Inhibition of Glucocorticoid Receptor-mediated Transcriptional Activation by p38 Mitogen-activated Protein (MAP) Kinase*

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Zoltán Szatmáry‡, Michael J. Garabedian, and Jan Vilček§

From the Department of Microbiology, New York University School of Medicine, New York, New York 10016

Tumor necrosis factor (TNF) promotes certain immune and inflammatory responses, whereas glucocorticoids exert immunosuppressive and anti-inflammatory actions. We show that TNF treatment produced a modest inhibition of glucocorticoid receptor (GR)-mediated transcriptional activation of a mouse mammary tumor virus (MMTV) promoter-driven luciferase construct in HeLa cells. The mitogen-activated protein (MAP) kinases, p38 and c-Jun N-terminal kinase (JNK), are important mediators of target gene activation by TNF, and JNK activation was earlier shown to inhibit GR-mediated transcriptional activation by direct phosphorylation of GR at Ser-246. Transfection of HeLa cells with MKK6b(E), a constitutively active specific upstream activator of p38, led to a potent inhibition of GR activation of the MMTV promoter-driven luciferase construct. A similar inhibition of activation of the MMTV promoter-driven luciferase construct was seen in HeLa cells transfected with MKK7(D), a constitutively functional activator of JNK. Data from “domain swap” experiments using GR chimeras indicated that the main target of the p38-mediated (but not JNK-mediated) inhibition is the ligand-binding domain of GR (spanning amino acids 525–795), whereas the constitutively active N-terminal AF-1 region (spanning amino acids 106–237) is dispensable for the inhibitory effect of p38. We also demonstrate that activated p38 targets the GR ligand-binding domain indirectly. Suppression of GR function by activated p38 and JNK MAP kinases may be physiologically important as a mechanism of resistance to glucocorticoids seen in many patients with chronic inflammatory conditions.

Tumor necrosis factor (TNF)1 and glucocorticoids have opposite effects on inflammatory and immune responses. TNF and its signaling intermediates promote many immune and inflammatory processes (1–3), whereas glucocorticoids are generally immunosuppressive and they inhibit pro-inflammatory events, including bacterial lipopolysaccharide-induced TNF production (4, 5). Glucocorticoid receptor (GR) is a ligand-dependent transcription factor with a modular structure, the principal functions of which (transcriptional activation, DNA binding, and ligand binding) are localized to specific domains (6). The unliganded GR exists in the cytoplasm as a large heteromeric complex that comprises hsp90 and other stabilizing proteins (7). Binding of glucocorticoids 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 in regulatory regions of target genes (6).

TNF plays a role in immune responses important in host defenses against infectious agents (1–3, 8). One possible outcome of TNF signaling is apoptosis, resulting from the activation of the caspase cascade. More commonly, TNF signaling activates the expression of genes with pro-inflammatory and anti-apoptotic activities. TNF-induced gene expression is largely mediated by activation of NF-κB and AP-1 transcription factors (1–3, 8, 9). A mutually antagonistic relationship exists between NF-κB and GR in that NF-κB can suppress the function of GR and GR effectively inhibits NF-κB activation (10–12). Activation of AP-1 family transcription factors by TNF is mediated by the c-Jun N-terminal kinase (JNK) and p38 MAP kinase (1, 3, 8). Inhibition of the synthesis of pro-inflammatory cytokines, including TNF and interleukin-1, and of other inflammatory mediators is thought to be the main mechanism whereby glucocorticoids exert their immunosuppressive and anti-inflammatory activities (4, 5). These actions are at least partly mediated by interference with transcription factors, especially NF-κB and AP-1 (12–14). NF-κB inhibition is in part due to the induction by glucocorticoids of IκB, the inhibitory subunit of NF-κB (10). The anti-inflammatory actions of glucocorticoids have also been attributed to direct interactions between GR and NF-κB or AP-1 (12, 14) and to the activation of some genes with anti-inflammatory action (15).

