where MKK7(D), a specific activator of JNK, was used instead of MKK6b(E), resulted in a similar outcome (data not shown).

Presence of MMTV LTR accessory binding sites inhibits stimulation of GR-mediated transcriptional activation in the context of TAT GREs

To complement the result with the MT-Luc reporter construct with deleted accessory binding sites, in the next set of experiments we applied an opposite approach. Namely, we compared the effect of activated p38 (Fig. 3) or JNK (data not shown) on GR-mediated transcriptional activation using TAT3-Luc and a recombinant TM-Luc reporter construct with the TAT GREs placed in the context of the MMTV LTR accessory binding sites (Guido et al. 1996) (Fig. 3A). The cells were co-transfected with MKK6b(E) along with TAT3-Luc or TM-Luc, treated with dexam-

ethasone for 16 h; and luciferase assay was performed, as previously. Dexamethasone treatment resulted in an almost 40-fold stimulation of TAT3-Luc (Fig. 3B, left-hand bars), and approximately 80-fold stimulation of TM-Luc reporter activity (Fig. 3B, bars 5–6). This is in agreement with the reported positive roles of factors bound nearby to the GR's transcriptional activity (Biola et al. 2001). While MKK6b(E) co-transfection essentially doubled the dexamethasone-stimulated GR activity on TAT3 GREs, addition of the extra transcription factor binding sites from the MMTV LTR almost completely eliminated this effect. The lack of the effect of MKK6(E) was not due to failure to activate p38, as evidenced by its increased phosphorylation and uniform expression pattern (Fig. 3C).

We obtained a similar result using MKK7(D), instead of MKK6b(E), in transfection experiments (data not shown). This is consistent with our previous finding that the presence of accessory binding sites from MMTV LTR (as NF1 and/or OCT-1/-2) contributes, at least partially, to the p38- and JNK-mediated inhibition of GR activity.

Influence of GRE sequences, either in isolation or in the context of MMTV LTR accessory binding sites, on the effect of p38 activation on GR transactivation

To demonstrate the sequence-specific effects of the GREs on p38-mediated actions, the data generated with TAT3-Luc and MMTV-Luc reporter constructs were compared to data obtained with MT-Luc and TM-Luc recombinant

