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Separating transrepression and transactivation: A distressing divorce for the glucocorticoid receptor?

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d) Abbreviations: AP, activator protein; ARE, AU rich element; ATF, activating transcription factor; CBP, CREB binding protein; CC10, Clara cell secretory 10 kDa protein; CINC, cytokine induced neutrophil chemoattractant; C/EBP, CCAAT/enhancer binding protein; COX, cyclooxygenase; DBD, DNA binding domain; Dok, downstream of tyrosine kinase; ERK, extracellular regulated kinase; GILZ, glucocorticoid-inducible leucine zipper; GM-CSF, granulocyte/macrophage colony-stimulating factor; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GRIP, glucocorticoid receptor interacting protein; HAT, histone acetyl transferase; HDAC, histone deacetylase; IL, interleukin; IκB, inhibitor of κB; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LBD, ligand binding domain; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase (MAPK); MKP, MAPK phosphatase; NF, nuclear factor; nGRE, negative glucocorticoid response element; P/CAF, p300 CBP associated factor; PEPCK, phoshoenol pyruvate carboxykinase; PL, phospholipase; Pol, RNA polymerase; POMC, pro-opiomelanocortin; RANKL, receptor activator of NF-κB ligand; SLAP, Src-like adaptor protein; SLPI, secretory leucocyte protease inhibitor; SRC, steroid receptor coactivator; SRE, serum response element; STAT, signal transducer and activator of transcription; TAT, tyrosine amino transferase; TGF, transforming growth factor; TNF, tumor necrosis factor; TTP, tristetraprolin; UTR, untranslated region.

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ABSTRACT

Glucocorticoids (corticosteroids) are highly effective in combating inflammation in the context of a variety of diseases. However, clinical utility can be compromised by the development of side effects, many of which are attributed to the ability of the glucocorticoid receptor (GR) to induce the transcription of, or transactivate, certain genes. By contrast the anti-inflammatory effects of glucocorticoids are to a very large extent due to their ability to reduce the expression of pro-inflammatory genes. This effect has been predominantly attributed to the repression of key inflammatory transcription factors, including AP-1 and NF-κB, and is termed transrepression. The ability to functionally separate these transcriptional functions of GR has prompted a search for dissociated GR ligands that can differentially induce transrepression, but not transactivation. In this review, we present evidence that post-transcriptional mechanisms of action are highly important to the anti-inflammatory actions of glucocorticoids. Furthermore, we present the case that mechanistically distinct forms of glucocorticoid-inducible gene expression are critical to the development of anti-inflammatory effects by repressing inflammatory signaling pathways and inflammatory gene expression at multiple levels. Considerable care is therefore required to avoid loss of anti-inflammatory effectiveness in the development of novel transactivation-defective ligands of GR.

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Despite half a century of clinical use and being among the most effective and widely used medications for the treatment of inflammatory diseases (Barnes, 2006; Rhen and Cidlowski, 2005), we do not have a complete picture of how glucocorticoids (corticosteroids or glucocorticosteroids) operate as anti-inflammatory agents. Classically, glucocorticoids, such as the synthetic dexamethasone, bind the ligand binding domain (LBD) of the glucocorticoid receptor (GR) to promote nuclear translocation (Pratt *et al.*, 2004). In the nucleus, the DNA binding domain (DBD) directs dimerization on imperfect DNA palindromes (consensus: 5' - GGT ACA NNN TGT TCT - 3') known as simple glucocorticoid response elements (GREs) (Fig. 1A) to transcriptionally activate (transactivate) genes including tyrosine amino transferase (TAT) or tryptophan oxygenase (Danesch *et al.*, 1987; Jantzen *et al.*, 1987; Rhen and Cidlowski, 2005). Importantly, roles for glucocorticoid-inducible genes, such as phosphoenol pyruvate carboxykinase (PEPCK) in regulating gluconeogenesis, or TAT in amino acid catabolism, suggested that transactivation by GR was key to the metabolic effects of glucocorticoids (see (Schacke *et al.*, 2002) for a comprehensive review). By contrast, genes including; lipocortin I (*aka* Annexin I), p11/calpactin binding protein (S100A10), which inhibit phospholipase (PL) A_2 activity, secretory leucocyte protease inhibitor (SLPI), a protease inhibitor, or the type II interleukin (IL)-1 receptor, a molecular decoy, were also identified as glucocorticoid-inducible and may contribute towards the anti-inflammatory properties of glucocorticoids (Abbinante-Nissen *et al.*, 1995; Flower and Rothwell, 1994; Re *et al.*, 1994; Yao *et al.*, 1999). However, despite such findings, the principal reason for the effectiveness of glucocorticoids as anti-inflammatory agents lies in the ability to reduce inflammatory gene expression and this was not primarily attributed to transactivation by GR (Barnes, 2006; Rhen and Cidlowski, 2005).