JNK and p38 MAP kinases are proline-directed serine/threonine kinases activated in response to cellular stress (hypersmotic shock, UV radiation, oxidative, or chemical stress) and pro-inflammatory cytokines, such as TNF or interleukin-1 (16, 17). Three principal MAP kinase families, the extracellular signal-regulated kinases (ERK), JNK, and p38, are defined by their structural properties and unique phosphorylation sites (18). 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 (19, 20), whereas activators of p38 are MKK3 and MKK6 (21). Earlier we demonstrated that JNK and ERK inhibited GR-mediated transcriptional activation, which could be attributed to GR phosphorylation at Ser-246 by JNK but not ERK (22). P38 kinase failed to phosphorylate a GR fragment comprising Ser-
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246 in vitro, but the effect of p38 on GR-mediated transcriptional activation has not been examined (22).

In the present study, we examined the effect of TNF on glucocorticoid signaling. TNF action results in a rapid activation of p38 and JNK, and these MAP kinases are responsible for the activation of downstream target genes by TNF (8, 23). We demonstrate that p38 activation by its specific upstream activator MKK6b leads to a potent inhibition of GR transcriptional activity. We show that the p38-mediated inhibition targets actions mediated by the ligand-binding domain (LBD) of GR. In agreement with earlier data (22, 24), we show that activation of JNK by its upstream activator, MKK7, greatly diminished GR transcriptional activity. In the intact organism, suppression of GR function by activated p38 and JNK MAP kinases may represent one of the mechanisms responsible for unresponsiveness to glucocorticoids that develops in many patients with chronic inflammatory conditions (25, 26).

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa human cervical carcinoma cells (ATCC, CCL2) were maintained in Dulbecco’s modified Eagle’s medium with 2 mm l-glutamine (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (HyClone), and a mixture of 100 units/ml penicillin and 100 μg/ml streptomycin (Gemini Bio-Products). Hormone treatments were done in Dulbecco’s modified Eagle’s medium/10% charcoal-treated fetal bovine serum containing 100 nm dexamethasone.

Reagents—Dexamethasone was from Sigma, and recombinant human TNF was provided by Masafumi Tsujimoto of the Suntory Institute for Biomedical Research (Osaka, Japan). SB203580 was from Tocris Cookson Inc. To detect endogenous GR, we used polyclonal anti-GR prepared in one of our laboratories (27). The rabbit polyclonal anti-p38 (C-20) and anti-JNK1,3 (C-17) sera were from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-p38 (recognizing p38 dually phosphorylated on Thr-180 and Tyr-182) and monoclonal anti-phospho-JNK antibodies (recognizing JNKs dually phosphorylated on Thr-183 and Tyr-185) were from New England Bioslabs. The anti-HA mouse monoclonal antibody (clone 12CA5) was from Roche Applied Science.

Plasmids—Full-length human MKK6b(E) and MKK7(D) sequences cloned into the pcDNA3 mammalian expression vector were gifts from Dr. Jiahui Han, Scripps Research Institute. The MKK6b(E) and MKK7(D) constructs encode constitutive active kinases containing mutations of the critical Ser and Thr residues in their activation loops to Gln or Asp, respectively. The pHAGAL4-GR525–795 construct, encoding the ligand-binding domain of GR fused to the GAL4 DNA-binding domain and HA tag, was prepared by subcloning the XhoI/XbaI fragment of pHAGAL4-GR525–795 into pcDNA3/HAGAL4. pHAGAL4-GR106–227 contains the GR N-terminal AP-1 domain linked to GAL4 DNA-binding domain (DBD) and HA tag. The mutant pHAGAL4-GRLBDT547A was generated using the QuiKChange site-directed mutagenesis kit (Stratagene), according to manufacturer’s instructions. All generated plasmids were verified by sequencing. MMTV-Luc reporter construct, containing composite glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity. The p4xβ luciferase construct, containing multimerized glucocorticoid-response element from mouse mammary tumor virus long terminal repeat upstream of the luciferase gene, was used to assess GR transcriptional activity.
was not due to a reduction in GR expression as the level of receptor protein was not significantly affected by MKK6b(E) (Fig. 2B, top panel). However, dexamethasone treatment slightly reduced GR protein levels (Fig. 2B, top panel), likely due to receptor down-regulation by the ligand (31). In addition, pretreatment with SB203580, a specific p38 inhibitor (30, 32), reversed the inhibition of GR transactivation by MKK6b(E), confirming that the inhibitory effect was indeed attributable to MKK6b(E)-mediated p38 activation (data not shown). Altogether, these results demonstrate that sustained specific activation of p38 results in a strong inhibition of GR transcriptional activation.