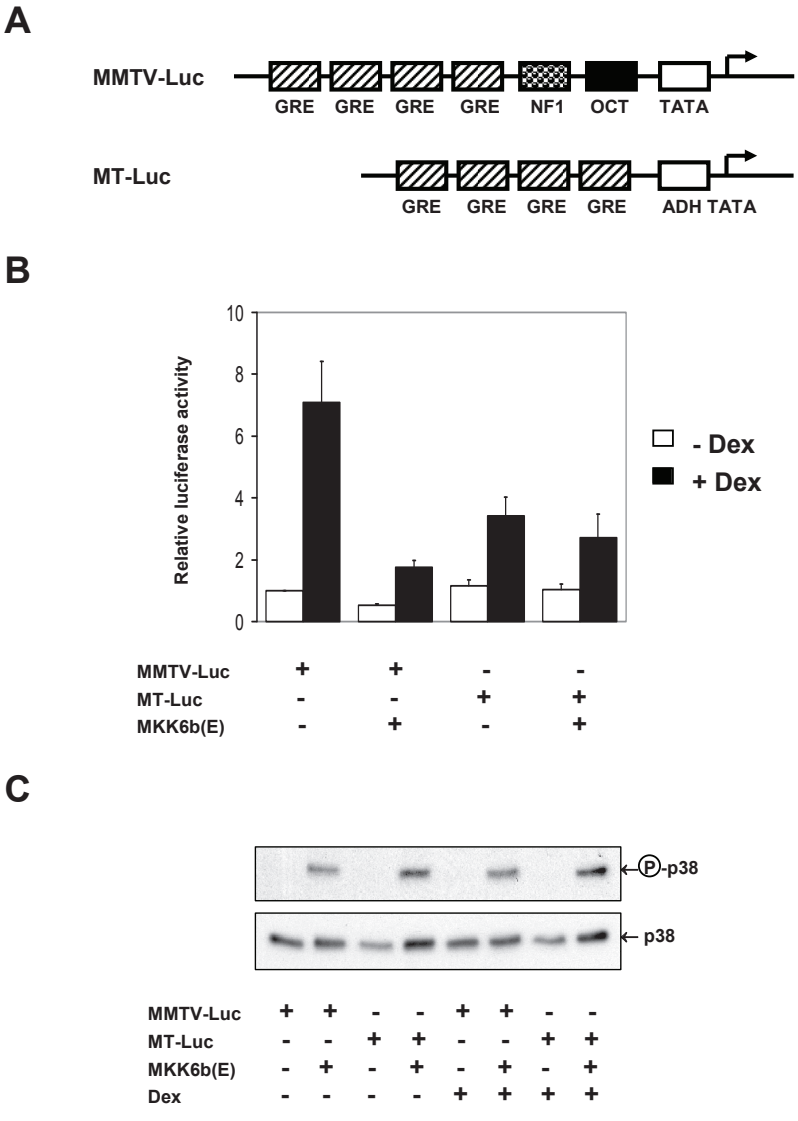

Figure 2. Deletion of accessory binding sites from the MMTV construct diminishes GR transactivation and its inhibition by p38. **A.** Schematic representation of the original MMTV-Luc reporter construct and the recombinant MT-Luc in which the accessory binding sites have been removed. This was achieved by replacing the TAT GREs in the TAT3-Luc reporter with the MMTV GRE-containing sequences (described in Guido et al. (1996), see "Materials and Methods"). **B.** HeLa cells were transfected with 0.1 μg of a pMMTV-Luc or MT-Luc reporter construct, and 1.35 μg of MKK6b(E), as indicated. Empty pcDNA3 was used to equalize the total amount of transfected DNA. Cells were treated with 100 nM dexamethasone (+Dex) for 16 hours or left untreated (-Dex). Luciferase activities were determined and normalized to total protein concentration. Results represent means ± S.E. from three independent transfections, each performed in duplicate. **C.** Equal amounts of protein were separated by SDS/PAGE, transferred and probed with an antibody against phospho-p38 (top panel, circled letter P indicates phosphorylation). The phospho-p38 immunoblot was then stripped and re-probed with an anti-p38 (bottom panel).

reporter constructs, respectively (Fig. 4). The effect of the GRE sequence is evident. Activation of p38 by its specific upstream activator, MKK6b(E), stimulated GR transactivation by 200% in the context of TAT GREs, but had no effect in the context of MMTV GREs without the accessory binding sites (Fig. 4A). On the other hand, activated p38 inhibited MMTV promoter-driven GR-mediated transcription, whereas slightly stimulated GR activity from TAT GREs when they were placed in the context of the MMTV LTR accessory binding sites (Fig. 4B). Similar comparison, except that MKK7(D) was used instead of MKK6b(E), resulted in a similar outcome (data not shown). The distance of the GREs from the transcription-start site and also their relative phase on the DNA-helix was identical in the two compared constructs, respectively (which was achieved by subcloning them into the same position in identical expression vectors).

Substitution for the DNA-binding domain of the GR by heterologous LexA DNA-binding domain eliminates the effect of activated p38 on GR-mediated transcriptional activation

To further characterize the effect of DNA-binding to p38-mediated modulation of GR activity, we substituted for the DNA-binding domain of the GR by heterologous LexA DNA-binding domain. The NLxC construct, containing the full-length N- and C-terminal domains from rat GR linked to heterologous LexA DNA-binding domain, was ectopically expressed in HeLa cells, along with the LexA-responsive pΔ4x-LALO reporter construct (Fig. 5A). Dexamethasone-treatment for 16 h resulted in a marked stimulation of lucirerase activity, but co-transfection with MKK6b(E) failed to inhibit the transcriptional activity of the NLxC construct, pointing out the role of DNA-bind-

