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Suppression of Granulocyte-Macrophage Colony-Stimulating Factor Expression by Glucocorticoids Involves Inhibition of Enhancer Function by the Glucocorticoid Receptor Binding to Composite NF-AT/Activator Protein-1 Elements

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Increased expression of a number of cytokines including GM-CSF is associated with chronic inflammatory conditions such as bronchial asthma. Glucocorticoid therapy results in suppression of cytokine levels by a mechanism(s) not yet fully understood. We have examined regulation of GM-CSF expression by the synthetic glucocorticoid dexamethasone in human T cells. Transient transfection assays with reporter constructs revealed that dexamethasone inhibited the function of the GM-CSF enhancer, but had no effect on regulation of GM-CSF expression occurring through the proximal promoter. Activation of the GM-CSF enhancer involves cooperative interaction between the transcription factors NF-AT and AP-1. We demonstrate here that glucocorticoid-mediated inhibition of enhancer function involves glucocorticoid receptor (GR) binding to the NF-AT/AP-1 sites. These elements, which do not constitute recognizable glucocorticoid response elements, support binding of the GR, primarily as a dimer. This binding correlates with the ability of dexamethasone to inhibit enhancer activity of the NF-AT/AP-1 elements, suggesting a competition between NF-AT/AP-1 proteins and GR. The Journal of Immunology, 2001, 167: 2502–2510.

Granulocyte-macrophage CSF is a member of a family of soluble glycoprotein growth factors known as the CSFs (1). It is produced by T cells, monocyte-macrophages, mast cells, fibroblasts, and endothelial and epithelial cells and affects both hemopoietic precursors and differentiated macrophages and granulocytes (2, 3). Overexpression of GM-CSF is a feature of allergic conditions such as bronchial asthma (4), and elevated levels of GM-CSF are found in bronchoalveolar lavage samples and bronchial biopsies taken from asthmatic subjects (5, 6). Glucocorticoids are a potent therapy for a wide range of inflammatory diseases and are the mainstay of treatment for chronic asthma (7). However, their mechanism of action in asthma remains largely unknown. Treatment with glucocorticoids leads to suppression of the cellular infiltration of asthmatic Airways (8, 9) and to suppression of proinflammatory cytokines including GM-CSF (10–12).

Transcriptional regulation of GM-CSF in T cells involves both the proximal promoter region and the distal enhancer (2, 13, 14). A number of regulatory elements have been defined within the GM-CSF proximal promoter. These include the conserved lymphokine element (CLE)30, which is the most proximal to the transcription start site and is bound by the transcription factors NF-AT, AP-1, Ets1, and Elf1 (15–17). Further upstream are the CLE1 and the CLE2/GC box, which can bind NF-κB and Sp1 (18, 19). Transactivation of the GM-CSF promoter occurs through the synergistic action of Ets1, NF-κB, and AP-1 (20). Additionally, we have previously reported a double palindromic regulatory element within the GM-CSF promoter that acts as an enhancer (21).

The human GM-CSF enhancer region is a 716-bp fragment located 7 kb downstream of the IL-3 gene and 2.6 kb upstream of the GM-CSF gene (22). Transfections with a construct containing the enhancer linked to the proximal promoter demonstrated the potent effects on GM-CSF transcription that this region confers (22). The enhancer was also found to up-regulate IL-3 expression (22). Further investigation led to the discovery of a T cell-specific enhancer 14 kb upstream of the IL-3 gene, and it is this element, rather than the GM-CSF enhancer, that is believed to regulate IL-3 expression in vivo (23). Cockerill et al. (24) have recently shown that the human enhancer is required for correctly regulated GM-CSF expression in vivo.