**Activation of Endogenous JNK by Its Specific Activator MKK7(D) Inhibits GR-mediated Transcriptional Activation**

Earlier work showed that JNK phosphorylates GR at Ser-246, leading to an inhibition of GR-mediated transcription (22, 24). To confirm the effect of JNK activation on endogenous GR activity and to compare it with the effect of p38 activation, we transfected HeLa cells with MKK7(D), a specific constitutive activator of JNK (30, 33), along with the MMTV-Luc reporter construct used in the preceding experiments. MKK7(D) decreased dexamethasone-stimulated and constitutive GR transcriptional activation in a dose-dependent manner, with the highest dose of MKK7(D) inhibiting dexamethasone-stimulated GR transcription by 80% (Fig. 3A). For comparison, one group was co-transfected with MMTV-Luc and the p38 activator MKK6b(E) used in the previous experiment (Fig. 3A, right-hand bars), which resulted in a slightly higher inhibition of GR transactivation than co-transfection with MKK7(D). MKK7(D)-mediated activation of JNK is evident from a dose-dependent increase in the phosphorylation of JNK p54 and especially JNK p46 (Fig. 3B, middle panel). MKK7(D) co-expression did not significantly affect the GR protein level (Fig. 3B, top panel) or the level of JNK proteins (Fig. 3B, bottom panel). As also seen in the previous experiment shown in Fig. 2B, we observed a slight dexamethasone-induced down-regulation of GR (Fig. 3B, top panel). Thus, in agreement with previous findings (22, 24), we observed a potent inhibitory effect of JNK activation on GR transcriptional activation.

**Role of the Ligand-binding Domain/AF-2 of GR in p38-mediated Inhibition**—It is well known that different functions can be localized to specific GR regions (34). The DBD is localized in the center of the molecule (spanning amino acids 440–525 in the rat GR), whereas the C-terminus of the ligand binding domain amino acids 525–795 is the LBD. At least two regions possess intrinsic transcriptional activation functions. AF-1, located at the N terminus, is hormone-independent and constitutive. In contrast, AF-2, which maps to the C terminus, is hormone-dependent. To identify the regions of GR that might be involved in the observed inhibition, the AF domains of rat GR were linked to the heterologous GAL4 DNA-binding domain and were assessed separately for their ability to be repressed by p38 or JNK.

The HAGAL4-GR525–795 construct, containing the ligand-dependent AF-2/LBD domain linked to the HA-tagged heterologous GAL4 DNA-binding domain (Fig. 4A), was co-transfected into HeLa cells with a GAL4-luciferase reporter construct. Dexamethasone treatment resulted in a greater than 100-fold stimulation of reporter activity, which was potently inhibited by co-transfection with the p38 activator, MKK6b(E) (Fig. 4B).
This inhibitory effect was completely reversed by treatment with the p38 inhibitor, SB203580 (32), confirming that the effect was indeed due to activated p38 (data not shown). In contrast, co-transfection with the selective JNK activator, MKK7(D), produced a much weaker inhibitory effect, consistent with earlier data showing that the inhibitory action of JNK activation produced by the HAGAL4-GR106–237 construct is not attributable to phosphorylation of Ser-246 (22, 24), which is not present in the HAGAL4-GR525–795 construct. Uniform expression of the ectopically expressed construct was ascertained by immunoblot with anti-HA antibodies (Fig. 4C, top panel). In addition, we confirmed that transfection with the p38 and JNK activators indeed resulted in a selectively increased phosphorylation of p38 and JNK, respectively, without affecting the cellular levels of p38 or JNK proteins (Fig. 4C, panels 2–5). Taken together, these results show that at least one of the major targets of the inhibition mediated by p38 (but not JNK activation) is the AF-2/LBD region.