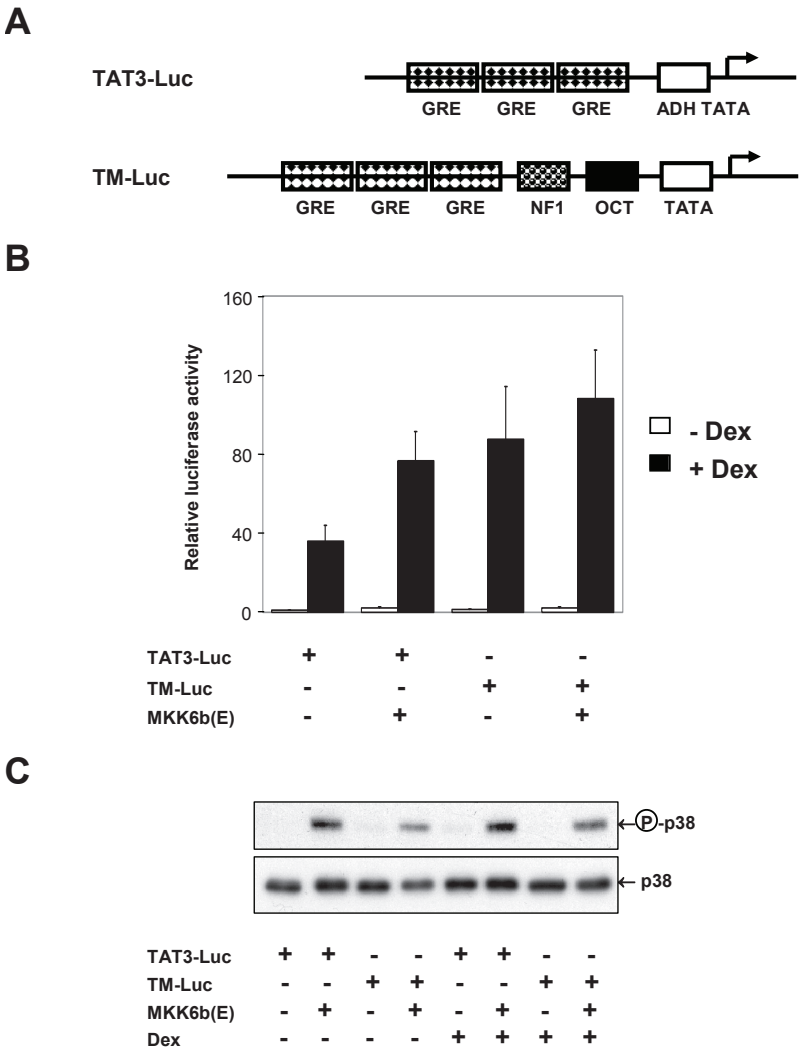

Figure 3. Presence of MMTV LTR accessory binding sites inhibits stimulation of GR transactivation by p38 in the context of TAT GREs. **A.** Schematic representation of the parental TAT3-Luc reporter construct and the recombinant TM-Luc construct. The TM-Luc reporter contains the distal TAT GREs inserted into the same context as the MMTV GRE sequences in the parental MMTV-Luc construct. **B.** HeLa cells were transfected as described in the legend for Fig. 2, except that TAT3-Luc and TM-Luc were used as reporter constructs. Luciferase activities were determined and normalized to total protein concentration. Results shown are the average of three independent experiments performed in duplicate ± S.E. **C.** Lysates were immunoblotted with an antibody against phospho-p38 (top panel) and p38 (bottom panel), similarly as in Fig. 2. Dex, dexamethasone.

ing in the effects of activated p38 on GR transactivation (Fig. 5B). Uniform expression of the ectopically expressed construct was ascertained by immunoblot with antibody against LexA DBD (Fig. 5C, top panel). As in the previous experiments, transfection with MKK6b(E) resulted in increased p38 phosphorylation without a concomitant change in p38 protein levels (Fig. 5C, middle and bottom panel, respectively).

Inhibitor of JNK, SP600125, promoter-dependently enhances transcription of endogenous, dexamethasone-induced GILZ mRNA, but not dexamethasone-induced MT-I mRNA

We have clearly demonstrated that the effect of activated p38 on GR-mediated transcription depends on promoter-context. Similarly, we found that the effect of JNK activation on GR-dependent transcription, by transfection of its specific upstream activator MKK7(D), is promoter context-dependent, as well (data not shown). In order to assess whether the effects of p38 and JNK in modulation of GR activity could be more generalized, we used different experimental setting. We examined the effect of various stress stimuli, capable to activate both p38 and JNK (Alpert et al. 1999), on transcription of two endogenously expressed GR-responsive proteins, namely glucocorticoid-induced leucine zipper (GILZ) and methallothionein-I (MT-I). Mouse 3T3 cells were treated for 90 min with hyperosmolar sorbitol (hyperosmotic stress), H_2O_2 (oxidative stress), or the sulfhydryl reagent arsenite (chemical stress) followed by treatment with dexamethasone for 1 h. All three agents completely inhibited GILZ and MT-I transcription induced by dexamethasone (data not shown). SB203580 and SP600125, the specific inhibitors of p38 (Cuenda et al. 1995; Alpert et al. 1999) and JNK (Bennett et al. 2001; Han et al. 2001), respectively, failed to reverse their effect; indicating an induction of an additional inhibitory pathway(s)