Transrepression and anti-inflammatory effects of glucocorticoids

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Transrepression at negative GREs. Initial examples of transcriptional repression (transrepression) by GR included the pro-opiomelanocortin (POMC) and prolactin gene promoters where *cis*-acting simple negative GRE sites (nGREs) were proposed to mediate repression via direct binding of GR to DNA (Fig. 1A) (Drouin *et al.*, 1989b; Drouin *et al.*, 1989a; Sakai *et al.*, 1988). Thus binding of GR to a simple nGRE that overlaps the TATA box in the osteocalcin promoter was presumed to block transcription (Meyer *et al.*, 1997; Stromstedt *et al.*, 1991). However, nGRE sites correspond poorly to the GRE consensus and, in the case of POMC, prolactin and other neuroendocrine genes, repression is more latterly attributed to tethering mechanisms in which positive transcriptional regulators that bind DNA are then targeted by GR (Chandran *et al.*, 1999; Martens *et al.*, 2005; Subramaniam *et al.*, 1998) (Fig. 1A).

Transrepression of inflammatory gene transcription. In the context of inflammatory gene promoters, consensus *cis*-acting sequences by which GR binds DNA and directly exerts transrepression are not generally described. Instead, such genes show binding sites for transcription factors including; activator protein (AP)-1, the functionally related activating transcription factors (ATFs), CCAAT/enhancer binding proteins (C/EBPs) and, in particular, nuclear factor (NF)-κB. Importantly, it is these sites, which are key to transcriptional activation, that are also necessary for glucocorticoid-dependent inhibition of inflammatory transcription (Barnes, 2006). Thus glucocorticoid-dependent repression of interleukin (IL)-8, inducible nitric oxide synthase (iNOS), or rat cytokine-induced neutrophil chemoattractant (CINC/gro) expression correlated with transcriptional inhibition principally via NF-κB sites (Kleinert *et al.*, 1996; Mukaida *et al.*, 1994; Ohtsuka *et al.*, 1996). Whilst in these studies glucocorticoids repressed NF-κB DNA binding activity, the repression of intercellular adhesion molecule

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(ICAM)-1 and E-selectin expression also involved NF-κB, but did not involve reduced NF-κB DNA binding (Brostjan *et al.*, 1997; Van de Stolpe *et al.*, 1994). Such discrepancies are widely reported and are likely to depend on the repressive mechanism(s) prevailing in any given situation. Thus in A549 pulmonary cells, dexamethasone shows little inhibition of IL-1β-induced NF-κB DNA binding induced for up to two hours, whereas stimulation for 6 h, or following long (24 h) glucocorticoid pre-treatments, significantly reduces NF-κB DNA binding (Newton *et al.*, 1998a). Such effects may involve reduced expression of the NF-κB subunits p50 (NFκB1) (Newton *et al.*, 1998a; Simpson and Morris, 1999), or possibly p65 (RelA) (Kurokouchi *et al.*, 2000; Simpson and Morris, 1999), and/or induced expression of the NF-κB inhibitor, IκBα (see below).

Since protein synthesis inhibitors prevent the repression of NF-κB DNA binding by dexamethasone, new gene expression is implicated and indeed glucocorticoids can increase IκBα transcription and expression to reduce NF-κB DNA binding (Auphan *et al.*, 1995; Scheinman *et al.*, 1995a). However, the induction of IκBα is not necessary for repression of NF-κB-dependent transcription in many cells (Heck *et al.*, 1997; Wissink *et al.*, 1998). For example, in pulmonary type II and endothelial cells stimulated with tumor necrosis factor (TNF) α, IL-1β, or lipopolysaccaride (LPS), IκBα expression was not increased and NF-κB DNA binding was unaltered over time frames in which glucocorticoid-dependent repression of gene expression occurred (Brostjan *et al.*, 1996; Newton *et al.*, 1998a; Ray *et al.*, 1997).