The GM-CSF enhancer contains four composite NF-AT/AP-1 elements, three of which demonstrate cooperative binding of recombiant NF-ATp and Fos/Iun. The fourth site binds NF-ATp and Fos/Iun independently. In the three sites that demonstrate cooperative binding function as enhancers (25), both NF-AT and AP-1 components differ in their affinity for the individual elements as determined by band retardation assays. It has been hypothesized that the spacing of the NF-AT and AP-1 sites is important for cooperative binding and enhancer function. Recent data has suggested that transcriptional repression of GM-CSF by 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) occurs through the inhibition of nuclear receptor; GRE, glucocorticoid response element; PDRIu, phorbol dibutyrate; Ion, ionomycin; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; NR, nuclear receptor; AF-2, activation function 2; TR, thyroid hormone receptor; RAR, retinoic acid receptor; HDAC, histone deacetylase; RSV, Rolls sarcoma virus.
Plasmids

pHGM617 contains the first 617 bp of the GM-CSF proximal promoter linked to the chloramphenicol acetyl transferase (CAT) gene (21). pGMBS716 contains the GM-CSF enhancer region, −2.6 to −3.3 kb, in addition to 617 bp of the promoter (22). To generate pGM716E1b, the GM-CSF enhancer region was obtained by digesting pHGMBS716 with HindIII and XhoI, which was then ligated into HindIII/XhoI-digested pHE1AT. pGM170, pGM30, pGM420, and pGM550 contain the respective NF-AT/AP-1 sites from the GM-CSF enhancer (25). Vectors were constructed by digesting pHGMBS716 with BglII to remove the enhancer, which was then replaced by ligation of oligonucleotides corresponding to each of the four sites into the reporter plasmid. Constructs containing head-to-tail dimers linked to 617 bp of the GM-CSF promoter were verified by sequence analysis using an Applied Biosystems 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Oligonucleotide duplexes used for reporter plasmid construction and as probes and competitors in band retardation assays had the following sequences. The upper strand sequence is given, with complementary single-stranded regions used for cloning shown in lower case: GM170, gatcCTGGAGGTCTACAAAGCCCTGTTTTCCTACAG; GM330, gatcGCCATCTGGAGGACTCGAGCATG; GCT420, gatcGGTGGACTACCTTTTCATGCCACATGT; GM550, gatcCTTTATATGACTTTGGTCTCTCCTTCC.
pDNA3.Flag GR was generated by PCR amplification of full-length human GR with oligonucleotides caggatcTCTGAGGCATCACTAAAGGTTTTATATGATTTC (antisense), ligating the resultant cDNA downstream of the Flag epitope in the vector pcDNAFlag2. Expression vectors used in cotransfections were pRSV.GR (32), pRSV.c-Fos, pRSV.c-Jun (38), and pRSV.NF-ATC (39).

Transfections

Transfections and CAT assays were conducted as previously described (21). Briefly, 4 × 10^5 cells were transfected with 10 μg of reporter plasmid DNA plus or minus 250 ng to 1 μg of expression plasmid DNA. Electroporation was conducted at 300 mV, 960 μF, = 2, with a Gene Pulser (Bio-Rad, Hercules, CA). Samples were activated and treated with dexamethasone 10 min posttransfection as indicated. Duplicate samples for each stimulus were conducted. Cells were incubated at 37°C; 5% CO₂ in humidified air for 20 h, harvested by centrifugation, and cell lysates were assayed for CAT activity. As a control for transfection efficiency and the effects of steroid treatment, samples were cotransfected with 2 μg of pcMV-β-gal, and β-galactosidase assays were performed using standard procedures (40). A293 cells were transfected by calcium phosphate precipitation as previously described (41).

Immunopurification of Flag-tagged GR

Flag-tagged GR was prepared from A293 cells transfected with the cDNA3.Flag GR vector according to the protocol described (42). Cells were lysed in IPH buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF), and debris was pelleted. Supernatants were incubated with Flag agarose for 30 min at 4°C before centrifugation. Cleared lysate was washed in I PH buffer. Flag protein was eluted from beads using acid glycine according to the manufacturer’s protocol and neutralized in 10 mM Tris-Cl, pH 8. Eluted protein was stored at −20°C. Western blots were conducted as previously described (41) and probed with anti-Flag (Kodak, Rochester, NY) or anti-GR antisera (Transduction Laboratories, Lexington, KY).