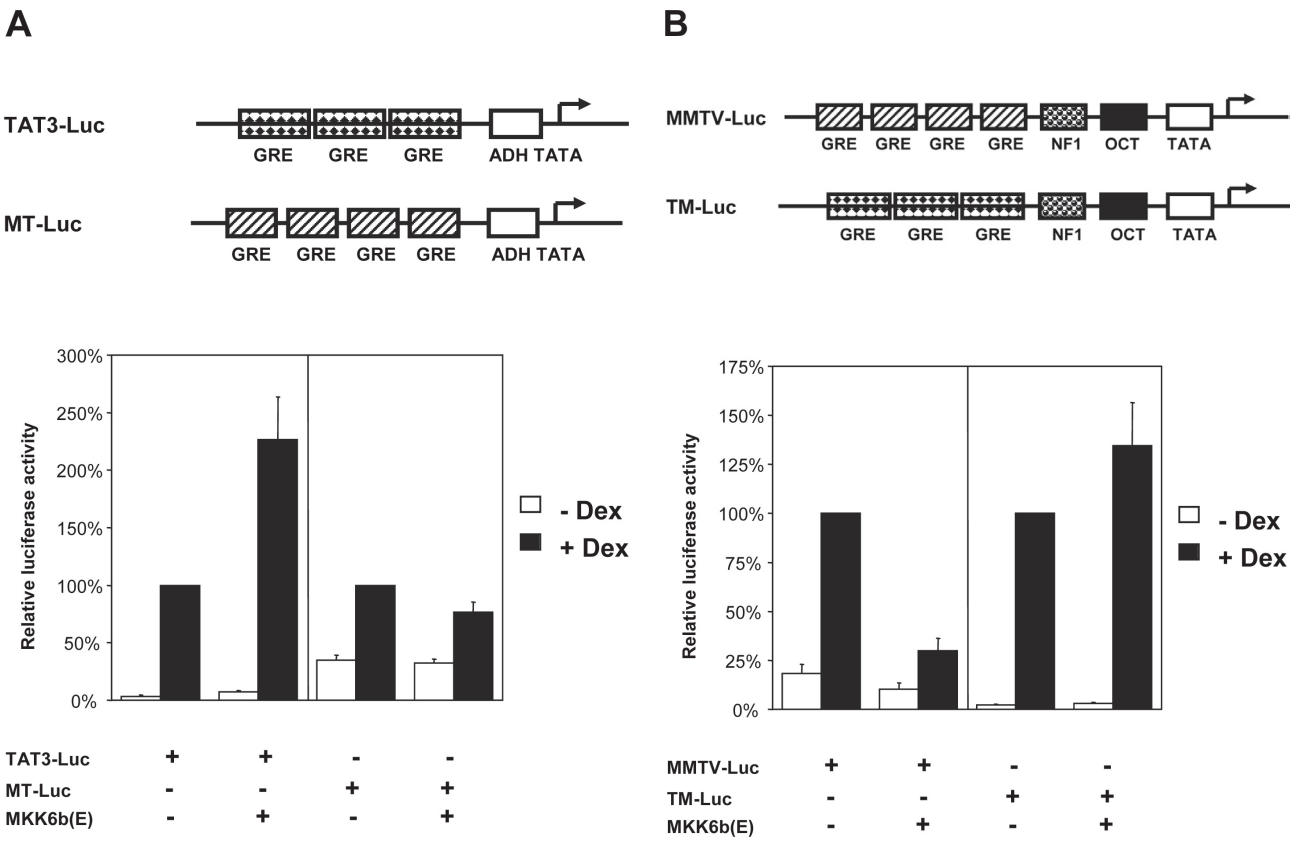

Figure 4. Influence of GRE sequences, either in isolation or in the context of MMTV LTR accessory binding sites, on the effect of p38 activation on GR transactivation. The data generated with the TAT3-Luc and MT-Luc constructs (GREs from the parental MMTV-Luc construct placed in the context of the minimal ADH-promoter using an identical pΔODLO expression vector) are cross-compared in **A**; whereas results using MMTV-Luc and TM-Luc (i.e., GREs from the parental TAT3-Luc construct placed within the context of MMTV LTR accessory binding sites using an identical pGL3-Basic vector) are shown in **B**, respectively. For better comparison, luciferase activities induced by dexamethasone (Dex) without MKK6b(E) co-transfection were set as 100%. The data shown are means ± S.E from three independent experiments, each performed in duplicate.

by these agents (data not shown). Surprisingly, SP600125, a specific inhibitor of JNK, enhanced transcription of an endogenous GILZ induced by dexamethasone (Fig. 6A), but failed to do so with dexamethasone-induced MT-I transcription (Fig. 6B). This finding further emphasizes the importance of the promoter-context in the JNK-mediated effect(s) on GR-dependent transcriptional activation.

Discussion

We reported earlier that activation of p38 or JNK inhibits GR-mediated transcriptional activation of the MMTV promoter-driven luciferase construct (MMTV-Luc) in HeLa cells (Rogatsky et al. 1998; Szatmary et al. 2004). While JNK inhibits GR transcriptional activation by direct receptor phosphorylation at the N-terminus (Rogatsky et al. 1998), p38 does so by indirectly targeting the LBD (Rogatsky et al. 1998; Szatmary et al. 2004).

In order to assess the general importance of the effects of activated p38 and JNK in modulation of GR activity, we examined the effect(s) of various stress stimuli, capable of activating both p38 and JNK (Alpert et al. 1999), on transcription of two endogenously expressed GR-responsive genes, namely GILZ and MT-I. All three agents completely inhibited their dexamethasone-induced transcription (data not shown). (However, we were unable to prove that this was mediated by p38 and/or JNK, since their specific inhibitors failed to reverse this inhibition (data not shown). This does not rule out the involvement of p38 and/or JNK in the observed effect of these stress stimuli, only indicates a presence of an additional inhibitory pathway(s).)

