The DNA Binding-independent Function of the Glucocorticoid Receptor Mediates Repression of A P-1-dependent Genes in Skin

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Abstract. The glucocorticoid receptor (GR) mediates the biological effects of glucocorticoids (GCs) through activation or repression of gene expression, either by DNA binding or via interaction with other transcription factors, such as A P-1. Work in tissue culture cells on the regulation of A P-1–dependent genes, such as collagenase (MMP-13) and stromelysin (MMP-3) has suggested that the antitumor and antiinflammatory activity of GCs is mediated, at least in part, by GR-mediated downmodulation of A P-1. Here, we have identified phorbol ester-induced expression of MMP-3 and MMP-13 in mouse skin as the first example of an in vivo system to measure negative interference between A P-1 and GR in the animal. Cell type-specific induction of these genes by tumor promoters is abolished by GCs. Importantly, this is also the case in GRdim mice expressing a DNA binding-defective mutant version of GR. In contrast, the newly identified target genes in skin, plasma glutathione peroxidase and HSP-27, were induced by GC in wild-type, but not in GRdim mice. Thus, these data suggest that the DNA binding-independent function of the GR is dispensable for repression of A P-1 activity in vivo and responsible for the antitumor-promoting activity of GCs.

Key words: tumor promotion • mouse skin • AP-1 • matrix metalloproteinase • collagenase • glucocorticoid receptor • GRdim

Glucocorticoids (GCs) are in widespread medical use to inhibit inflammatory processes. Furthermore, they are known to block experimentally induced tumorigenesis, such as phorbol ester-induced multiplet carcinogenesis in mouse skin (Belman and Troll, 1972). These biological activities of GCs are mediated by positive or negative regulation of gene expression via binding to the glucocorticoid receptor (GR), a member of the steroid hormone receptor superfamily, which acts as a ligand-induced transcription factor (Beato et al., 1995).

Different modes of action of transcriptional regulation by GR have been described. Upon ligand-induced dimerization the GR binds to conserved DNA motifs known as glucocorticoid response elements (GRE) and negative acting GREs (nGREs), respectively, to positively or negatively regulate gene expression (Beato et al., 1995; Karin, 1998). Second, mutual interference between GR and other transcription factors, such as A P-1 and NFκB, does not require DNA binding of the GR, but rather is mediated by interaction with these factors (Beato et al., 1995; Karin, 1998). The physiological importance of the dimerization- and DNA binding-independent repression function of GR was documented in homozygous mice carrying a DNA binding-defective mutant of GR (GRdim). In these mice, DNA binding and, as a consequence, transcriptional regulation of target genes containing GREs and nGREs was impaired, whereas repression of A P-1–mediated gene expression in primary embryonic fibroblasts was normal (Reichardt et al., 1998). In contrast to GR-deficient mice, GRdim mice survive until adulthood (Cole et al., 1995; Reichardt et al., 1998), suggesting that the capacity of the
Materials and Methods

Animals

The dorsal skin of 7-9-wk-old female C57BL/6J mice (BRL) or GRdim mice (Richard et al., 1998) was shaved 4 d before experimentation. Mice were treated topically with 1-10 nmol TPA or with 50 μg dexamethasone (Sigma Chemical Co.) dissolved in 200 μl acetone. The animals were killed 0-6 h after application.

Northern Blot Analysis

Skin tissues were homogenized and RNA was prepared as described previously (Richard et al., 1998) and analyzed by Northern blot (Gack et al., 1995). Immunochemistry was performed using a polyclonal rabbit anti-human GR antibody (M20; Santa Cruz), followed by an ABC staining procedure (ABC Rabbit IgG Kit; Vector Labs, Inc.) according to the manufacturer’s instructions.

In Situ Hybridization and Immunohistochemistry

6-μm paraffin sections from skin biopsies were subjected to situ hybridization using 35S-UTP-labeled sense and antisense probes of MMP-13- and MMP-3 as described in Gack et al. (1995). Immunohistochemistry was performed using a polyclonal rabbit anti–mouse GR antibody (M20; Santa Cruz), followed by an ABC staining procedure (ABC Rabbit IgG Kit; Vector Labs, Inc.) according to the manufacturer’s instructions.

cDNA Expression Array Hybridization

A tlas™ mouse cDNA expression array I filters were hybridized with radiolabeled first strand cDNA following the specifications of the manufacturer (CLONTECH). Differences in expression patterns were analyzed using A1S software and Array Vision software module (Imaging Research).

Results

Rapid Induction of MMP-13 and MMP-3 Gene Expression by TPA in Mouse Skin

To establish an in vivo experimental system suitable to measure A P-1-dependent gene expression and to study mutual interference between A P-1 and GR in the animal, we first determined expression of MMP-3 and MMP-13 in mouse skin after treatment with TPA. In mock-treated animals, transcription levels of MMP-3 and MMP-13 were barely detectable (Fig. 1 a). Upon application of TPA, an up to 100-fold induction of both genes was observed.