Band retardation assays

Recombinant human GR was obtained from Affinity BioReagents (Golden, CO) (43). 410 μg KSG, 1 ml 0.1 M DTT, 0.1 mM EDTA, 5 mM DTT, 0.2 mM PMSF, 15 μg/ml leupeptin) before use. Jurkat nuclear extract, prepared as previously described (21), and Flag GR protein, were used in band retardation assays. Oligonucleotide probes were labeled with [γ-^32P]ATP with T4 polynucleotide kinase according to standard procedures (40). Then 0.5 μl of diluted GR, or 5 μg Jurkat nuclear extract, was incubated with 7.6 fmol [^32P]end-labeled oligonucleotide duplex, plus 80 ng poly(dI-dC) (500-fold excess) in buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.5 mM Na₂EDTA, 50 mM NaCl, 0.5 mM DTT, 4% glycerol) for 15 min at room temperature. Where indicated, 152 fmol (20-fold excess) unlabeled specific competitor DNA was added to the binding reaction. Complexes were resolved on 5% polyacrylamide gels run in 0.3X Tris/borate/EDTA buffer (40). The GRE controlling expression of the IL-1β promoter (~161 bp from the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) was used as a positive control for GR binding and has the following sequence (5′−3′): GATCGTATGTTGTTACAAA CGTGTCTTAAACA (45). GM-CSF oligonucleotides used only in EMSAs
were: 550(-AP-1), gatcTCTTATGCCCCGCGCAGCTTTCCTCCTTTCA; 550(-NF-AT) gatcTCTTATTATGACTCTTGCGCTAGTCCTTTCA; 550.5/H11032, gatcTCTTATTATGACTCTT; 550.3/H11032, gatcGCTTTCCTCCTTTCA; and 550.3(-NF-AT), gatcGCTTTAAGAAT TTGA.

Results

GM-CSF mRNA transcription is repressed by glucocorticoids

The effects of the synthetic glucocorticoid dexamethasone on GM-CSF expression from two human T cell lines were investigated. HUT 78 and Jurkat cells were stimulated with PDBu and Ion for 20 h in the presence of various concentrations of dexamethasone. Dexamethasone treatment inhibited GM-CSF expression in a dose-responsive manner as determined by RT-PCR. β-actin levels were unaffected by this treatment (Fig. 1, A and B). In both cell types, treatment with 10⁻⁶ M dexamethasone caused a >80% repression of transcription as compared with treatment with PDBu/Ion alone.

Inhibition of GM-CSF expression by dexamethasone is mediated by the enhancer region

To address whether the inhibitory effects of dexamethasone are mediated through the proximal promoter, HUT 78 and Jurkat cells were transfected with the reporter construct pHGM617, which contains the proximal 617 bp of the GM-CSF promoter driving expression of the CAT gene. Cells were activated 10 min post-transfection in the presence or absence of 10⁻⁶ M dexamethasone and were harvested after 20 h. Cell extracts analyzed for CAT activity. Dexamethasone treatment had no effect on expression of the reporter gene in either HUT 78 (Fig. 2A) or Jurkat cells (Fig. 2B). Furthermore, overexpression of GR by cotransfection with the GR expression vector pRSV.GR in activated, dexamethasone-treated cells had no repressive effect (Fig. 2B).

Transient transfection analysis has previously shown that in addition to the proximal promoter, transcriptional regulation is influenced by an enhancer region located 3 kb upstream of the transcription start site. We tested the steroid sensitivity of the enhancer using the reporter plasmid pHGM716 that contains the enhancer directly upstream of the proximal promoter. Dexamethasone treatment resulted in a 38.4% inhibition and a 23.9% inhibition of CAT activity in HUT 78 and Jurkat cells, respectively (Fig. 2, A and B), suggesting that the 716-bp enhancer region is capable of mediating steroid responsiveness. Suppression is GR dependent, as increasing GR levels by cotransfection of pRSV.GR increased the level of