Among the presently known glucocorticoid-responsive genes very few contain simple GREs. The majority harbors imperfect GREs in the context of additional binding sites for other transcription factors – called composite GRE; allowing more exact tuning of gene regulation and permitting additional signal transduction pathways to influence the transcriptional activity mediated through the glucocorticoid-response element. In transcription assays, the MMTV-Luc is the most extensively used reporter construct containing a composite GRE.

The simple GRE is exemplified by the TAT3-Luc construct. We have shown here that p38 or JNK activation stimulates GR-dependent transcription from the TAT3-Luc construct in the same cells (Fig. 1); indicating that the effect of the p38 and JNK on the responsiveness of the GR is determined by the promoter-context. Biola et al. (Biola et al. 2001) found similar promoter-dependency of IL-2-mediated inhibition of GR-dependent transcriptional activation. Similarly, overexpression of STAT-5 (signal transducer and activator of transcription-5) in prolactin-activated COS-7 cells resulted in an increased activity of the β-casein gene promoter upon treatment with glucocorticoids, whereas the MMTV promoter activity was decreased under similar conditions (Stocklin et al. 1996). Moreover, mutation analysis revealed that GR hyperphosphorylation increased the magnitude of the hormone-induced response of the GR on TAT GREs, whereas had no effect on its transactivation potential in the context of the MMTV LTR (Webster et al. 1997; Bodwell et al. 1998; Wang et al. 2002). In spite of these findings, the underlying mechanism of this promoter context-dependency has not been investigated.

It has been shown, that GR function is regulated by post-translational covalent addition of a small ubiquitin-related modifier-1 (SUMO-1) (Le Drean et al. 2002). In contrast to ubiquitin, which targets its protein substrates to the proteasome, SUMO-1 does not signal proteolysis. Instead, it appears to play multiple roles in protein stabilization and translocation, nuclear body formation, and modulation of transcriptional activity of certain transcription factors (Le Drean et al. 2002). Overexpression of SUMO-1 stimulated GR's transactivation capacity on a simple promoter, but this effect required multiple copies of consensus GREs *per* promoter ($p(GRE)_{x4}$-TK-Luc). On the contrary, in the context of the MMTV LTR (pMMTV-Luc), SUMO-1 had no effect on GR transactivation. To rule out sumoylation in the observed stimulatory effect of activated p38 and JNK on transcription from TAT3-Luc, we performed experiments with TAT1-Luc, containing only a single copy of the TAT's distal GRE linked to a luciferase gene. Using this construct, instead of TAT3-Luc, resulted in less stimulation of the transcriptional activity, but the outcome, regarding the effects of MKK6b(E) and MKK7(D), was qualitatively identical (data not shown); excluding the involvement of sumoylation in the observed effects.

In the light of these results we can further clarify the mechanism by which JNK and p38 exert their inhibitory effects in the context of composite GREs; and we can rule out that activation of JNK or p38 prevents binding of the glucocorticoid hormone to the GR's LBD or retains the GR in the cytoplasm, since it did not prevent dexamethasone from stimulating the TAT3 promoter. Therefore, the cellular localization where p38 and JNK modulate the activity of the GR is most likely in the nucleus. Using a milder detergent (1% Nonidet P-40, instead of 2% SDS) we found that the activation of p38 caused the ectopically expressed LBD construct to move to an insoluble fraction (data not shown), indicating a formation of an inhibitory complex, in which the LBD and/or DNA-binding might play a role. Similar mechanism was found in the repression of cAMP-responsive genes by insulin (Nakajima et al. 1996). Nakajima et al. demonstrated that this occurs through promotion of an interaction between $pp90_{RSK}$ and the CBP coactivator, which interfered with CBP activity. However, we did not detect fluctuations of the accessible endogenous GR protein

levels as a result of p38 or JNK activation (Rogatsky et al. 1998; Szatmary et al. 2004).

To further dissect the underlying mechanism of the observed promoter-dependency, we tested a model, in which the actions of p38 and JNK rely on other transcription factors bound nearby, in the context of the composite MMTV promoter (Figs. 2 and 3, data not shown). The inhibition of p38 (Fig. 2) and JNK (data not shown) on GR-responsive transcriptional activation was eliminated by removing the accessory binding sites for these transcription factors, indicating their involvement in the inhibitory effects. In addition, placing the TAT GREs in the same position as the MMTV GREs within the MMTV LTR (that is, in the context of the MMTV LTR accessory binding sites), counteracted their p38- (Fig. 3) and JNK-dependent (data not shown) stimulatory effect on GR activity.

