Repression of *Interleukin-5* transcription by the glucocorticoid receptor targets GATA3 signalling and involves histone deacetylase recruitment.

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Abstract

Glucocorticoids are the mainstay of asthma therapy and mediate the repression of a number of cytokine genes, such as Interleukin (IL)-4, IL-5, IL-13 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), which are central to the pathogenesis of asthmatic airways inflammation. The glucocorticoid receptor (GR) mediates repression by a number of diverse mechanisms. We have previously suggested that one such repressive activity is by direct binding of GR to elements within the GM-CSF enhancer that are recognised by the NF-AT/AP-1 complex. We reasoned that since many cytokine genes activated in asthma are transcriptionally regulated by the recruitment of this complex to DNA, their binding sites might provide a target for GR to mediate its repressive effects. Here, we show that transcriptional repression of the Interleukin-5 gene involves recruitment of GR to a DNA region located within the IL-5 proximal promoter, which is bound by NF-AT and AP-1 proteins. GR recruitment had a profound effect upon the activation capacity of GATA3, which has a binding site close to the NF-AT/AP-1 domain in both IL-5 and IL-13 promoters. Repression by GR involves co-repressor recruitment, since treatment of transfected cells with the deacetylase inhibitor Trichostatin A (TSA) caused a partial relief of repression. Additionally, repression could be augmented by co-transfection of cells with a histone deacetylase (HDAC1). These data suggest that the local recruitment of GR causes repression by inhibiting transcriptional activation by GATA3, a key tissue-specific determinant of expression of Th2 cytokines.
Introduction

Transcriptional regulation by the glucocorticoid receptor (GR) is known to take place by a number of diverse mechanisms. GR was initially demonstrated to mediate transcriptional activation of the murine mammary tumour virus (MMTV) gene by binding as a dimer to a consensus response element, the GRE (1). Subsequently however, the array of mechanisms utilised by GR in controlling gene expression has expanded, and in particular, details of transcriptional repression by GR have emerged. These mechanisms involve both DNA binding (2,3), the demonstration of the negative GRE (nGRE) (4), the recruitment of either coactivator or co-repressor complexes to a common subunit (5), the ability to sequester non-DNA bound proteins (6,7), and the regulation of kinase activity (8). It is now clear that some of these systems may operate in conjunction with one another, thereby imparting both variability and complexity of transcriptional regulation upon a target promoter.

The genes encoding IL-4, IL-5, IL-13 and GM-CSF lie in close proximity on human chromosome 5q (9). The products of these genes are pro-inflammatory cytokines which mediate both asthmatic and allergic responses. Our previous work has described a mechanism for transcriptional repression of the GM-CSF gene by glucocorticoids which involves binding of GR to NF-AT/AP-1 sites within the GM-CSF enhancer (10). The NF-AT/AP-1 complex mediates signalling through the T cell receptor and acts upon numerous genes including IL-4, -5, -13 and GM-CSF (11). Specificity of transcriptional regulation is governed, at least in part, by factors such as GATA3 (12) and c-Maf (13) which precisely target selected genes rather than having ubiquitous functions. To
provide further evidence that NF-AT/AP-1 sites might be a site of action of glucocorticoids, we have used the \textit{IL}-5 promoter. This promoter has a number of NF-AT/AP-1 sites in addition to a GATA3 response element (14). GATA3 is a key determinant of T cell differentiation, which has been proposed to control chromatin conformation at the 5q locus. In addition to \textit{IL}-5, GATA3 also binds the \textit{IL}-13 proximal promoter (15). As GATA and AP-1 proteins have previously been demonstrated to functionally interact (16), we tested the hypothesis that glucocorticoid repression might effect GATA3-dependent transcriptional activation.

Here we demonstrate that GR mediates \textit{IL}-5 repression, at least in part by acting through an NF-AT/AP-1 site in the proximal promoter. This site has a consensus NF-AT recognition sequence yet lacks an obvious AP-1 site. GR was able to bind to this site \textit{in vitro}. Repressive activity could be augmented by co-expression of a histone deacetylase, while treatment of cells with the HDAC inhibitor TSA partially relieved repression. These data suggested that repression was an active process. This NFAT/AP-1 site juxtaposes a crucial GATA site in the proximal promoter of \textit{IL}-5. This proximity prompted us to examine the effect of glucocorticoids on GATA3-mediated activation. Our data demonstrate that glucocorticoids had a profound repressive effect and suggest that GATA3, a key driver of T cell differentiation and \textit{IL}-5 and \textit{IL}-13 transcription, may be a target for the repressive effect of glucocorticoids on pro-inflammatory Th2 cytokine genes.
Experimental procedures

Purification of CD4\(^+\)ve T cells

Venous blood was taken from healthy human volunteers using heparin as an anticoagulant. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway) according to the manufacturer's instructions. CD4\(^+\) T cells were purified using positive selection (Dynal, Oslo, Norway). CD4\(^+\) purity was greater than 95%. Cells were resuspended at 1X10\(^6\)/ml in RPMI1640 supplemented with 10%FCS and IL-2 (50U/ml). Ethical approval for the use of human volunteers for this study was provided by the Institutional ethical review committee.