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Dexamethasone (Dex) inhibits GM-CSF transcription in T cell lines. HUT 78 (A) and Jurkat T cells (B) were stimulated with PDBu + Ion, in the presence of the indicated concentrations of dexamethasone, for 20 h. RNA was isolated, RT-PCR was performed, and products were migrated on agarose gels. Band intensity was quantitated using ImageQuant software. Data are expressed as the percentage of GM-CSF: β-actin ratio in cells treated with PDBu/Ion in the absence of dexamethasone and represent the average of two independent experiments.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Dexamethasone (Dex)-mediated inhibition of GM-CSF expression involves the enhancer region. The HUT 78 (A) or Jurkat (B) T cell line were transfected with 10 µg pHGM617 or pHGM716, plus 1 µg pRSV.GR or pRSV control (for Jurkat cells), stimulated with PDBu and Ion with or without 10⁻⁶ M dexamethasone for 20 h. Cell extracts were assayed for CAT activity. The results have been normalized to a value of 100 for the activated sample and in each case are the mean of three independent experiments. Error bars indicate SE.
Activation of the GM-CSF enhancer by NF-AT/AP-1 is suppressed by GR

To confirm that the enhancer region confers glucocorticoid responsiveness, a plasmid was constructed in which the 716-bp enhancer drives expression from the minimal viral promoter E1b. The construct had minimal activity when assayed by transient transfection in Jurkat cells (Fig. 3A). Cotransfection of expression vectors encoding AP-1, or NF-AT components (RSV.c-Fos, RSV.c-Jun, and RSV.NF-ATc), resulted in activation of the enhancer in a dose-responsive, synergistic manner (Fig. 3A). Activation of the enhancer by AP-1/NF-ATc can be suppressed in part by dexamethasone treatment (24.1% inhibition; Fig. 3B). Increasing the level of GR within the cell by cotransfection with pRSV.GR resulted in increased suppression of pGM716.E1b activity (44.5% inhibition; Fig. 3B). The level of suppression was of the same magnitude as that observed when the enhancer was in context of the endogenous GM-CSF promoter (Fig. 2B), suggesting that the enhancer contains both steroid-responsive and unresponsive regions and was dose responsive to pRSV.GR levels (data not shown). Suppression was ligand dependent as cells cotransfected with pRSV.GR gave the same level of CAT activity as those cotransfected with the control vector pRSV (Fig. 3B). Steroid treatment had no effect on CAT activity of the parental pE1b vector (data not shown).

The GM-CSF enhancer NF-AT/AP-1 elements differ in their ability to enhance transcription and in their steroid responsiveness

Having demonstrated that the enhancer imparts steroid responsiveness upon an exogenous promoter, experiments were conducted to dissect the responsive elements within this 716-bp fragment. To test the hypothesis that GR might act, at least in part, by interfering with NF-AT/AP-1 components, vectors were generated in which the individual 30-bp enhancer NF-AT/AP-1 elements were cloned upstream of the GM-CSF promoter as head-to-tail dimers. Transfection assays demonstrated the differential abilities of the individual elements to act as enhancers (Fig. 4). Element GM420 was the most potent enhancer, followed by GM330 and GM550. Element GM170 did not enhance the activity of the GM-CSF proximal promoter. Our data are in accordance with the published observations on the ability of the individual elements to act as enhancers (25). The steroid responsiveness of each of the enhancer elements was tested. Both GM550 and GM330 were found to be steroid responsive, 47.5 and 37.2% inhibition, respectively, in samples cotransfected with pRSV.GR (Fig. 4). GM170 and GM420 were both unresponsive to steroid treatment, as was the control construct pHGM that contains only the proximal promoter.

GR binds to all four of the NF-AT/AP-1 elements within the GM-CSF enhancer

The observation that dexamethasone-mediated suppression of GM-CSF occurs in part through the enhancer region led us to analyze the mechanism of this suppression. The ability of the GR to bind to the NF-AT/AP-1 elements within the GM-CSF enhancer was investigated using band retardation assays. None of the elements contain recognizable GREs. Synthetic oligonucleotide duplexes corresponding to the four elements were synthesized and assessed for the ability of GR to bind. The GRE from the MMTV LTR (−187 to −161 bp) was used as a positive control for GR binding and as a specific competitor for GR binding to the enhancer elements. Three of the four NF-AT/AP-1 elements supported binding of recombinant GR (Fig. 5A), with very weak binding observed to the fourth element, GM420.