Comparison of the effects of the GRE sequences, either in isolation (Fig. 4A) or in the context of the MMTV accessory binding sites (Fig. 4B), clearly demonstrated that the GR responsiveness depends on the bound GRE sequence, as well. Moreover, replacing the GREs by LexA-binding sites prevented the activated p38 and JNK from inhibiting, resp. stimulating the glucocorticoid-induced activity of the GR fusion protein (Fig. 5, data not shown). In agreement with these findings, single base pair mutations in composite GREs of the prolactin and proliferin genes, as well as in the DNA-binding domain of the GR, switch between GR-repressible and GR-inducible states (Sakai et al. 1988; Starr et al. 1996; van Tilborg et al. 2000; Rogatsky et al. 2001).

One potential explanation could be that allosteric modulation of GR conformation by the respective GRE influences the interaction with coactivator proteins (Fig. 7). Indeed,

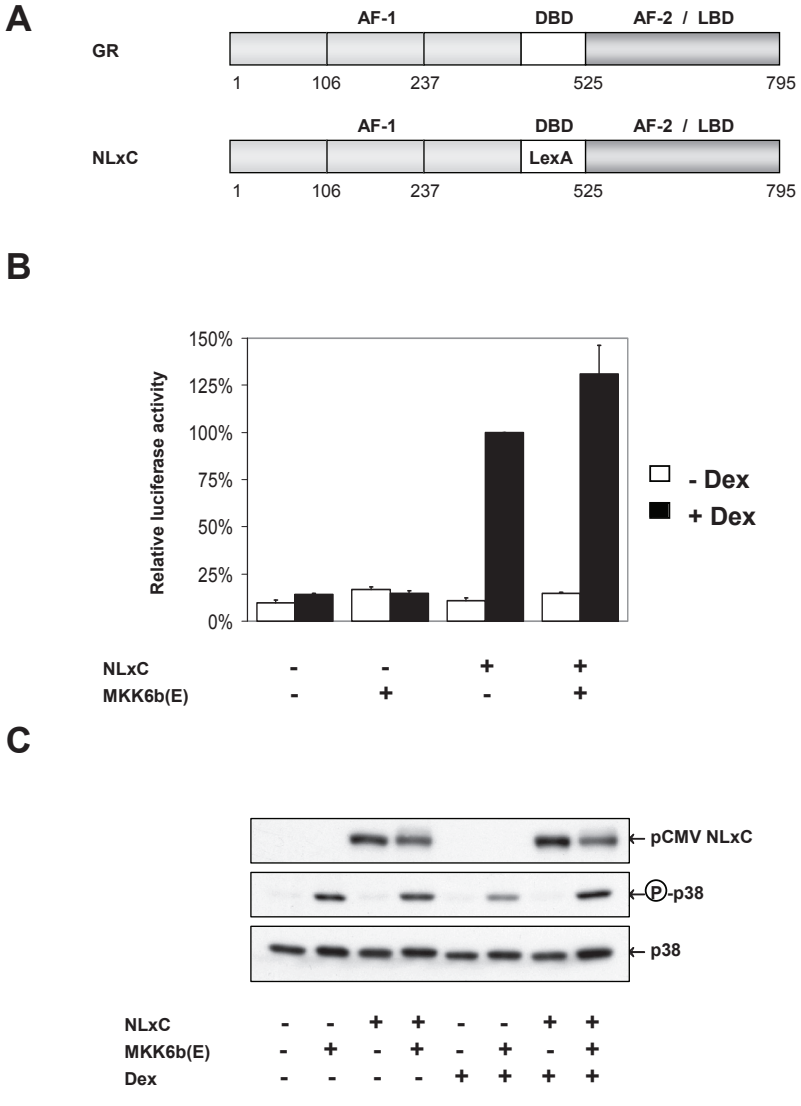

Figure 5. The heterologous LexA DNA-binding domain eliminates the effect of activated p38. **A.** Schematic representation of the GR construct consisting of the full-length N- and C-terminal domains from rat GR linked to the heterologous LexA DNA-binding domain. **B.** The NLxC construct was ectopically expressed in HeLA cells using 1.35 μg of a pCMV NLxC construct. In addition, each dish received 0.1 μg of a pΔ4x-LALO reporter construct in combination with 1.35 μg of MKK6b(E) (or empty pcDNA3), as indicated. On the following day, cells were treated with 100 nM dexamethasone (+Dex) for 16 h or left untreated (-Dex). Luciferase activities were determined and normalized to total protein concentration. We found that activated p38 stimulates CMV promoter-driven NLxC expression (Szatmary et al. 2004), therefore the transfected amounts of the NLxC construct were adjusted accordingly; this adjustment, however, did not result in a qualitatively different outcome of the GR activity assay. The data shown are means ± S.E. of three independent experiments, each performed in duplicate. **C.** Immunoblot analyses were performed using an anti-LexA DBD antibody to detect the NLxC construct (top panel), as well as anti-phospho-p38 (middle panel, circled letter P indicates phosphorylation) and anti-p38 (bottom panel) antibodies, as indicated.

experiments employing partial protease digestion of estrogen receptor α (ERα) bound to various types of ER elements (EREs) revealed distinct cleavage products depending on the individual ERE, suggesting that the response element has an effect on receptor conformation (Klinge et al. 2001; Barkhem et al. 2002). Recent data suggest that different types of GREs may induce distinct conformational changes of the GR structure that could influence receptor-coactivator interactions, among others (Lefstin and Yamamoto 1998; van Tilborg et al. 2000; Rogatsky et al. 2001).

