

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

Jan P. Tuckermann,* Holger M. Reichardt,[‡] Rosa Arribas,[‡] K. Hartmut Richter,* Günther Schütz,[‡] and Peter Angel*

*Division of Signal Transduction and Growth Control, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany; and

[‡]Division of Molecular Biology of the Cell I, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany

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 AP-1. Work in tissue culture cells on the regulation of AP-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 AP-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 AP-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 GR^{dim} 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 GR^{dim} mice. Thus, these data suggest that the DNA binding-independent function of the GR is dispensable for repression of AP-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 • GR^{dim}

GLUCOCORTICOIDS (GCs)¹ are in widespread medical use to inhibit inflammatory processes. Furthermore, they are known to block experimentally induced tumorigenesis, such as phorbol ester-induced multi-step 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).

Address correspondence to Peter Angel, Division of Signal Transduction and Growth Control, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel.: 49 6221-42-4570. Fax: 49 6221-42-4554. E-mail: p.angel@dkfz-heidelberg.de

Jan P. Tuckermann's present address is Division of Molecular Biology of the Cell I, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany.

1. *Abbreviations used in this paper:* GCs, glucocorticoids; GR, glucocorticoid receptor; GR^{dim}, mice homozygous for a mutation in the endogenous GR gene; GRE, GR responsive element; MMP, matrix metalloproteinase; PGX-3, plasma glutathione peroxidase-3; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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 AP-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 (GR^{dim}). In these mice, DNA binding and, as a consequence, transcriptional regulation of target genes containing GREs and nGREs was impaired, whereas repression of AP-1-mediated gene expression in primary embryonic fibroblasts was normal (Reichardt et al., 1998). In contrast to GR-deficient mice, GR^{dim} mice survive until adulthood (Cole et al., 1995; Reichardt et al., 1998), suggesting that the capacity of the

GR to interfere with other transcription factors plays an essential role for normal functions in the body.

Down-modulation of the activity of transcription factors, such as AP-1 and NF κ B, is thought to mediate the antiinflammatory and antitumor promoting activities of GCs. Although different lines of evidence have suggested an important role of both, the transactivation and the antiapoptotic activities of NF κ B in cell transformation (Foo and Nolan, 1999), a direct role of NF κ B in the induction of experimentally induced carcinogenesis in skin, has not yet been demonstrated. In contrast, the lack of malignant progression during skin tumor development in *c-fos*^{-/-} mice (Saez et al., 1995) strongly underlines the requirement of AP-1-regulated gene expression in this process. High constitutive expression levels of AP-1 target genes, such as interstitial collagenase (MMP-13) and stromelysin-1 (MMP-3), were observed in skin tumors (Basset et al., 1997). In tissue culture cells, repression of interstitial collagenase and stromelysin expression by dexamethasone is mediated through negative interference between AP-1 and GR (Beato et al., 1995; Dumont et al., 1998; Karin, 1998). Whether the DNA binding-dependent or -independent function of the GR is required for repression of tumor promotion and phorbol ester-induced gene expression in the animal is still unclear.

Here, we have measured phorbol ester- and GC-induced changes in MMP-13 and MMP-3 gene expression in mouse skin to analyze mutual interference between AP-1 and GR in vivo. Both genes contain functional AP-1 sites, which are essential for upregulation by many growth stimulating agents in tissue culture (Angel and Karin, 1991). MMP-13 appears to represent the most selective reporter gene to measure AP-1 activity: in *c-fos* transgenic mice, expression of MMP-13 is enhanced, whereas in *c-fos* knockout mice, the level of MMP-13 transcripts is reduced (Gack et al., 1994; Porte et al., 1999). Second, phorbol ester-dependent induction of MMP-13 is almost completely lost in fibroblasts from *c-fos* and *c-jun* knockout embryos (Hu et al., 1994; Schreiber et al., 1995). Finally, the AP-1 site in the murine MMP-13 promoter is necessary and sufficient for 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-dependent induction (Porte et al., 1999), demonstrating that other transcription factors, including NF κ B, whose activities have been found to be affected by phorbol esters (Nelsen et al., 1988), do not contribute to phorbol ester-dependent induction of this gene.