To further characterize the GR complexes bound to the GM-CSF elements, we generated Flag-tagged GR protein by transfection of A293 cells with a CMV.Flag GR vector, and we immunopurified the Flag.GR protein (Fig. 5B). By a number of functional
and biochemical assays this protein behaves as wild-type GR (data not shown). We compared this material with Jurkat nuclear extract by EMSA using both consensus GRE and the GM550 sequence. Nuclear extract and Flag GR generated complexes of identical mobility with both oligonucleotides, and both protein sources behaved identically in cross-competition assays (Fig. 5C). We take these data as strong evidence that the complex causing retardation of the GRE and GM550 oligonucleotides in the Jurkat extract is GR.

To compare the ability of each of the NF-AT/AP-1 elements to support GR binding, a series of band retardation assays were performed. The NF-AT/AP-1 elements were labeled and assessed for their ability to bind GR, and the unlabeled elements were used as competitors for GR binding (Fig. 6). Strongest competition in each case was provided by consensus GRE and GM550, which abolished GR binding to all of the elements (Fig. 6, A and B). GM550 supported the strongest binding of GR, and GM420 supported the weakest (Fig. 6B). Using cumulative competition and binding data, the relative binding affinity of GR for each element was measured. Detailed analysis of the relative GR binding affinities was performed on a Molecular Dynamics Phosphorimager employing ImageQuant software (Molecular Dynamics, Sunnyvale, CA). This analysis allowed the relative affinity of GR for each element to be calculated by plotting the percentage of probe bound against the cold competitor used (data not shown). This analysis confirmed that GM550 supported the strongest binding of GR. GR had approximately the same affinity for GM170 and GM330 (Fig. 6A), although lower than for GM550. GR binding to GM420 was very weak in comparison to the other elements. The ability of GM550 to support GR binding was almost as great as that of the GRE (88.3 ± 3.1% the affinity of GR for GRE).

**GR binds independently to both the NF-AT and AP-1 sites within the GM550 element**

Because none of the four NFAT/API1 elements contained consensus GREs, we chose GM550 to further investigate the site of GR binding. Oligonucleotides in which either the NF-AT or AP-1 site was deleted were synthesized and labeled for competition studies with the GRE and GM550 oligonucleotides. The results are shown in Fig. 6C. The GM550 oligonucleotide retained the ability to support the binding of GR to the GM550 oligonucleotide, and the ability of GM550 to support GR binding was almost as great as that of the GRE (88.3 ± 3.1% the affinity of GR for GRE).

**FIGURE 4.** The four NF-AT/AP-1 elements of the GM-CSF enhancer differ in their ability to function as enhancers and in their steroid responsiveness. Jurkat cells were cotransfected with 10 μg of the promoter construct pGM617 or the promoter plus individual enhancer element constructs pGM170, pGM330, pGM420, or pGM550, plus 1 μg pRSV.GR or pRSV control. Samples were stimulated and treated with dexamethasone (Dex; 10^{-6} M), and cell extracts were assayed for CAT activity. The results have been normalized to a value of 100 for the activated pGM617 sample and are the mean of four experiments with error bars representing SE.

**FIGURE 5.** GR binds to all four of the GM-CSF enhancer NF-AT/AP-1 elements. A, Recombinant GR was incubated with labeled oligonucleotides representing the four NF-AT/AP-1 elements of the GM-CSF enhancer as detailed in Materials and Methods. Unlabeled GRE (20-fold excess) was used as a specific competitor. B, Immunopurified Flag.GR was electrophoresed on a 10% SDS polyacrylamide gel and subjected to Western blotting with anti-GR and anti-Flag antisera. A control whole-cell extract from cells transfected with an empty expression vector is also shown. C, Immunopurified Flag GR (lanes 2–4 and 9–11) and 5 μg Jurkat nuclear extract (NE) (lanes 5–7 and 12–14) were incubated with labeled GRE (lanes 1–7) and element GM550 (lanes 8–14). A 20-fold excess of unlabeled GRE and GM550 were used as a specific competitors where indicated.
NF-AT and the nonconsensus AP-1 sites, suggesting a complex mechanism of transcriptional repression at this site.