It has been proposed that the recruitment of p160 coactivators by GR is promoter-dependent. Grenier et al. (2004) have recently shown that selective inhibition of GR coactivators SRC-1 and SRC-2 (GRIP1/TIF2) by RNA interference inhibits MMTV-driven luciferase transcription, whereas inhibition of SRC-3 (RAC3/ACTR/AIB1) has no effect. On the other hand, in the context of a simple promoter ((GRE)2-TATA), silencing of the SRC-1 and the RAC3/ACTR/AIB1, but not GRIP1/TIF2, inhibited GR-mediated transactivation. These results were further confirmed by overexpression experiments. It seems likely that the promoter-context influences the affinity of the GR for its cofactors (Barkhem et al. 2002; Li et al. 2003; Grenier et al. 2004). It has been suggested, that GR bound to a minimal promoter recruits SRC-1 and SRC-3/RAC3/ACTR/AIB1, but SRC-2/GRIP1/TIF2 is excluded; whereas GR bound to an MMTV recruits SRC-1e and SRC-2/GRIP1/TIF2, while SRC-1a and SRC-3/RAC3/ACTR/AIB1 are excluded. In this scenario, activation of p38 or JNK might inactivate SRC-2/GRIP1/TIF2, which in turn would repress MMTV activation; on the other hand, the activity of SRC-3 (or another coactivator present exclusively in the context of the simple GRE) might be further stimulated by activated p38 or JNK, therefore enhancing the transcription from TAT3-Luc (Fig. 7). Interestingly, cytoplasmic localization of the p160 coactivators depends on their phosphorylation status (Grenier et al. 2004), therefore they might be the direct targets of these MAP kinases.

In addition, we found unexpectedly that SP600125, a specific inhibitor of JNK, enhanced GILZ transcription induced by dexamethasone (Fig. 6A), but not MT-I transcription (Fig. 6B); further emphasizing the importance of the promoter-context in the effect(s) of JNK. SB203580, a specific inhibitor of p38, had no effect on dexamethasone-induced transcription of either GILZ (Fig. 6A) or MT-I (Fig. 6B); suggesting, that there might be a more pronounced residual (constitutive) activity of JNK than of p38. GILZ is among the most responsive genes up-regulated by glucocorticoids, and there is a speculation that it might be a general mediator of their anti-inflammatory and immunosuppressive actions