Stimulation of CD4\(^+\)ve T cells

CD4\(^+\)ve T cells were stimulated in 24 well microtitre plates coated with anti-CD3 (OKT-3) 1µg/ml. Where indicated, Dexamethasone (Sigma, Poole, UK) was added to a final concentration of 10\(^{-7}\)M immediately following plating. Cultures were incubated for 18 hours at 37\(^\circ\)C then cells were harvested for RNA extraction.

Differentiation of human Th2 cells

Th2 cells were generated from naïve T cells following the protocol outlined in Cousins et al (17). Day 28 Th2 cells were used in this study, Cells were activated using anti-CD3/anti-CD28 either alone or in the presence of 10\(^{-7}\)M dexamethasone for 18hr then harvested for RNA extraction.
Cell lines and culture conditions

Jurkat cells were grown in RPMI 1640 (Gibco BRL) medium, HEK293 cells in DMEM, and HeLa cells were grown in MEM, all supplemented with 10% FCS (Sigma, Poole, UK), L-glutamine (2mM), penicillin (100 IU/ml), and streptomycin (100µg/ml), at 37°C and 5% CO₂ in humidified air. Where indicated, cells were activated with 100 ng/ml phorbol dibutyrate (PDBu) and 1 µg/ml ionomycin (Ion) (Calbiochem). Dexamethasone (Sigma, Poole, UK) was stored at a concentration of 10⁻²M in ethanol, then further diluted in the appropriate culture medium, and added to cells to give the relevant final concentration. TSA (Sigma, Poole, UK) was used at a final concentration of 33x10⁻⁹M.

RT-PCR

Total cellular RNA was isolated using a Qiagen RNeasy kit (Qiagen, Chatsworth, Ca) according to the manufacturer’s instructions. 250ng of RNA per sample was reverse transcribed in a total volume of 60µl using a random hexamer primer (Pharmacia) as previously described (10). IL-4, -5, -13, RAD50 and 18S ribosomal RNA levels were measured by semi-quantitative PCR. PCR conditions were an initial 95°C 10 minutes, followed by cycles of 95°C, 58.5°C and 74°C for one minute each. PCR for 18S RNA was carried out as an internal control (12 cycles). Primer sequences: 18S RNA sense, CACCACACCTTCTACAATGAGCTGC; anti-sense, ACAGCCTGGATAGCAACGTACATGG. IL-5 sense GAGGATGCTTCTGCATTTGAGTTTG, anti-sense, GTCAATGTATTTCTTTATTAAGGACAAG. IL-4 sense AACACAACCTGAGGAGGAAACCTTCTGC, anti-sense
CTCTCTCATGATCGTCTTTAGCCTTTC; IL-13 sense
ATCGAGAAGACCCAGAGGATGCTGAGC anti-sense
TTTTACCCCTCCTAACCTCCTTCCC. RAD50 sense
AAGTCATTTAGTTGTACGTAGCTCAGTCCC anti-sense
ATGAAATTAAAGCCTTGGATAAGCCG. (MWG Biotech). PCR products were
analysed by electrophoresis on 2.5% agarose gels run in glycine buffer (200
mM glycine, 15mM NaOH, 2 mM Na$_3$EDTA). Images were quantified using
Image Quant software. Real time qPCR was performed using an Applied
Biosystems 7900 system and FAM labelled 'Assay-on-Demand' reagent sets
(Applera) for: IL5: Hs00174200_m1; IL13: Hs00174379_m1, GM-CSF:
Hs00171266_m1 and RAD50: Hs00194871_m1. PCR reactions were
multiplexed using a VIC labeled 18S probeset, and analyzed using SDS
software (Applied Biosystems).