### Discussion

The mechanism of transcriptional activation by nuclear hormone receptors (NRs) has been the subject of intense scrutiny. Some NRs, which are constitutively present within the nucleus, contain a conserved transactivation domain known as activation function 2 (AF-2) that undergoes a ligand-dependent conformational change (47, 48). A large number of cofactors have been identified that interact with AF-2 of NRs in a ligand-dependent manner and that function in a combinatorial manner to reorganize chromatin templates (49). Ligand binding leads to the dissociation of corepressors and the recruitment of coactivators by NRs (reviewed in Ref. 50). The thyroid hormone receptor (TR) and retinoic acid receptor (RAR) are able to repress transcription both in the absence, or presence, of their respective ligands through their interaction with specific corepressors. Nonliganded TR or RAR interact with the nuclear receptor corepressor, ligand binding causing a reduction in affinity for the corepressor (51, 52).

Unlike the other NRs, the GR translocates to the nucleus after ligand binding (53). Transcriptional activation by GR involves homodimeric binding to positive acting GREs that have a defined consensus sequence (54). GR binding to positive GREs induces a distinct conformational change in the GR DNA-binding domain (55, 56), allowing coactivator recruitment through the carboxy-terminal AF-2 (57, 58). In contrast, negative GREs have less conserved sequences. A common feature of negative GREs is that GR does not necessarily bind DNA as a homodimer. On the pro-opiomelanocortin-negative GRE, GR has been suggested to bind as a trimer (32); furthermore, mutations that prevent the GR forming a homodimer do not interfere with its ability to repress both the collagenase and the proliner genes (30, 59). In addition, GR with a point mutation in the D loop of the GR DNA-binding domain, required for dimerization, fails to bind DNA and cannot transactivate GRE-dependent promoters (60). Consequently, when GR acts to repress transcription, the conformational change required for coactivator recruitment is not achieved and may allow the potential recruitment of corepressors by ligand-bound GR (61). It is possible that repression by GR binding to DNA as a dimer may occur if the DNA-GR interaction does not cause the conformational change required for activation. Our data suggests that inhibition of GM-CSF enhancer function involves binding of GR dimers. Adcock and colleagues have suggested an additional mechanism of GR action (62), showing an ability of the receptor to recruit the corepressor histone deacetylase 2 (HDAC2) and proposing that the GR/deacetylase complex is also part of a CREB-binding protein-containing complex. DNA binding by GR was not involved in this mechanism. Therefore, these surprising data infer that both histone acetyl transferase and deacetylase activities exist within the same complex, raising the question of competition for specific histone substrates. Additionally, they propose a role for GR in the stabilization of histone/DNA contacts. More recently, a transcriptional corepressor, SMRT/HDAC1-associated repressor protein, has been isolated that interacts both with the steroid receptor RNA coactivator steroid receptor RNA activator and with HDAC1 and 2, thereby providing direct evidence for recruitment of a repressor complex to ligand-bound GR (35).

Transcriptional interference of the GR with other transcription factors has been demonstrated to repress the function of both AP-1 and NF-κB (30, 38, 63, 64). The importance of GR transrepression has been demonstrated by generation of dimerization-deficient GR mice using the point mutation in the D loop discussed above (65).
The mice are viable, unlike GR-deficient mice (66), able to transrepress AP-1-driven genes, but are unable to transactivate GRE-dependent genes due to the inability of their GR to bind DNA as a dimer.