The AF-1 Domain of GR Is Dispensable for the Inhibitory Effect of p38—HeLa cells were co-transfected with the constitutively nuclear and transcriptionally active HAGAL4-GR106–237 construct comprising the hormone-independent AF-1 region of GR (Fig. 5A), the MAP kinase activators MKK6b(E) or MKK7(D), and the GAL4-luciferase reporter construct, along with appropriate controls, as indicated (Fig. 5B). The cells were allowed to recover for 24 h, and luciferase activities were quantified to assess the constitutive transcriptional activity mediated by the transfected GR construct in the absence of dexamethasone treatment. Transfection of the HAGAL4-GR106–237 construct resulted in a marked (about 20-fold) stimulation of luciferase activity (Fig. 5B). Neither MKK6b(E) nor MKK7(D) had a significant inhibitory activity on the transcriptional activation produced by the HAGAL4-GR106–237 construct, indicating that the N-terminal AF-1 domain is dispensable for the inhibitory effect of activated p38 or JNK. This lack of inhibition was not due to the failure of MKK6b(E) or MKK7(D) to activate p38 or JNK, respectively, as evidenced by the increased selective phosphorylation of the appropriate target proteins without a significant change in the respective overall protein levels (Fig. 5C). Failure of MKK6b(E) or MKK7(D) to significantly affect the transcriptional activity of the HAGAL4-GR106–237 construct also shows that the GAL4 DBD is not affected by p38 (or JNK) activation and that transfection of cells with MKK6b(E) or MKK7(D) does not result in a general transcriptional repression. The failure of JNK activation to affect transcriptional activity of the HAGAL4-GR106–237 construct is not unexpected because Ser-246, earlier shown to be the target of JNK phosphorylation, is not present in the HAGAL4-GR525–795 construct.
of the inhibitory action of JNK (22, 24), lies outside the region encompassed by this construct.

**Activated p38 Targets the GR AF-2/LBD Region Indirectly**—Transcription factors have been identified as direct targets of p38 phosphorylation (35); therefore, it seemed plausible that the AF-2/LBD region of GR might be a direct substrate of p38. Only one potential p38 MAP kinase consensus sequence was identified in the AF-2/LBD region (a threonine at position 547 followed by a proline). To determine whether this site is required for the inhibitory action of p38, we prepared a mutant HAGAL4-GR525–795 construct in which Thr-547 was changed to a nonphosphorylatable alanine residue (HAGAL4-GR-LBDT547A) by site-directed mutagenesis (Fig. 6A). Point mutation of Thr-547 failed to reverse the inhibitory effect of MKK6b(E), suggesting that the inhibition does not involve a direct phosphorylation of the AF-2/LBD of GR by p38 at this residue (Fig. 6B). As in the previous experiments, transfection with MKK6b(E) resulted in increased p38 phosphorylation without a concomitant change in p38 protein levels (Fig. 6C). These results suggest that p38 affects the AF-2/LBD region indirectly.

**DISCUSSION**

Among the many demonstrated immunosuppressive and anti-inflammatory actions of glucocorticoids is their ability to inhibit TNF synthesis by blocking translation of the TNF protein (4, 5). Glucocorticoids are also known to suppress TNF actions, including NF-κB-mediated transcription and activation of JNK (4, 11, 12). To determine whether TNF reciprocally inhibits glucocorticoid actions, we examined the effect of TNF on GR-mediated transcriptional activation in cells transfected with the MMTV-Luc reporter construct (Fig. 1). We found that treatment with TNF suppresses GR signaling, but the inhibition was relatively modest, only up to −30% (Fig. 1). Interleukin-1, another inflammatory cytokine that stimulates p38 and JNK, was shown to produce a similar moderate inhibition.
(−35%) of GR-mediated transcription in a line of mouse L929 cells (36). In contrast to this moderate inhibitory effect, activation of either p38 or JNK by co-transfection of their specific immediate upstream activators, MKK6b(E) or MKK7(D), respectively, resulted in a potent inhibition of GR transactivation in the same cell line (Figs. 2 and 3). A likely reason why TNF was less effective in suppressing GR transcriptional activation than transfection of cells with the upstream activators of p38 or JNK is that TNF induces a rapid but transient p38 and JNK activation that subsides by about 1 h (37, 38), whereas p38 and JNK activation in cells transfected with MKK6b(E) or MKK7(D) is much more sustained and still pronounced after 16–24 h (Figs. 2B, 3B, 4C, 5C, and 6C). Sustained p38 or JNK activation may be needed for an efficient inhibition of GR transactivation. However, the inhibitory effect of TNF may be more pronounced under physiological conditions because in the intact organism, the response of cells and tissues to TNF is likely to be more gradual and sustained (38).