Plasmids
The nomenclature of all plasmids is base position relative to the transcriptional
start site of the gene. p-451+-35.IL-5.CAT was previously described (18). GM-
CSF vectors were also previously described (10). p-130+-35.IL-5.CAT was
generated by PCR using oligonucleotides: sense 5'
tacgctgcagTAAGATATAAGGCATTGGAAAC and antisense 5'
tacctcagAACGTCTGCGTTTGC. The template used was p-451+-35.IL-
5.CAT. Amplimers were digested with PstI and SalI (Helena Biosciences) and
cloned into pBL3.CAT which had been linearised with the same enzymes.
Upstream regulatory regions encompassing bases -130 to -39, were cloned
upstream of the TATA box in the minimal promoter E1b.CAT. This fragment
was derived as a PCR amplimer using oligonucleotides 5’ sense
tacgctgcagTAAGATATAAGGCATTGGAAAC and 5’ antisense
tacgtctagaTTTGAGGAAATGAATAATTCTAACC. The -130 to -39 region of the proximal promoter was further divided into three domains, -130 to -90 sense 5’
ctagTAAGATATAAGGCATTGGAAACATTATTTTCACGATATGC, the IL-5
GATA.CLE0 domain, from -80 to -39 sense 5’
ctagGCATTCTCTATCTGATTTAGAAATTATTTCTCTCAAA, and the IL-5
CLEO, from -63 to -39, sense 5’ctagGTAGAAATTATTTCTCTCAAA.
The complementary antisense oligo’s were used in each case preserving the ctag overhang of the cloning site. These individual elements were cloned as single copies upstream of the minimal E1b promoter. p-225--+50.IL-13.CAT was generated by PCR using oligonucleotides: sense
5’catgggatccACAATCTCTGACCTGGACTAAGGC and antisense 5’
catgcgtgcacCAACAGGAGAGGATTGAGG. The resulting 930 bp amplimer was digested with BglII and Sall and the 275bp promoter/exon1 fragment was cloned into the BamHI and Sall sites of pBL3.CAT. All construct sequences were verified by sequence analysis using an Applied Biosystems 377 automated sequencer. Expression vectors used in co-transfections were,
pcDNA3.Flag GR (10), pRSV.c-Fos, pRSV.c-Jun (7), pRSV.NF-ATc (19),
CMV.Flag.HDAC1 (20), CS2+MT.Sin3A, and CMV.GATA3.Flag (18).

Transfections

Transfections and CAT assays were carried out as previously described (10). Jurkat T cells were grown in 10% FCS until the day prior to transfection, then transferred to media containing serum that had been stripped with charcoal to
remove endogenous steroid, and maintained in this media post-transfection. 4 x $10^6$ Jurkat T were transfected with 5$\mu$g of reporter plasmid DNA plus or minus 1 to 3$\mu$g of expression plasmid DNA. Electroporation was carried out at 300mV, 960 $\mu$Fd, $\infty$ Ohms, with a Gene Pulser (Bio-Rad). Samples were activated and treated with dexamethasone 10 minutes post-transfection as indicated. Cells were incubated at 37°C, 5% CO$_2$, in humidified air for 20 hours, harvested by centrifugation, and cell lysates assayed for CAT activity. HEK293 and HeLa cells were transfected by calcium phosphate precipitation as previously described (21). For HeLa cells, 1$\mu$g of reporter plasmid was used along with the same amount of expression vectors for the different transcription factors. Plasmid concentrations were equilibrated with an empty CMV expression vector. For histone deacetylase inhibition assays, TSA was added to cell cultures at a final concentration of 33nM where indicated.

**Electrophoretic mobility shift assays.**

Electrophoretic mobility shift assays were performed as previously described using purified Flag-tagged GR protein (10). Oligonucleotide probes were labelled with $[^{32}\text{P}]$-ATP with T4 polynucleotide kinase according to standard procedures (22). 0.5$\mu$l of GR, was incubated with 7.6fmol $^{32}$P end labelled oligonucleotide duplex, plus 100ng poly(dI.dC) (500 fold excess) in binding buffer (10mM Tris.HCl, pH 7.5/ 1mM MgCl$_2$/ 0.5mM Na$_3$EDTA/ 50mM NaCl/ 0.5mM DTT, 4% glycerol), for 15 minutes at room temperature. Where indicated, 152fmol (20 fold excess) unlabelled specific competitor DNA was added to the binding reaction. Complexes were resolved on 5% polyacrylamide gels run in 0.3X Tris Borate/EDTA buffer (22). The glucocorticoid response element (GRE) corresponding to -187 to -161 from the mouse mammary
tumour virus (MMTV) long terminal repeat (LTR) was used as a positive control for GR binding, and has the following sequence 5': GATCGTTTATGGTTACAAACTGTTCTTAAAACA (23). The IL-5 -130 to -90 oligo used in EMSA was 5': ctagTAAGATATAAGGCATTGGAAACATTTAGTTTCACGATATGC.