In this paper, we have analyzed regions of the GM-CSF gene for glucocorticoid responsiveness and have identified a potential mechanism for transcriptional repression of this cytokine. This mechanism may be relevant to glucocorticoid action in asthma. Initial analysis of the GM-CSF proximal promoter, which mediates activation by known GR targets such as AP-1 and NF-κB (15, 18), demonstrated that this region was insensitive to steroid. The interaction between the GR and AP-1 components is complex and does not always result in a negative effect on transcription. The effect on transcription of the composite GRE contained in the proliferin gene is modulated by the AP-1 components present (59). GR acts synergistically with a Jun homodimer, or represses transcription in the presence of a Fos-Jun heterodimer. There are a number of other examples of GR acting either in a synergistic or repressive manner with AP-1 components, including regulation of dexamethasone-induced transcriptional activation of the MMTV LTR (67) and regulation of the neurotensin/neuromedin N gene (68). Regulation of the IL-2 gene occurs through a cooperative mechanism involving NF-AT and AP-1 interaction at the promoter. Inhibition by glucocorticoids involves disruption of this cooperativity (69) through the GR binding to AP-1 that disrupts binding to NF-AT (70). NF-AT and AP-1 can bind to the CLE0 element in the GM-CSF promoter (15). However, NF-AT binding is very weak, and no cooperative binding with AP-1 occurs (71). Therefore, it is unlikely that glucocorticoids inhibit GM-CSF expression in the same way that they inhibit IL-2 promoter activation. Recent data (72) has shown that GR regulates activity of the IL-4 promoter through complex formation with, and inhibition of function of, NF-ATc. This illustrates the potential for multiple functional regulatory events focussed at the NF-AT/AP-1 sites.

Our data indicate that repression takes place, at least in part, through the GM-CSF enhancer region and that GR binds to the composite NF-AT/AP-1 elements leading to repression of enhancer function. Binding occurs despite the lack of consensus GREs within the NF-AT/AP-1 elements. This is reminiscent of the VDR-mediated inhibition of GM-CSF enhancer function. VDR inhibits GM-CSF expression through binding to one of the NF-AT/AP-1 elements within the GM-CSF enhancer (26). Binding is to a nonconsensus VDR recognition sequence and is unusual in that the

FIGURE 7. GR binds independently to both NF-AT and AP-1 halves of element GM550. Oligonucleotides mutated at either the AP-1 or NF-AT sites of GM550 were synthesized, along with half sites and an NF-AT mutated half site (A). The full-length oligonucleotides, used either as probes or competitors in EMSA, demonstrated the ability to be bound by GR (B). Use of half site oligonucleotides either as probes or competitors demonstrated that each half site was capable of being bound by GR and that a NF-AT mutated half site was unable to act as a competitor (C).
receptor binds as a monomer and not as a heterodimer in partnership with the retinoid X receptor. Further analysis revealed that binding of the VDR monomer prevents NF-AT access to its binding site and interacts with c-Jun, thereby stabilizing the affinity of the AP-1 interaction (27).

Following the observation that steroid treatment represses the function of the GM-CSF enhancer in a GR-dependent manner we conducted a more detailed analysis of this region. The four NF-AT/AP-1 elements of the enhancer display differential ability to bind NF-AT/AP-1 components, to act as enhancer elements (Fig. 4), and to support GR binding (Fig. 5). None of the elements contain consensus GREs, and they only display partial relatedness to one another with respect to NF-AT/AP-1 site spacing and primary sequence (25). Our data suggest that the suppressive effects of GR are mediated primarily through GM550 and GM330, to which NF-AT/AP-1 bind with the weakest affinities (25). The functional ability of steroid to inhibit enhancer function correlated with the ability of the individual enhancer elements to support binding of GR. GR exhibited the strongest affinity for GM550 (Fig. 5), and dexamethasone treatment inhibited the enhancer function of this element to the greatest extent (Fig. 4). In more detailed analysis, GR was found to bind independently to both the NF-AT and AP-1 halves of the element (Fig. 7). Interestingly, GM550 was the element demonstrated to bind VDR and whose function was inhibited by 1,25(OH)_{2}D_{3} treatment (26). Our data suggest that the mechanism of repression at this site is different to that of vitamin D, due to the additional ability of GR to bind to the AP-1 site. The fact that dexamethasone treatment did not result in a greater suppression of enhancer activity is probably due to the unresponsiveness of GM420. GR bound to this element very weakly (Figs. 5 and 6), and dexamethasone had no effect on its ability to function in the context on GR structure may allow the recruitment of a corepressor such as HDAC2 or, alternatively, the inability to recruit a coactivator. In summary, this study suggests a complex mechanism for transcriptional repression of GM-CSF by GR brought about by the ability of the receptor to compete with NF-AT and AP-1 components for binding sites within the GM-CSF enhancer.

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References
INHIBITION OF GM-CSF BY GLUCOCORTICOIDS

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