Similarly, glucocorticoid-dependent repression of the collagenase 1 promoter was localized to an AP-1 site that is key to transcriptional activation and alone this conferred glucocorticoid

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sensitivity (Jonat *et al.*, 1990; Schule *et al.*, 1990). Furthermore, whilst direct interaction between AP-1 and GR was proposed to account for repression, one study noted no effect on AP-1 DNA binding (Jonat *et al.*, 1990), whereas another reported inhibition (Yang-Yen *et al.*, 1990). However, subsequent analysis of AP-1 site occupancy revealed no effect of glucocorticoids and direct interference of transcription (i.e. a tethering nGRE) represents the current model of inhibition (Fig. 1A) (Konig *et al.*, 1992).

Nuclear events mediate transrepression. As with AP-1, a direct interaction between NF-κB and GR, without DNA binding by GR, is suggested to account for transrepression by glucocorticoids (Caldenhoven *et al.*, 1995; Ray and Prefontaine, 1994; Scheinman *et al.*, 1995b). Importantly, this process is independent of IκBα expression, NF-κB DNA binding (De Bosscher *et al.*, 1997; Heck *et al.*, 1997), or NF-κB site occupancy (Nissen and Yamamoto, 2000), and is described as a tethering nGRE (Fig. 1A)(De Bosscher *et al.*, 2003). An apparent consequence of direct interference was a mutual antagonism between GR and that both AP-1 and NF-κB, which was suggested to result from competition for co-activators, in particular, CREB binding protein (CBP) (Kamei *et al.*, 1996; McKay and Cidlowski, 1998; McKay and Cidlowski, 2000; Sheppard *et al.*, 1998). However, other studies dispel this notion and instead suggest that interference with the basal transcriptional apparatus explains transrepression, possibly via recruitment of the p160 family member, GR interacting protein (GRIP) (De Bosscher *et al.*, 2000; De Bosscher *et al.*, 2001; De Bosscher *et al.*, 2003; Rogatsky *et al.*, 2001; Rogatsky *et al.*, 2002). Alternatively, phosphorylation of the C-terminal domain of RNA polymerase (Pol) II is prevented by glucocorticoids and this may mediate promoter-selective inhibition of NF-κB-dependent transcription via the GR-dependent loss of a regulatory kinase complex (Luecke and Yamamoto,

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2005; Nissen and Yamamoto, 2000). Similarly, and in keeping with the fact that histone acetylation is required for activated transcription (Adcock *et al.*, 2004), glucocorticoids may also decrease the acetylation at inflammatory gene promoters by reducing CBP-associated histone acetylase (HAT) activity and recruiting histone deacetylase (HDAC) 2 to the NF-κB (p65)-CBP complex (Ito *et al.*, 2000; Ito *et al.*, 2001). This produces a transcriptionally less favorable promoter conformation and may occur via deacetylation of GR to promote interaction with NF-κB (Ito *et al.*, 2006). Interestingly, the tethering of GR and recruitment of HDAC2 to deacetylate histone H4 also explains repression at the POMC promoter and this suggests a general physiological relevance for such a mechanism (Fig. 1B) (Bilodeau *et al.*, 2006).

GR mutants, dissociated steroids and the *dim* mouse. Since the metabolic side effects of glucocorticoids were largely ascribed to transcriptional activation, whereas repression of inflammatory gene transcription was attributed to transrepression, there is considerable interest in GR ligands that dissociate these two functions (Uings and Farrow, 2005). Clues to this possibility came from GR mutants that do not transactivate classical GRE-dependent transcription, yet transrepress AP-1- and NF-κB-dependent transcription (Heck *et al.*, 1994; Heck *et al.*, 1997; Liden *et al.*, 1997; Tao *et al.*, 2001; Yang-Yen *et al.*, 1990). For example, mutation of alanine 458 to threonine (A458T) within the dimerization, or D, loop of GR allows transrepression, but prevents dimerization, DNA binding and simple GRE-mediated transcription (Dahlman-Wright *et al.*, 1991; Heck *et al.*, 1994). Furthermore, replacement of wild type GR with this (*dim*) mutant yields mice ($GR^{dim/dim}$), which are defective in dexamethasone-induced GRE-dependent transcription and endogenous TAT gene expression, yet are competent at repression of AP-1-dependent and classic inflammatory genes (Reichardt *et al.*, 1998; Reichardt *et al.*, 2001;

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Tuckermann *et al.*, 1999). Similarly, GR mutants, which cannot bind co-activators or transactivate, but can still transrepress NF-κB, further indicates that functional dissociation is achievable (Wu *et al.*, 2004).