**Immunoprecipitations**

Immunoprecipitations were performed as previously described (10) with modifications. HEK293 cells were transfected with CMV.hGRα, CS2+MT.Sin3A and CMV.Flag.HDAC1 or CMV.Flag.GATA3 as a negative control. Whole cell lysates were prepared in IPH buffer (50mM Tris.Cl pH8.0, 150mM NaCl, 5mM EDTA, 0.5% EDTA, 1mM PMSF) and Flag-tagged proteins were immunoprecipitated by incubation with Flag. Agarose beads (Sigma, Poole, UK) at 4°C. Immunoprecipitates were captured by centrifugation, beads were washed 4 times with IPH containing 500mM NaCl, with thorough mixing of samples at each wash. Immunoprecipitates were, then re-suspended and electrophoresed through 8% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked in TBS/Tween/4% milk then probed with anti GR antisera (Transduction Laboratories), anti myc-tag (NEB), or anti Flag (Sigma). The location of immunoreactive complexes was revealed by ECL (Amersham) and autoradiography.

**Results**

**Th2 cytokine transcription is repressed by glucocorticoids**

The effects of the synthetic glucocorticoid dexamethasone on expression of genes within the human 5q locus was analysed using primary human CD4+ T
cells and primary human Th2 cells. Cells were stimulated with anti-CD3/anti-
CD28 for 18 hours either alone, or in the presence of 10^{-7}M dexamethasone
before harvest and cDNA preparation. RT-PCR analysis demonstrated that
dexamethasone treatment inhibited IL-4, IL-5, IL-13 and GM-CSF, while having
no effect on either RAD50 RNA or 18S ribosomal RNA expression (Fig. 1A).
These data were confirmed using quantitative real time PCR using cDNA
derived from the same source of cells. PCR reactions were normalised for 18S
ribosomal RNA expression, cytokine gene expression was significantly
repressed by glucocorticoid treatment compared to RAD50 (Fig.1C). Data
derived from freshly isolated CD4^+ cells, containing both naïve and memory
populations, was similar to that generated from differentiated Th2 cells. These
data demonstrate that glucocorticoids are able to act broadly to mediate
repression of 5q cytokine genes.

Repression of IL-5 transcription is mediated by the proximal promoter.
Although glucocorticoids have numerous potential functional targets, we wished
to assess effects mediated through interactions of GR with DNA. The IL-5
promoter was therefore functionally tested for its capacity to mediate
glucocorticoid responsiveness. Two CAT reporter vectors were generated
driven by either 447 or 130bp of IL-5 promoter DNA (Fig. 2A). Jurkat T cells
were transiently co-transfected with NF-ATc, c-Fos and c-Jun expression
vectors, treated with or without 10^{-7}M dexamethasone and activated with
PDBu/Ion for 20 hours. A combination of NF-AT and AP-1 proteins could
transcriptionally upregulate both IL-5 promoter fusions, and this upregulation
could be repressed by treatment with dexamethasone. By contrast, a
consensus GRE, driving CAT expression under control of the minimal SV40 promoter was potently activated by glucocorticoids (Fig. 2B). These data suggest that a GR-responsive domain resides within the most proximal 130 bp to the *IL-5* transcriptional start site.

**Repression of the IL-5 promoter is mediated by an NF-AT/AP-1 response element.**

We have previously determined that repression of the *GM-CSF* enhancer by glucocorticoids mapped to a series of NF-AT/AP-1 sites. The proximal 130 bp of the *IL-5* promoter, which mediated dexamethasone responsiveness is also regulated by the NF-AT/AP-1 complex. The -130--+35 domain also contains previously described response elements for GATA3, as well as CLE0 binding proteins. These three subdomains were isolated from the *IL-5* promoter and were cloned upstream of the TATA box of the *E1b* minimal promoter (Fig. 3A). In these experiments we asked whether a given *IL-5* region, once activated by NF-AT/AP-1, could be repressed by glucocorticoids. The transcriptional activity of each activated construct was ascribed a value of 100% and the effect of glucocorticoids was assessed relative to this value. The data demonstrate that the minimal domain mediating repression mapped between -130 and -90 relative to the *IL-5* transcriptional start site (Fig. 3B). By contrast, the GATA.CLE0 element (-85--39) and the CLE0 (-63--39) were slightly activated by the addition of glucocorticoids. The potent downregulation of endogenous message demonstrated in Figure 1 suggests that the inhibitory effect of glucocorticoids overrides any local activation at the GATA/CLE0 site.
Glucocorticoid receptor binds to the \textit{IL-5} NF-AT/AP-1 response element \textit{in vitro}.