Figure 1. TPA induces MMP-13 and MMP-3 in skin. a, TPA (10 nmol) in acetone was applied to the back skin. After 0, 1, 4, and 6 h, mice were killed and RNA from skin was prepared. M M P-13, MMP-3, c-jun, and c-fos expression was analyzed by Northern blot analysis. b, TPA (0.1, 1, 10, and 50 nmol) was applied as described in a, and animals were killed after 6 h and analyzed by Northern blot analysis. Rhybridization with a cDNA fragment of 18S RNA was performed serving as a loading control.
within four to six hours. Induction of both genes is dose-dependent, reaching maximal levels at 10 nmol, whereas 1 nmol of TPA was not sufficient for potent induction (Fig. 1b). Furthermore, upregulation was preceded by transcriptional activation of the main regulators of MMP-3 and MMP-13 gene expression, c-jun and c-fos. Induction of these immediate early genes was already detectable within one hour and declined at four hours in the case of c-jun, and between four and six hours for c-fos (Fig. 1a).

To identify the specific cell type responsible for enhanced MMP-13 and MMP-3 gene expression in response to TPA, in situ hybridization analysis of parallel transversal sections of skin biopsies was performed. Whereas in untreated skin, no significant signals for the expression of MMP-13 and MMP-3 could be detected (data not shown), both genes were highly induced in a cell type-specific manner upon phorbol ester treatment (Fig. 2). MMP-13 transcripts were observed in the epidermis only in a subset of basal keratinocytes (Fig. 2, a and b). MMP-3 expression was found exclusively in monocytic cells in the dermal...
compartment (Fig. 2, c and d). Interestingly, in contrast to primary and immortalized skin fibroblasts showing enhanced expression of both genes in response to TPA (Clark et al., 1985; Wilhelm et al., 1987), neither MMP-13 nor MMP-3 transcripts were found in fibroblasts of the dermis (Fig. 2, b and d).

**Repression of TPA-induced Genes by Glucocorticoids in Skin**

We next wanted to investigate whether GR could interfere with AP-1 activity under these conditions. Since the expression pattern of GR in murine skin has not yet been described, we first wanted to confirm the presence of GR in MMP-3- and MMP-13-expressing cells in the skin by immunohistochemistry. Strong expression of GR protein was observed throughout the epidermis (Fig. 2, e and f) and in mononuclear cells in the dermis (Fig. 2 f). Weaker signals for GR protein were observed in dermal fibroblasts (Fig. 2 f). These data show that GR is expressed by a broad range of cells in the skin, including MMP-13 and MMP-3-positive cells. Therefore, TPA-induced expression of MMP-13 and MMP-3 in skin in the presence or absence of GC is an appropriate system to measure GR-specific inhibition of gene expression in vivo. Consequently, we asked whether TPA-induced expression of MMP-13 and MMP-3 is repressed by dexamethasone in these cells. We found a complete inhibition of induction of both genes in skin after concomitant treatment with TPA and dexamethasone in skin (Fig. 3 a).

**Repression of Gene Transcription by the GR Does Not Require DNA Binding of the Receptor**

To analyze in more detail the molecular mechanism responsible for the repression of AP-1-dependent genes by GC in skin, we took advantage of GR dim mice. These mice carry a DNA binding-defective GR. Previously, transient transfection studies in tissue culture cells suggested that the DNA binding function of the GR is not required for transrepression of AP-1-mediated transcription (Heck et al., 1994). However, in light of the lack of induction of MMP-3 and MMP-13 expression by TPA in dermal fibroblasts in vivo (Fig. 2) and the complex regulatory processes present in skin, it is still possible that both DNA binding-dependent and -independent functions of GR are required for repression of TPA-induced expression of MMP-3 and MMP-13 in skin cells. To determine the importance of either one of these functions of GR, we analyzed the expression of both genes in GR dim mice. Expression of both MMP-3 and MMP-13 is dramatically induced upon TPA treatment in GR dim mice, similar to wild-type mice. In situ hybridization analysis confirmed that induction of these genes in GR dim mice originates from basal keratinocytes and mononuclear cells (data not shown), resembling the pattern of expression in wild-type mice (Fig. 2). Interestingly, induction was completely repressed by dexamethasone in both wild-type and GR dim mice (Fig. 3 a), which strongly suggests that the DNA binding-dependent function of the GR is not required for repression of TPA-induced MMP gene expression in vivo.