GR function may also be dissociated by ligands, including derivatives of the antagonist, RU486, which display limited simple GRE transactivation ability, but can transrepress AP-1 reporters (Heck *et al.*, 1994). Likewise, the steroidal compound, RU24858, can be a poor transactivator, but can efficiently repress AP-1-dependent responses (Vayssiere *et al.*, 1997). Importantly, these compounds also repress NF-κB-dependent transcription and show anti-inflammatory properties *in vivo* (Belvisi *et al.*, 2001; Vanden Berghe *et al.*, 1999; Vayssiere *et al.*, 1997). However, despite these encouraging data, RU24848 also induces side effects, including loss of body weight and bone mass (Belvisi *et al.*, 2001). Furthermore, in human eosinophils, RU24858 induced glucocorticoid-dependent genes, including lipocortin 1, to a similar extent as dexamethasone (Janka-Junttila *et al.*, 2006). Conversely, in osteoblastic cells RU24858 and related compounds were poor inducers, relative to prednisolone, of receptor activator of NF-κB ligand (RANKL), a gene that promotes bone resorption (Humphrey *et al.*, 2006). Thus the gene-specific and cell-type dependent transactivation ability of RU24858, even at simple GREs, suggests the existence of further regulatory determinants other than binding of ligand (Chivers *et al.*, 2006; Eberhardt *et al.*, 2005; Tanigawa *et al.*, 2002; Vanden Berghe *et al.*, 1999; Vayssiere *et al.*, 1997).

Non-classical GR-dependent transactivation

Combinatorial transactivation. In considering transcriptional activation by GR, it is important to note transactivation from non-simple GREs. Thus GR and AP-1 may synergize at composite

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and tethering promoter sites (Fig. 1A) (Pearce *et al.*, 1998; Pearce and Yamamoto, 1993; Teurich and Angel, 1995). Likewise, glucocorticoids can positively regulate NF-κB-dependent responses and synergy between NF-κB and GR occurs at appropriately spaced sites in artificial constructs and real genes (Hofmann and Schmitz, 2002; Wang *et al.*, 1997; Webster *et al.*, 2002). Furthermore, even at simple GREs, GR has long been known to synergize with other transcription factors, whilst positive combinatorial responses between GR and a variety of factors including; signal transducer and activator of transcription (STAT) 1, STAT3, STAT5, C/EBP, Ets, Egr-1 and AP-2 are widely described (Aittomaki *et al.*, 2000; Mullick *et al.*, 2001; Schule *et al.*, 1988; Strahle *et al.*, 1988; Tai *et al.*, 2002). Thus IL-6 and dexamethasone combination, as could occur in the resolution or treatment of inflammation, synergistically induces the rat serine protease inhibitor-3 and the α2-macroglobulin genes via STAT3 and C/EBP α. (Kordula and Travis, 1996; Lerner *et al.*, 2003; Takeda *et al.*, 1998). As the later did not involve GR DNA binding, a positive tethering GRE (Fig. 1A) is indicated in the context of a multiprotein complex that also contains c-Jun (Lerner *et al.*, 2003). Similar synergy between STAT5 and GR may variously be described as a positive composite or a tethering GRE depending on the requirement for GR DNA binding (Cella *et al.*, 1998; Stocklin *et al.*, 1996). Furthermore as C/EBPβ/STAT5 co-operativity requires GR (Wyszomierski and Rosen, 2001), and C/EBP proteins are glucocorticoid-inducible and can synergize with the GR, there is obvious potential for dramatic modulation of the transcriptional response by glucocorticoids (Gotoh *et al.*, 1997; Kordula and Travis, 1996; Strahle *et al.*, 1988). Likewise, co-operative interaction between GR, STAT5 and isoforms of nuclear factor (NF) 1 also highlights the importance of multiprotein complexes (Mukhopadhyay *et al.*, 2001). Thus interaction between transcription factors, co-activators, including CBP and the steroid receptor cofactor (SRC) p160 family of proteins, allows

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differential and combinatorial responses at different promoters (Freedman, 1999; Kabotyanski *et al.*, 2006; Kamei *et al.*, 1996; Lerner *et al.*, 2003; Wyszomierski and Rosen, 2001) .