By the analysis of MMP-13 and MMP-3 gene expression in skin of phorbol ester- and GC-treated wild-type and GR^{dim} mice, we could demonstrate that AP-1 activity is repressed by the GR in vivo. Since induction of both genes is considered a hallmark of altered gene expression during mouse skin carcinogenesis, these data strongly suggest that the ability of GR to undergo protein-protein interaction with transcription factors, such as AP-1, is responsible for the antitumor promoting activity of GCs.

Materials and Methods

Animals

The dorsal skin of 7–9-wk-old female C57BL/6J mice (BRL) or GR^{dim} mice (Reichardt et al., 1998) was shaved 4 d before experimentation. Mice

were treated topically with 1–10 nmol TPA or with 50 μ g dexamethasone (Sigma Chemical Co.) dissolved in 200 μ l acetone. The animals were killed 0–6 h after application.

Northern Blot Analysis

Skin tissues were homogenized and RNA was prepared as described previously (Reichardt et al., 1998) and analyzed by Northern blot (Gack et al., 1994; Schreiber et al., 1995). The probes for 18 S rRNA, HSP-27, and plasma glutathione peroxidase-3 (PGX-3) were obtained by reverse transcriptase PCR using a mouse skin RNA preparation.

In Situ Hybridization and Immunohistochemistry

6- μ m paraffin sections from skin biopsies were subjected to in 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

AtlasTM mouse cDNA expression array I filters were hybridized with radioactively labeled first strand cDNA following the specifications of the manufacturer (CLONTECH). Differences in expression patterns were analyzed using AIS 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 AP-1-dependent gene expression and to study mutual interference between AP-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

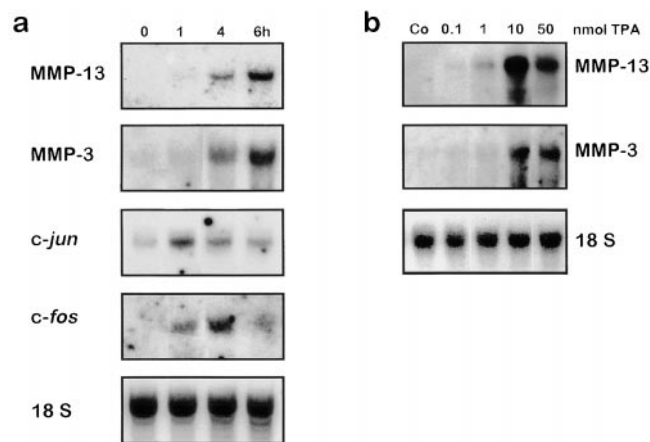


Figure 1. TPA induces MMP-13 and MMP-3 in skin. a, TPA (10 nmol) in acetone was applied to the back skin. After 0, 1, 4, and 6 h, mice were killed and RNA from skin was prepared. MMP-13, MMP-3, *c-jun*, and *c-fos* expression was analyzed by Northern blot analysis. b, TPA (0.1, 1, 10, and 50 nmol) was applied as described in a, and animals were killed after 6 h and analyzed by Northern blot analysis. Rehybridization with a cDNA fragment of 18 S RNA was performed serving as a loading control.