GR has been reported to either activate (Jonat et al., 1990) or repress c-jun expression (Wei et al., 1998) in tissue culture cells, depending on the cell type. To confirm that GR-dependent repression of MMP-3 and MMP-13 is not required by a loss of c-jun or c-fos expression upon hormone treatment, we measured the level of c-fos and c-jun transcripts in unstimulated and stimulated mouse skin. Significant basal level of c-jun transcripts can be detected in wild-type and GR dim mice, which became further enhanced upon TPA treatment (Fig. 3 b). Induction of c-fos was even more pronounced. Expression of c-jun, but not c-fos, was significantly induced by dexamethasone in both wild-type and GR dim mice, which might be mediated by the DNA binding-independent, positive function of GR on Jun/Jun homodimers (Miner and Yamamoto, 1992; Teurich and Angel, 1995) binding to the c-jun promoter. Most importantly, dexamethasone reduced TPA-induced expression of c-fos and c-jun only slightly (Fig. 3 b). The presence of c-jun and c-fos transcripts in TPA- and dexamethasone-treated animals confirmed that repression of phorbol ester-induced MMP-3 and MMP-13 expression is most likely due to inhibition of AP-1 activity by GR and cannot be explained by a loss of expression of the critical AP-1 components c-jun and c-Fos.

**Positive Regulation of PGX-3 and HSP27 by GC in Mouse Skin Is Absent in GR dim Mice**

To prove abrogation of GR-mediated transactivation function in GR dim mice, we aimed to detect differences in GC-dependent regulation of GR target genes in skin cells of wild-type and GR dim mice. Therefore, we performed gene expression profiling on a mouse Atlas™ cDNA expression array. Filters containing spotted DNA from 588 known genes were hybridized in parallel with radiolabeled cDNA derived from RNA of skin from untreated and dexamethasone-treated mice. Among the differentially expressed genes (data not shown), two examples, PGX-3 and HSP-27, were analyzed by Northern blot analysis (Fig. 4). In the skin of wild-type mice, expression of both PGX-3 and HSP-27 was significantly upregulated (4.3- and 2.9-fold, respectively) six hours after dexamethasone treatment. Enhanced levels of PGX-3, but not HSP-27, already were detectable after 1.5 hours (data not shown). Impor-
Importantly, the phorbol ester-induced expression of MMP-13 and MMP-3 was efficiently inhibited by GCs in a cell type-specific manner. To analyze if DNA binding of the GR is involved in this downregulation of MMP-13 and MMP-3 in vivo, we made use of GR<sup>dim</sup> mice. To address the question whether gene activation by GCs is indeed impaired in GR<sup>dim</sup> mice, we identified GC-induced genes in the skin using a high-density filter screening approach. PGX-3 and HSP27 were among the strongest induced genes and as yet have not been described to be responsive to GCs. For both PGX-3 and HSP27, we could show a failure of GC-mediated upregulation in GR<sup>dim</sup> mice.

In contrast to loss of GC-dependent gene activation in GR<sup>dim</sup> mice, TPA-mediated expression of AP-1 target genes, such as MMP-13, MMP-3, and MMP-9 (gelatinase B; data not shown) is efficiently downregulated by dexamethasone. Obviously, the DNA binding function of GR and subsequent transcriptional activation of GRE-dependent genes is not required for transrepression of AP-1 in vivo. The presence of c-fos and c-jun transcripts upon cotreatment with TPA and dexamethasone suggests that the de novo synthesis of both AP-1 components is not abolished by GCs. Specifically, induction of c-jun by TPA, which requires the activity of MAP kinases, such as JNK, to hyperphosphorylate preexisting c-jun protein, was not repressed by GCs. Thus, inhibition of the JNK pathway by GR, which was described in tissue culture cells (Caëlies et al., 1997), may not play a major role in vivo, at least in skin.

In transgenic mice, overexpression of c-jun and c-fos, as well as tissue-specific expression of the AP-1 target genes, human collagenase and stromelysin, induces, or at least enhances, tumorigenesis (Wang et al., 1991, 1995; D’Arimiento et al., 1995; Sternlicht et al., 1999). Downregulation of AP-1 target gene expression has been proposed to be a crucial event in GC-mediated inhibition of tumor formation in the multiple stage model of carcinogenesis in skin (J onat et al., 1990). Here, we demonstrate that TPA, one of the most potent and best characterized tumor promoters in mouse skin carcinogenesis, induces MMP-13 and MMP-3 very rapidly at the same dosage, which is, when applied periodically, optimal to mediate tumor formation (Fürstenberger and Kopp-Schneider, 1995). The critical role of AP-1 in MMP-13 and MMP-3 expression and the downmodulation of expression of both genes by GCs strongly suggest that transrepression of AP-1 activity is a key feature of antitumor promoting activity of GCs. The finding that this activity of GR does not require the DNA binding function might be highly valuable in the search for better therapeutical strategies of GC application in skin carcinogenesis and related diseases.

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