In order to understand the mechanism of repression, we asked whether purified GR was capable of directly binding to this -130--90 NF-AT/AP-1 element by using a band retardation assay. In this experiment we compared the capacity of GR to bind to \textit{IL-5} and to a consensus MMTV GRE, and secondly the capacity of the \textit{IL-5} element to act as a competitor of GR binding to the GRE and \textit{vice versa}. We observed comparable DNA binding ability of GR to the two sites and each individual site showed almost equivalent activity as a competitor (Fig 4). These data suggest that in this \textit{in vitro} scenario, GR was capable of binding directly to the -130--90 \textit{IL-5} element.

\textbf{Repression of \textit{IL-5} is mediated by histone deacetylases.}

GR is known to mediate its repressive effects by a variety of mechanisms. Many classes of transcription factor impose their transcriptional effects through the recruitment of proteins that have the ability to modify nucleosomal histone tails, particularly the amino termini of H3 and H4. Acetylation and deacetylation have been demonstrated to be involved in gene activation and repression respectively. We asked whether the repressive activity of GR at the \textit{IL-5} promoter was active, or whether it merely blocked the accessibility of the positively acting NF-AT/AP-1 complex to DNA. We addressed this in two ways, first, by the inclusion of the HDAC inhibitor TSA in our repression experiments, and secondly by HDAC overexpression. We reasoned that if there was no active component, TSA would have no effect on repressive capacity. TSA was however able to relieve GR-mediated transcriptional repression of reporter
constructs driven by the minimal 41bp NF-AT/AP-1 response element (-130--90.IL-5.CAT), the IL-5 promoter (-130+-35.IL-5.CAT), and the GM-CSF enhancer driving CAT expression from the minimal promoter E1b. (GM-CSF.E1b.CAT) (Fig. 5a). These data imply that histone deacetylases are involved in the repression of IL-5. HDAC1 was able to augment GR mediated repression when overexpressed in transient transfections using both IL-5 and GM-CSF reporter constructs (Fig. 5B). Taken together, these data suggest that GR promotes active repression which is facilitated by co-repressor recruitment.

**GR interacts with HDAC1.**

To determine whether GR was detectable in a complex with HDAC1, we overexpressed both proteins and Sin3A, a HDAC co-factor, in HEK293 cells and performed co-immunoprecipitation experiments. GR immunoreactivity was present in Flag immunoprecipitates from Flag HDAC1 expressing cells, as was myc tagged Sin3A. By contrast, no GR was detected in Flag immunoprecipitates from cells expressing the negative control, Flag.GATA3 (Fig. 6). These data suggest that GR may form a complex with histone deacetylases. The interaction between GR and HDAC1 appeared to be stable since it was able to withstand vortexing in 500mMNaCl/IPH wash buffer.

**Glucocorticoids potently repress GATA3-mediated activation of the IL-5 and IL-13 promoters.**

PCR of cDNA derived from the primary CD4+ and Th2 cells demonstrated that cytokine messenger RNA expression was potently repressed by glucocorticoids (Fig.1). By contrast, the transfections demonstrated only a partial repressive
activity mediated by GR binding to NF-AT/AP-1 response elements. In these experiments we envisaged competition between NF-AT/AP-1 and GR for binding to the same DNA element. The consequence of this competition would be that the two distinct classes of transcription factors would recruit co-activator (via NF-AT/AP-1) or co-repressor (via GR) complexes to the same DNA site, thereby imparting conflicting regulatory signals upon the transcriptional apparatus. To address how this site occupancy would influence repression by GR, we used the fact that the *IL-5* promoter can be activated by GATA3, whose binding site juxtaposes the -130 to -90 NF-AT/AP-1 site. We reasoned that binding of GATA3 to its site, and activation via this route would leave the NF-AT/AP-1 site vacant to permit unimpeded DNA binding by GR. This was done in parallel with transfection of a combination of both NF-AT/AP-1 and GATA3. We performed these co-transfection experiments on both *IL-5* -130 to +35 and *IL-13* -227 to +50 promoters, which show similar organization with respect to the proximity of NF-AT/AP-1 and GATA3 response elements. With both promoters, NF-AT/AP-1-mediated activation was only partially repressible by glucocorticoids (Fig 7). When the NF-AT response element was vacant, as when the constructs were activated by GATA3, treatment with glucocorticoids caused an almost complete repression. We observed no ability of GR to interact with GATA3 as assessed by co-immunoprecipitation (Fig.6) and we had previously shown that GR does not mediate repression by acting through the GATA.CLE0 site (Fig 3). When both GATA3 and NFAT/AP-1 proteins were used as the activator, the constructs reverted to being only partially repressible by steroids. The data suggest that at both *IL-5* and *IL-13* proximal promoters, the most profound effect of glucocorticoids is upon GATA3-dependent
transcriptional activation, and that NF-AT/AP-1 impairs this repression by competing with GR for DNA binding.