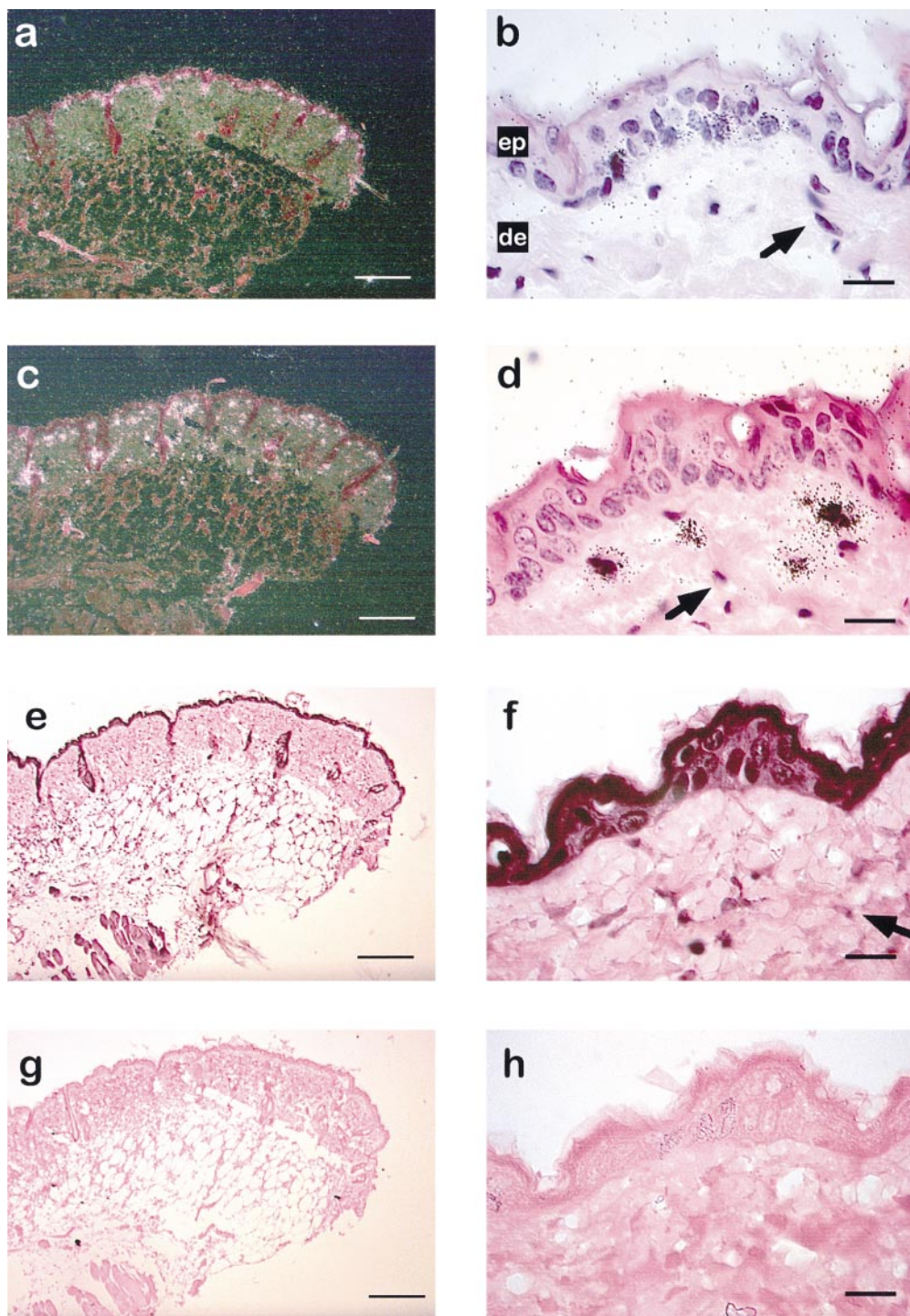


Figure 2. Cell type-specific expression of MMP-13, MMP-3, and GR in skin of TPA-treated mice. In situ hybridization for MMP-13 (a and b) and MMP-3 (c and d) on transverse sections of the skin from female C57BL/6 mice treated for 6 h with TPA. Dark-field (a) and bright-field (b) illumination of a section hybridized with a MMP-13 specific riboprobe. The arrow indicates nuclei from fibroblasts. Dark-field illumination (c) and bright-field (d) illumination of a skin section hybridized with a MMP-3 specific riboprobe. e–h, Immunohistochemical analysis of GR expression using anti-mouse GR antibody (e and g) or secondary anti-rabbit IgG antibody alone (g and h). The arrow indicates nuclei from fibroblasts. ep, Epidermis; de, dermis. Bars: (a, c, e, and g) 100 μm ; (b, d, f, and h) 20 μm .

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. 1 b). 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. 1 a).