Dimerization-independent transcription. Depending on conditions and exclusion criteria, global gene expression analyses reveal that 1 - 20% of sequences are regulated by glucocorticoids and of these 45 - 70% are induced (Donn *et al.*, 2007; Galon *et al.*, 2002; Planey *et al.*, 2003; Wang *et al.*, 2003). Rogatsky et al. identified around 90 directly dexamethasone-inducible genes of which 10 were analyzed in cell lines expressing either wild type or mutant GRs (Rogatsky *et al.*, 2003). Whilst all genes showed glucocorticoid-inducibility by wild type GR, mutants of the two GR activation functions (AF1 and AF2) or the *dim* mutant resulted in expression of different gene subsets. For example, with *dim* GR, 4 out of 10 genes were induced to greater than 50% of their normal response, and only 3 of the 10 genes showed less than 20% of the wild type response. This suggests a requirement for different aspects of GR function by different glucocorticoid-inducible genes. Consequently, whilst preventing classical GRE-dependent transcription and TAT induction, $GR^{dim/dim}$ mice may allow induction of other glucocorticoid-inducible genes to exert either anti-inflammatory or side effects. This is illustrated by phenylethanolamine *N*-methyltransferase (Tai *et al.*, 2002), which is required for epinephrine synthesis, and the anti-inflammatory mitogen activated protein kinase (MAPK) phosphatase (MKP-1) (Kassel *et al.*, 2001), as these are both glucocorticoid-inducible by the GR *dim* mutant (Abraham *et al.*, 2006; Adams *et al.*, 2003). Likewise, survival of STAT5/GR interaction and resultant gene induction in $GR^{dim/dim}$ animals further questions the effect of dissociated GR ligands (Tronche *et al.*, 2004).

Transactivation plays an important anti-inflammatory role

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A partial role for transcriptional repression. Despite the discussion above, it is clear that the transcriptional repression does not fully explain the repression of inflammatory gene expression by glucocorticoids (Table 1). Thus, in A549 pulmonary cells, repression of NF-κB-dependent transcription by dexamethasone was no more than 40 - 50 % and this correlated with the transcription rate of NF-κB-dependent genes (Chivers *et al.*, 2006; Newton *et al.*, 1998a; Newton *et al.*, 1998b). Likewise, repression of IL-8 expression in primary airway epithelial cells, or transforming growth factor (TGF) β-induced IL-11 in A549 cells, by dexamethasone was not primarily via reduced transcription (Chang *et al.*, 2001; Wang *et al.*, 1999). Similarly, in primary human airways smooth muscle cells, dexamethasone again reduced NF-κB-dependent transcription by no more than 50%, yet many NF-κB-dependent genes, and the two bradykinin receptor genes, in which post-transcriptional processes predominated, were strongly repressed (Catley *et al.*, 2006; Haddad *et al.*, 2000). Moving over to inflammatory cells, analysis of the granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-5 promoters and both NF-κB- and AP-1-dependent transcription in primary human T cells and T cell lines also failed to document substantial a role for transrepression under conditions where endogenous gene expression was highly repressed by dexamethasone (Bergmann *et al.*, 2004; Staples *et al.*, 2003). Finally, in primary human monocytes, LPS induced IL-1β transcription, yet the repression by dexamethasone was primarily post-transcriptional (Kern *et al.*, 1988). Thus using endogenous GR in multiple cells, including primary human cells, only a partial role for transcriptional repression in the repression of inflammatory gene expression by glucocorticoids is documented.

Post-transcriptional repression and glucocorticoid-dependent gene expression. Since, a) total inhibition of transcription, for example by actinomycin D, when added after an inducing stimulus

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can be insufficient to repress inflammatory gene expression; and, b) glucocorticoids only partially inhibit transcription, it is not surprising that post-transcriptional events, in particular mRNA destabilization, are also central to glucocorticoid-dependent repression (Table 1) (Newton, 2000; Stellato, 2004). Importantly, such effects are frequently blocked by inhibitors of transcription or translation and a requirement for glucocorticoid-dependent gene expression is therefore indicated (Table 1). Indeed AU-rich elements (AREs), in the 3' untranslated regions (UTR) of unstable mRNAs can mediate both signal-induced mRNA stabilization and destablization by glucocorticoids (Fan *et al.*, 2005; Lasa *et al.*, 2001; Peppel *et al.*, 1991). Furthermore, the ARE binding protein tristetraprolin (TTP), which promotes mRNA deadenylation and destabilization (Lai *et al.*, 1999), may be induced by dexamethasone to post-transcriptionally repress inflammatory gene expression (Smoak and Cidlowski, 2006) (but see (Jalonen *et al.*, 2005).