Discussion

The cytokines, IL-4, IL-5 and IL-13 are expressed by the Th2 subclass of helper lymphocytes (24). Data from intracellular cytokine staining of primary Th2 cells has shown a rapid induction of synthesis upon cell activation, suggesting that the chromosome 5q cytokine locus is in a conformation that is permissive for gene expression, yet requisite of an external stimulus (17). The establishment of either a permissive or non-permissive environment for transcription in Th1 and Th2 cells during their differentiation from a naïve precursor provides a detailed paradigm for regulation of gene loci. Data from naïve primary murine T cells suggests that they are poised to permit rapid expression of small quantities of both IL-4 and IFNγ (25). During differentiation to Th2 cells, under the influence of external IL-4, the ability to express IFNγ is diminished and is accompanied by loss of local histone acetylation (25). By contrast development of the Th2 lineage is accompanied by increased histone acetylation at the IL-4 locus and an increase in locus accessibility as measured by the appearance of novel DNaseI hypersensitivity sites. One factor in particular, GATA3, has been postulated to be involved in establishment of these Th2 hypersensitive sites and in maintaining histone acetylation at the murine cytokine gene locus (26). In general, these hypersensitive sites tend not be located at promoters, although it is suggested that they co-localize with regions rich in potential GATA3 binding sites. In addition, the proximal promoters of IL-5 and IL-13, are bound by and transcriptionally upregulated by GATA3 (14,15). This data suggest that GATA3
has roles both proximal to and distal from transcriptional start sites of the target
genes perhaps reflecting an influence both on establishment and maintenance
of a locus-wide chromatin structure, as well as having a local, permissive
influence on transcription of specific genes.

Th2 cytokine synthesis *in vivo* is upregulated during both asthmatic and allergic
responses. Glucocorticoids are the mainstay of therapeutic intervention for
these conditions (27), and although steroids are known to alleviate the pro-
inflammatory actions of these cytokines their precise mode of action in
regulating cytokine function has not been fully dissected. Repressive roles of
the receptor have been shown to encompass a wide variety of mechanistic
actions including DNA binding, tethered, and sequestering activities (28).
Additionally, GR has been shown to upregulate transcription of regulatory
partners of transcription factors mediating pro-inflammatory responses (IκB)
(29), and to inhibit activatory kinases such as JNK (8). It is likely that these
activities can act either in isolation or in combination depending upon the DNA
context and the admixture of factors recruited to the promoter. It has therefore
been difficult to construct a common paradigm for repressive functions of GR,
rather, an alternative approach has been to dissect individual response
elements.

The nuclear hormone receptors appear to cycle on and off their target
promoters once bound by ligand (30). Studies on the estrogen receptor (ER)
also illustrated an exchange of the co-factor complement recruited during
successive periods of ER DNA binding (31). Since many of these co-factors
exert their effects by mediating covalent modifications of nucleosomal histone tails, it is postulated that such an exchange facility might permit a chain of different modifications to be implemented during successive stages of a transcriptional event. Secondly, cycling on and off DNA of the receptor might permit scanning of the local steroid ligand concentration such that any influence on transcriptional activity may be maintained if ligand is present, or rapidly uncoupled in order to terminate effects when ligand levels fall (32).