To identify the specific cell type responsible for en-

hanced 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 monocytic 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 cannot be explained by a loss of *c-jun* and/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 AtlasTM 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-

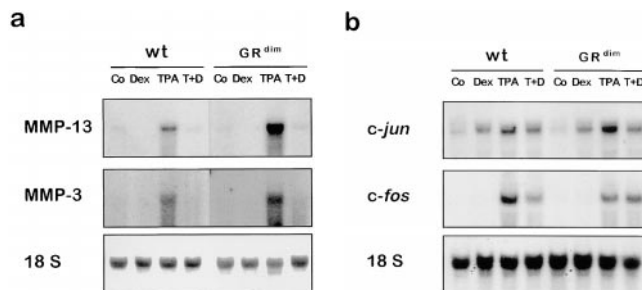


Figure 3. The DNA binding function of the GR is dispensable for repression of MMP-13 and MMP-3 induction in vivo. Wild-type (wt) or GR^{dim} mice were treated with acetone (Co), 50 μg dexamethasone (Dex), 10 nmol TPA (TPA), or 10 nmol TPA with 50 μg dexamethasone (T+D). Animals were killed after 6 h (a) or 1.5 h (b) and analyzed by Northern blot.

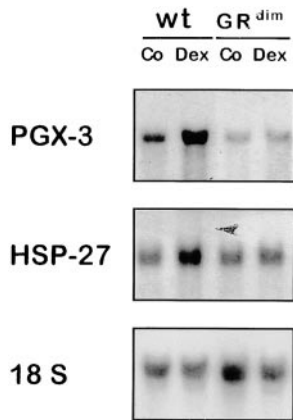


Figure 4. Induction of HSP-27 and plasma glutathione peroxidase 3 requires the DNA binding function of the GR. Control (Co) and dexamethasone (50 µg; Dex) treated wild-type (wt) or GR^{dim} mice were killed after 6 h and RNA from back skin was analyzed for PGX-3 and HSP-27 expression by Northern blot.

tantly, in GR^{dim} mice, almost no elevation in mRNA levels of PGX-3 and HSP-27 is detectable (Fig. 4).

Taken together, negative interference of AP-1 by GR does not require the DNA binding function in vivo, since in wild-type and in GR^{dim} mice, MMP-13 and MMP-3 are equally repressed, whereas genes positively regulated are activated by GC only in wild-type mice, but not in GR^{dim} mice.

Discussion

We established an experimental system to induce transcription of the AP-1-dependent genes MMP-13 (collagenase-3) and MMP-3 (stromelysin-1) in skin by the application of TPA. In line with the critical role of de novo synthesis of c-Jun and c-Fos for full transcriptional activation of interstitial collagenases (Angel and Karin, 1991), we found a rapid upregulation of *c-jun* and *c-fos* mRNA upon TPA treatment in skin (Kennard et al., 1995).

In contrast to the coordinate induction of both genes in tissue culture cells (Angel and Karin, 1991), the expression pattern of MMP-13 and MMP-3 genes in the skin was restricted to distinct cell types. MMP-13 was expressed upon TPA treatment in basal keratinocytes, which are the sites of expression of MMP-13 observed during wound healing in mice (Madlener et al., 1998). TPA-induced MMP-3 expression was mainly found in monocytic cells in the dermal compartment. However, we could not detect expression of MMP-3 in the basal layer of the epidermis by in situ hybridization, although others have observed MMP-3 expression in fractionated keratinocytes derived from TPA-treated epidermis (Krieg et al., 1988). During experimental wound healing, induction of MMP-3 has been found in some cells of the mesenchymal compartment of the skin and in basal keratinocytes after 24 hours (Madlener et al., 1998). Thus, most likely MMP-3 induced by TPA treatment only partially reflects the expression pattern observed during wounding. Surprisingly, in contrast to fibroblasts in tissue culture (Wilhelm et al., 1987; Madlener et al., 1998), we found no induction of either MMP-13 or MMP-3 in dermal fibroblasts upon TPA treatment of skin. The specific microenvironment composed of soluble factors and components of the extracellular matrix obviously does not allow the responsiveness of these cells to TPA by induced expression of MMPs. The molecular mechanism controlling cell type-specific upregulation of MMP-13 and MMP-3 transcripts in the skin remains to be elucidated.

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^{dim} mice. To address the question whether gene activation by GCs is indeed impaired in GR^{dim} 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^{dim} mice.

In contrast to loss of GC-dependent gene activation GR^{dim} 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 (Caellas 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'Armiento 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 (Jonat 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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