MKP-1, an anti-inflammatory glucocorticoid-inducible gene. The involvement of p38 MAPK in ARE-mediated stabilization of inflammatory genes provides a further mechanism for glucocorticoid-mediated destabilization via a process that requires glucocorticoid-induced gene expression (Clark, 2003; Lasa *et al.*, 2000; Lasa *et al.*, 2001; Winzen *et al.*, 1999). In this case, MKP-1, or dual specificity phosphatase 1, is strongly induced by glucocorticoids and dephosphorylates the active, phosphorylated form, of p38 MAPK to reduce both p38 activity and the stability of inflammatory gene mRNAs (Chen *et al.*, 2002; Kassel *et al.*, 2001; Lasa *et al.*, 2002; Zhao *et al.*, 2005) (Fig. 2). In addition, the p38 MAPK pathway may activate, or induce the expression of, inflammatory transcription factors including ATF-1, ATF-2 and AP-1 (see (Newton and Holden, 2003; Wesselborg *et al.*, 1997). Therefore induction of MKP-1 by glucocorticoids may also lead to transcriptional repression of inflammatory genes, such as E-selectin, that are regulated by AP-1 and/or ATF factors (Furst *et al.*, 2006). Likewise, p38 MAPK

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is an established positive regulator of NF-κB-dependent transcription (Newton and Holden, 2003; Wesselborg *et al.*, 1997), and in such cases glucocorticoid-induced MKP-1 expression is predicted to reduce expression of NF-κB-dependent genes.

However, MKP-1 specificity may not be limited to p38 MAPK as both the extracellular regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs) may be substrates for inhibition by this phosphatase (Chu *et al.*, 1996; Franklin and Kraft, 1997; Kassel *et al.*, 2001; Slack *et al.*, 2001). Thus in human airways smooth muscle cells the induction of MKP-1 by dexamethasone represses JNK phosphorylation and is partly responsible for the repression of GROα expression (Issa *et al.*, 2007). In addition, inhibition of JNK by MKP-1 directly represses AP-1-dependent transcription (Liu *et al.*, 1995), and prevents both Elk-1 and serum response element (SRE) activation (Wu *et al.*, 2005), again indicating a role in the repression of transcription. Furthermore, the JNKs, like the p38 MAPK (Lee *et al.*, 1994), are also implicated in the translational control of cytokine biosynthesis and indeed this process is also targeted by glucocorticoids (Swantek *et al.*, 1997). Thus bone marrow-derived macrophage from MKP-$1^{-/-}$ mice reveal impaired dexamethasone-dependent repression of many inflammatory genes and this illustrates the considerable anti-inflammatory potential of glucocorticoid-induced MKP-1 (Abraham *et al.*, 2006). Furthermore the robust induction of MKP-1 by dexamethasone in $GR^{dim/dim}$ macrophage, indicates that GR dimerization is not necessary and suggests that GR ligands, which do not induce simple GRE responses, may still induce MKP-1 (Abraham *et al.*, 2006). Indeed, despite poor activation of a simple GRE reporter, the induction of MKP-1 by RU24858 may account for the sensitivity of RU24858-dependent repression of cyclooxygenase (COX)-2, IL-8 and iNOS expression to inhibitors of transcription and translation (Chivers *et al.*, 2006; Korhonen *et al.*, 2002).

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Other glucocorticoid-inducible genes with anti-inflammatory potential. Partial glucocorticoid-dependent repression of inflammatory gene expression in MKP-$1^{-/-}$ animals suggests the existence of further repressive mechanisms (Abraham *et al.*, 2006). Indeed, whilst a number of glucocorticoid-inducible genes with anti-inflammatory effects have been mentioned, numerous others also exist (Fig. 2). For example, glucocorticoids induce the expression of Clara cell secretory 10 kDa protein (CC10) and thymosin β4 sulfoxide, which are protective in the context of pulmonary allergic inflammation and neutrophilic responses respectively (Chen *et al.*, 2001; Young *et al.*, 1999). Likewise, the repression of inflammatory gene expression may be targeted by glucocorticoid-inducible leucine zipper (GILZ), which is highly glucocorticoid-inducible in T cells, macrophage, mast cells and epithelial cells and represses both AP-1- and NF-κB-dependent transcription (Berrebi *et al.*, 2003; Eddleston *et al.*, 2007; Godot *et al.*, 2006; Mittelstadt and Ashwell, 2001). Similarly, inflammatory signal transduction may be targeted by Dexras1, which is a glucocorticoid-inducible Ras-like protein that prevents activation of ERK (Graham *et al.*, 2002; Kemppainen and Behrend, 1998). Furthermore, interaction with, and inhibition of, protein kinase C δ by Dexras1 (Nguyen and Watts, 2006), or up-regulation of the adaptor proteins downstream of tyrosine kinase (Dok)-1 and Src-like adaptor protein (SLAP) by dexamethasone may also reduce inflammatory signaling, antigen-induced ERK activation, Ca^{2+} transients or activation of Syk kinase Syk (Hiragun *et al.*, 2005; Hiragun *et al.*, 2006).