Our previous data on the GM-CSF gene suggested that one effect of GR localised to the GM-CSF enhancer, which acts as a composite response element for NF-AT/AP-1 complexes and has four binding sites for these factors (33). In addition, this enhancer contains a number of potential GATA sites and is the location of DNaseI hypersensitive site formation (34). We demonstrated the capacity for GR to bind directly to these NF-AT/AP-1 sites in vitro. Such an activity could potentially repress transcription by a number of mechanisms: Firstly, it may prevent binding of NF-AT/AP-1 complexes and consequent recruitment of co-activators. Secondly, GR may prevent co-activator recruitment, or it may induce a conformation on the NF-AT/AP-1 proteins that favours co-repressor recruitment. Thirdly, GR may directly recruit co-repressor complexes. We chose to determine whether a similar mechanism of action to that observed in the GM-CSF gene was operative in another gene located within the human 5q locus, IL-5, which plays a pivotal role in eosinophil function. We show that IL-5 mRNA expression is repressed by glucocorticoids and that a component of this repression maps to a 41bp response element that mediates NF-AT/AP-1 signalling. This region has also recently been suggested to be the
target of the WHSC protein, which acts as a transcriptional repressor and has homology to the SET domain proteins (35), indicative of potential histone methyl transferase activity. It has previously been suggested that the IL-4 gene is regulated by glucocorticoids and that this regulation involved HDAC recruitment (36). Furthermore, Adcock and colleagues (37) were the first to suggest the ability of GR to recruit the HDAC complex to cytokine promoters. While our data also suggest HDAC recruitment, the mode of recruitment and the consequences of this co-repressor recruitment differ from previous studies.

We have shown that the HDAC inhibitor TSA relieves GR mediated repression of IL-5, suggesting that HDAC complexes were recruited to the promoter. This view is supported by the finding that GR co-immunoprecipitates with Flag tagged HDAC1, although whether this was a direct interaction was not established. We also demonstrated that co-expression of HDAC1 augmented GR-dependent transcriptional repression. The recruitment of GR to both IL-5 and IL-13 proximal promoters profoundly influenced transcription mediated by GATA3. The local recruitment of GR may alter the ability of GATA3 either to bind to its target site, to cause transcriptional upregulation, or maintain an environment that is permissive for transcription. We are currently investigating these possibilities with Chromatin Immunoprecipitation in primary Th2 cells. These activities present an interesting paradox, that is, what happens to GR in order to permit recruitment of co-activator complexes following ligand binding in some circumstances, yet co-repressor complexes in others. This ability has been addressed previously. Yamamoto and colleagues demonstrated that recruitment of the co-activator GRIP1 to a tethered GR at the col3A promoter
led to repression (3). These data suggested that the architecture of this previously designated activator could be altered in order to permit repression. A second line of evidence involves the steroid RNA co-activator SRA, Evans and colleagues reported that a co-factor SHARP (SMRT/HDAC Associated Repressor Protein) bound directly to SRA and repressed GR- and SRA-mediated transcriptional activation of a GR-responsive reporter construct (5).

Our studies suggest ability to recruit particular co-factors may be related to the architecture of GR in a particular context. The emerging data on transcriptional regulation by nuclear hormone receptors serves to illustrate the complexity of co-activator and co-repressor complexes that can assemble at a given promoter, the variety of enzymatic activities that these factors carry and the dynamic nature of the transcription process.

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**Figure legends**

**Figure 1. Dexamethasone inhibits cytokine gene transcription.**

A. CD4\(^+\) T cells were maintained in overnight culture either in the absence (-) or presence (+) of 10\(^{-7}\)M Dexamethasone, cells were then harvested and RNA isolated and reverse transcribed. PCR was performed for IL-4, IL-5, IL-13, GM-CSF, RAD50 and 18S products were migrated on 2.5% agarose gels. **B.**

Position of genes within the human 5q cytokine locus. **C.** Real time PCR analysis of genes within the 5q locus was performed both on the same samples as in A, and upon cDNA from hTh2 cells differentiated for 28 days. Data shown are representative of 3 independent experiments for CD4\(^+\) and 2 independent experiments for Th2 cells.

**Figure 2. Dexamethasone-mediated inhibition of IL-5 expression involves the promoter region.** 3\(\mu\)g of plasmids bearing 5' deletion mutants of the human IL-5 promoter fused to a CAT reporter (A), were transiently transfected into Jurkat T cells. Cells were co-transfected with vectors encoding c-Fos, c-Jun and NF-ATc (1\(\mu\)g each), along with 1\(\mu\)g CMV.GR.Flag or pCMV control and stimulated with PDBu and Ion +/- 10\(^{-7}\) M dexamethasone for 20 hours. Cell extracts were prepared and assayed for CAT activity. A 2XGRE.SV.CAT construct was used as a control to demonstrate transactivation induced by glucocorticoids. The results are expressed as CAT activity relative to cells co-transfected with the named reporter vector in the absence of glucocorticoids. In each case results are the mean of at least 3 independent experiments. Error bars indicate standard error of the mean.
**Figure 3.** A 40bp element of the *IL-5* promoter is activated by NF-AT/AP-1 and mediates glucocorticoid responsiveness. A. Reporter plasmids used in the experiment. B. Reporter constructs were transiently transfected into Jurkat T-cells along with RSV.c-Fos, RSV.c-Jun, and RSV.NF-ATc, along with either CMV.GR.Flag or pCMV control. Cells were activated with PDBu and Ion in the presence (+) or absence (-) of dexamethasone (10^{-7} M). The results are expressed as percentage CAT activity relative to cells not treated with glucocorticoids, these samples having been ascribed a value of 100%, and are the mean of 4 experiments with error bars representing standard error of the mean.