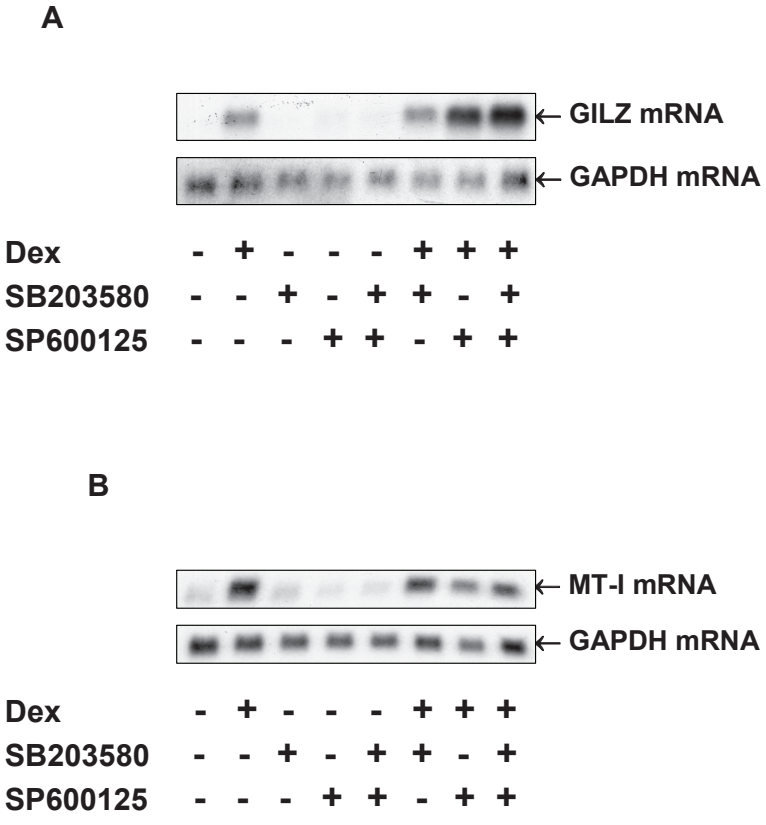

Figure 6. JNK inhibitor, SP600125, stimulates transcription of dexamethasone-induced GILZ mRNA, but not dexamethasone-induced MT-I mRNA. Mouse 3T3 cells were pre-treated for 1.5 h with 10 μM SB203580 or SP600125, or left untreated (i.e., treated with a DMSO vehicle); then 100 nM dexamethasone or control diluent (ethanol) was added, as indicated. After 1 h incubation, total RNA was isolated. Ten-μg of RNA was fractionated on 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled probes as described under "Materials and Methods". **A.** Stimulation of dexamethasone-induced GILZ mRNA by SP600125 is shown (top panel). Equal loading and transfer for each lane was determined by ethidium bromide staining of rRNAs (not shown), as well as by re-probing the membrane with GAPDH cDNA (bottom panel). **B.** Dexamethasone induced transcription of MT-I mRNA (top panel), similarly as transcription of GILZ mRNA (see above), but SP600125 had no effect in this promoter-context. Transcription of the control GAPDH mRNA (bottom panel) is also demonstrated.

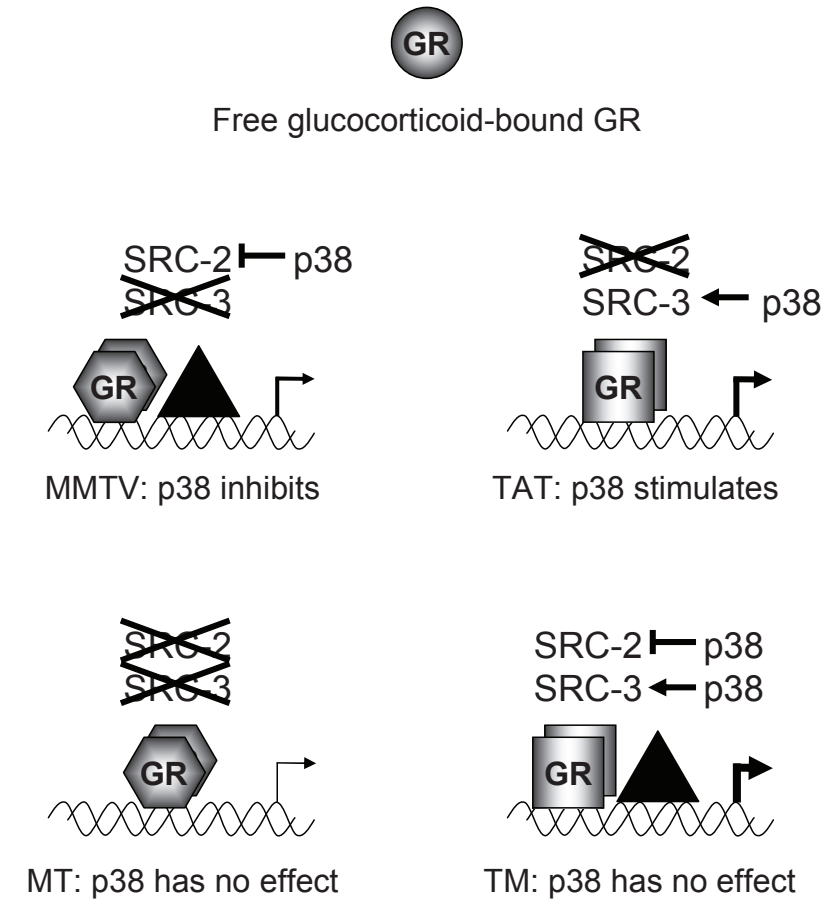

Figure 7. A hypothetical model for modulation of GR activity by activated p38 in various promoter-contexts. Adapted from Lefstin and Yamamoto (1998), according to Grenier et al. (2004). The schematic representations of the promoter constructs are shown in Figs. 1A, 2A, 3A, 4A and 4B. The arrows denote activation and the lines with bars inhibition.

(Mittelstadt and Ashwell 2001; Berrebi et al. 2003). It has been found recently, that GILZ promoter is a composite GRE, containing two octamer (OCT) binding sites in addition to six GREs and other response elements (Asselin-Labat et al. 2005).

In summary, our data indicate that the promoter-context plays an important role in regulation of GR-responsiveness to activation of p38 and/or JNK. Moreover, in addition to transcription factors bound nearby, interaction with the DNA itself might modulate GR activity, as well. A similar mechanism of promoter-specificity has been suggested for the transcription factor NF-κB (Leung et al. 2004). Therefore, GR-mediated transcriptional activities also should be evaluated according to the respective promoter-context(s).

Acknowledgements. We thank Jan Vilček, Michael J. Garabedian, Hans-Georg Wisniewski, Paul Schwenger, Steven Markus and Deborah Alpert for many helpful discussions and reagents.

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Received: May 10, 2011
Final version accepted: May 23, 2012