A further mechanism by which glucocorticoids exert anti-inflammatory and immunosuppressive effects is by preventing proliferative responses and promoting apoptosis of certain immune cells (Newton, 2000). These effects are in part due to the repression of critical proliferative or anti-

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apoptotic factors, for example IL-2, IL-3, GM-CSF and IL-5 in respect of T-cells and eosinophils. However, glucocorticoid-dependent arrest of lymphoid cells is also due to post-transcriptional mRNA destabilization of the G_1 progression factor, cyclin D_3 (Reisman and Thompson, 1995). As with the glucocorticoid-dependent repression of inflammatory genes, this effect is blocked by transcriptional inhibition suggesting once more an involvement of glucocorticoid-inducible genes. Similarly, the pro-apoptotic effect of glucocorticoids on thymocytes requires GR transactivation, which in this case is not sustained by the *dim* GR mutant (Reichardt *et al.*, 1998). Thus glucocorticoid-induced pro-apoptotic genes in thymocytes and T cells may also contribute to reduced inflammation (Wang *et al.*, 2003; Wang *et al.*, 2006b).

GR ligands and function

Dissociated GR ligands and differential GR function. Along with RU24858, numerous steroidal and non-steroidal ligands of GR show various degrees of dissociated function based on standard reporter assays (Barker *et al.*, 2006; De Bosscher *et al.*, 2005; Elmore *et al.*, 2001; Elmore *et al.*, 2004; Shah and Scanlan, 2004; Wang *et al.*, 2006a). Whilst a number of these compounds show anti-inflammatory activity, detailed descriptions in respect of inducible genes or side effects are not reported. However, Elmore *et al.*, characterized GR ligands in standard GRE transactivation and E-selectin promoter (transrepression) assays as well as on inflammatory and side effect genes (Elmore *et al.*, 2004). Thus certain high affinity ligands revealed considerable differences in potency in respect of transepression of the E-selectin promoter compared with repression of either IL-6 or collagenase expression. This suggests the existence of different mechanisms of inhibition in respect of each outcome and could reflect the distinct forms of transrepression for AP-1 and NF-κB that have been functionally established using both GR

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ligands and mutants (Bladh *et al.*, 2005; Martens *et al.*, 2005; Shah and Scanlan, 2004). However, an alternative explanation is that at least some of the observed "transrepression" is attributed to the glucocorticoid-dependent induction of genes (e.g. GILZ, MPK-1), which may mimic true transrepression. In respect of the compounds used in the Elmore *et al.* study, this concept is supported since classical GRE-dependent transactivation did not correlate with TAT or aromatase expression (Elmore *et al.*, 2004). Thus, despite poor simple GRE-dependent transactivation, these ligands, as occurs for RU24858 (Belvisi *et al.*, 2001; Chivers *et al.*, 2006), may induce genes that relate to side effects or anti-inflammatory properties.

Explaining the differential effects of ligands on GR function. Binding of ligand to GR elicits conformational change in the receptor (Bledsoe *et al.*, 2002; Kauppi *et al.*, 2003; Xu *et al.*, 1999). This may influence the precise interactions of GR within the nucleus, such that in the presence of antagonist, GR associates with the co-repressor NCoR, whereas binding of agonist recruits the co-activator, SRC-1 (Garside *et al.*, 2004; Kauppi *et al.*, 2003). Complexity is introduced to the system as the concentration-function relationship, between a ligand and a response may be modulated by the local concentration of GR, co-activators, or their splice variants, as well as the nature of the interaction with DNA (Chen *et al.*, 2000; Cho *et al.*, 2005; Meijer *et al.*, 2005). Indeed, mutually antagonistic effects of co-repressors and co-activators in respect of the dose-response relationship to GR agonists means that the relative intracellular concentrations of each will determine final responsiveness and such findings may explain the observed variability (potency and efficacy) of GR-dependent responses (Wang *et al.*, 2004). Furthermore interaction of nuclear hormone receptors with multiple chromatin remodelling enzymes, including CBP, p300, SRC-1, p300/CBP associated factor (P/CAF), as well as methylases and basal transcription factors (Freedman, 1999; McKenna and O'Malley, 2002; Uings and Farrow, 2005), means even

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for the simplest promoters that there are numerous interactions, which could be differentially modulated by the alternate conformations of GR bound by ligand. If the scope of transcriptional responses by GR, for example at simple GREs, composite GREs, tethering GREs (Fig. 1A), and the intermediates that lie in between these conceptual extremes is considered, then the complex differential effects of GR ligands in modulating transcription becomes difficult to predict.