**Figure 4.** Glucocorticoid receptor binds to the IL-5 NF-AT/AP-1 response element in vitro. Electrophoretic mobility shift assay showing the ability of GR to bind to a consensus GRE or the *IL-5* NF-AT/AP-1 site (Lanes 2 and 6 respectively). GR binding to each oligonucleotide was competed with a 20X excess of either self oligo (Lanes 3 and 7) or non-self specific competitor (Lanes 4 and 8). Gels were dried and the position of radioactive complexes revealed by autoradiography.

**Figure 5.** Glucocorticoid repression is mediated by histone deacetylase. A. The -130--90.IL-5.*E1b.*CAT construct which contains the NF-AT/AP domain from the *IL-5* promoter, the -130+-35.*IL-5.*CAT vector, and a construct driven by the GM-CSF enhancer (*GM-CSF.Enh.*E1b.CAT), and were transiently transfected into Jurkat T cells along with RSV.c-Fos, RSV.c-Jun, RSV.NF-ATc, and either CMV.GR.Flag or pCMV control. Cells were activated in the presence
of dexamethasone ($10^{-7}$M) with or without TSA ($33 \times 10^{-9}$M), CAT activity was calculated relative to the activated sample. **B.** To determine whether HDAC1 had a direct effect upon -130--90.IL-5.E1b.CAT and GM-CSF.Enh.E1b.CAT reporter constructs, CMV.HDAC1.Flag was co-transfected along with the expression vectors shown. The results are expressed as CAT activity relative to cells co-transfected with the named reporter vector and CMV and are the mean of 4 experiments with error bars representing standard error.

**Figure 6.** The glucocorticoid receptor forms a complex with HDAC1. HEK293 cells were transiently transfected with either CMV.Flag.HDAC1 or CMV.Flag.GATA3, as indicated, in the presence or absence of CMV.GR$\alpha$. Whole cell extracts were prepared in 100$\mu$l IPH (150mM NaCl). Proteins were Immunoprecipitated with anti Flag.agarose beads. Washed precipitates and inputs were electrophoresed and probed with antibodies directed to GR, myc and Flag. Immunoreactive bands were revealed with ECL (Amersham Pharmacia) and autoradiography.

**Figure 7.** Glucocorticoid-mediated repression of the IL-5 and IL-13 promoters impinges upon GATA3 signalling. HeLa cells were transiently transfected with IL-5 or IL-13 driven reporters and the transcription factors shown, and incubated in the presence or absence of glucocorticoids. Data are expressed as CAT activity relative to the NF-AT/AP1/GATA3 treated samples, which were ascribed a value of 100% activity, and are the mean of a minimum of three independent experiments. Error bars indicate standard error of the mean.
**Abbreviations:**

AP-1: Activator protein 1,

ER: Estrogen Receptor,

GR: Glucocorticoid Receptor,

GRE: Glucocorticoid response element,

GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor,

HDAC: Histone deacetylase,

IL: Interleukin,

Ion: Iononmycin,

NF-AT: Nuclear Factor of Activated T Cells,

PDBu: phorbol dibutyrate

TSA: Trichostatin A
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Fig 2

A

-447  +35
NF-AT/AP-1  NF-AT/AP-1  NF-AT/AP-1  GATA  CLEO  TATA  CAT

-130  +35
NF-AT/AP-1  GATA  CLEO  TATA  CAT

B

Control  +NFAT/AP-1  +NFAT/AP-1 + Dox  +Dexamethasone

| Relative CAT activity (%) | 100 | 80 | 60 | 40 | 20 | 0 |
|---------------------------|-----|----|----|----|----|---|
| -447 +35 IL-5 CAT         |     |    |    |    |    |   |
| -130 +35 IL-5 CAT         |     |    |    |    |    |   |
| 2XGCRE SV CAT             |     |    |    |    |    |   |
Repression of Interleukin-5 transcription by the glucocorticoid receptor targets GATA3 signalling and involves histone deacetylase recruitment

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