An inhibitory effect of JNK on GR transcription was demonstrated earlier. Our group showed that JNK (but not p38) produced direct phosphorylation of GR on Ser-246 in vitro and that selective activation of JNK in intact cells inhibited GR-mediated transcription (22). Subsequently, Itoh et al. (24) showed that JNK-mediated phosphorylation of Ser-226 in human GR (equivalent to Ser-246 in rat GR) enhances GR nuclear export, leading to termination of GR transcription. In contrast to JNK, the inhibitory action of p38 kinase on GR transcription has not been analyzed previously. To identify GR regions that may be the target of the inhibitory actions of p38, we used reporter constructs comprising either the C-terminal AF-2/LBD domain or the N-terminal hormone-independent AF-1 region linked to the heterologous GAL4 DBD (Figs. 4A and 5A).

We demonstrated that transcriptional activation mediated by the C-terminal AF-2/LBD domain was strongly inhibited by p38 activation elicited by MKK6b(E) (Fig. 4B). This inhibition was completely reversed by the p38 inhibitor, SB203580, confirming that the effect is indeed attributable to activated p38 (data not shown). However, JNK activation elicited by MKK7(D) produced only a weak inhibitory effect on the transcriptional activation mediated by the C-terminal AF-2/LBD
domain, indicating that p38 and JNK inhibit GR transcriptional activation by different mechanisms. No significant inhibition was seen with either MKK6b(E) or MKK7(D) in cells co-transfected with a reporter construct consisting of the AF-1 domain (residues 106–237) linked to the GAL4 DNA-binding domain (Fig. 5). The latter finding is consistent with reports that inhibition of GR-mediated transcriptional activation by JNK depends on GR phosphorylation at Ser-246 (22, 24), which lies outside the region encompassed by the HAGAL4-GR106–237 construct.

To further dissect the mechanism of the inhibitory action of p38 and to determine whether the C-terminal LBD/AF-2 domain may be the direct target of p38 phosphorylation, we prepared a mutant HAGAL4-GR525–795 construct in which an alanine residue was substituted for Thr-547 (the only potential p38 phosphorylation site within this region) by site-directed mutagenesis (Fig. 6A). The location of the point mutation is indicated with X. B, 1.35 μg of HAGAL4-GR525–795 or mutant GR construct was transfected into HeLa cells along with pGAL4-Luc and MKK6b(E), as indicated. Twenty-four hours after transfection, the cells were treated as indicated, and luciferase activities were determined and normalized to total protein concentration. The data shown are means ± S.E. of three experiments, each performed in duplicate. Dex, dexamethasone.

C, immunoblot analysis was performed using an anti-HA antibody to detect the HAGAL4-GR525–795 and the mutant GR constructs, and with anti-phospho-p38 and anti-p38 antibodies, as indicated. The circled letter p indicates phosphorylation.

Fig. 6. Direct phosphorylation of the AF-2/ligand-binding domain of GR by p38 is not required for inhibition. A, a nonphosphorylatable GR AF-2/ LBD mutant was generated from HAGAL4-GR525–795 in which Thr-547 was mutated to alanine, as diagrammed. The location of the point mutation is indicated with X. B, 1.35 μg of HAGAL4-GR525–795 or mutant GR construct was transfected into HeLa cells along with pGAL4-Luc and MKK6b(E), as indicated. Twenty-four hours after transfection, the cells were treated as indicated, and luciferase activities were determined and normalized to total protein concentration. The data shown are means ± S.E. of three experiments, each performed in duplicate. Dex, dexamethasone.

Dex, dexamethasone.
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clear receptors (51). Finally, p38 could induce or activate other transcription factors (16, 17, 23, 35) that might sequester critical coactivator(s) required for GR transcriptional activation, leading to inhibition of GR function by regulatory squelching (52).

In summary, we have clearly demonstrated that activation of p38 inhibits the transcriptional activation of GR. We showed that p38 targets the AF-2/LBD domain of GR but obtained evidence suggesting that p38 does not target AF-2/LBD directly. Our data indicate that p38 and JNK inhibit GR actions by distinct mechanisms, consistent with the earlier conclusion that phosphorylation of Ser-246 is responsible for the inhibitory effect of JNK (22, 24). The inhibitory effect of p38 and JNK on GR function may contribute to the antagonism between TNF and glucocorticoids. Perhaps more importantly, activation of p38 and JNK within inflammatory lesions may be one of the causes of refractoriness to steroid treatment that develops in many patients with chronic inflammatory diseases such as rheumatoid arthritis, Crohn’s disease, or asthma (25, 26, 36).

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