The prospect of therapeutically improved dissociated glucocorticoids?

The above discussion reveals multiple processes by which glucocorticoids acting via GR can affect gene expression. For example, GR, plus glucocorticoid, elicits positive transcriptional responses not only from simple GRE elements, but also in combination with other transcription factors and via promoter elements that do not necessarily involve GR dimerization or contact with DNA. Indeed a recent study of 548 known or potentially glucocorticoid-inducible genes revealed that most contain GRE sites that do not conform to the simple GRE palindrome and are more typically represented as composite elements (So *et al.*, 2007). Thus ligands that are silent at simple GREs may still induce transcriptional responses from these other promoters. Therefore in the design and characterization of novel "dissociated" GR ligands, it is important to consider which types of transcriptional responses relate to which functional outcomes. Indeed, referring back to the original paradigm for dissociation, if a ligand was silent in respect of all forms of transactivation, it is questionable whether such a ligand would show any anti-inflammatory potential at all. Reasons for this are two-fold. Firstly, if a GR ligand is silent in respect of positive transcriptional responses via tethering GREs there could also be issues as to the effectiveness of such a ligand in transrepression from tethering sites. Secondly, the existence of glucocorticoid-inducible genes that repress AP-1 and NF-κB raises a question as to the nature of

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"transrepression". Thus induction of MKP-1 or GILZ, to switch of AP-1 or NF-κB may appear as transrepression.

In terms of separating transactivation from transrepression to generate novel anti-inflammatory agents with improved safety profiles, it is clear that many, if not most, transactivation mechanisms are not represented in current screening protocols. Consequently, the dissociation which is actually achieved by existing compounds is unclear. Furthermore, accumulating evidence supports the concept that there are many glucocorticoid-inducible genes and that a number of these exert effects that are consistent with anti-inflammatory benefit (Fig. 2). Thus the collective induction of such genes offers the potential for multiple anti-inflammatory effects that are integrated at different regulatory levels (Fig. 2). Therefore, loss of any transactivational properties may reduce the expression of some anti-inflammatory genes and attenuate aspects anti-inflammatory effectiveness (Fig. 2). Whilst detailed accounts of anti-inflammatory efficacy *vs* expression of side effects genes remain to be reported, novel compounds that show reduced up-regulation of steroid-inducible genes may in fact show lessened side effect issues (Coghlan *et al.*, 2003; De Bosscher *et al.*, 2005; Schacke *et al.*, 2004). However, such benefits will need to be very carefully balanced against the possibility that anti-inflammatory functions will also be lost. Therefore final clinical efficacy will depend on the relative balance of both "desirable" and "undesirable" transactivation and transrepression events, which need to be carefully defined in order to optimize the design of future GR ligands. Consequently, rather than searching for compounds that discriminate between transactivation and transrepression, we propose the need to functionally screen in order to identify "differential" compounds that display the most favorable functional profiles. Finally, steroid hormones also show non-genomic modes of action and these

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are not considered in current strategies to design improved GR ligands (Losel *et al.*, 2003).

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Footnotes

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Legends for figures

Fig. 1. Schematics of the transcriptional responses elicited by the glucocorticoid receptor (GR). A, GR is recruited to transcriptional promoter regions by binding DNA as a homodimer in the context of simple GRE sites (i, iv), or in conjunction with other transcription factors (X, Y) at composite sites (ii, v), or finally via interaction with another transcription factor without actually contacting the DNA itself (i.e. tethering) (iii, vi). In each scenario there may be positive or negative effects on transcription and the GRE site is described accordingly. The nature of the response elicited by GR is communicated to the basal transcriptional machinery, represented here as TATA binding protein (TBP) and RNA polymerase II (Pol II). B, Communication between GR and the transcriptional machinery is achieved via the recruitment of co-activators and co-repressors to the transcription factor complex. i, To activate simple GRE-dependent transcription the co-activator CREB-binding protein (CBP) and a p160 protein family member (p160), often steroid receptor co-factor-1 (SRC-1), is bound by GR and links with the basal machinery. The intrinsic and the associated histone acetylase (HAT) activities of CBP, and others, results in histone, and transcription factor, acetylation. Subsequent opening of the chromatin structure around the promoter region facilitates transcriptional activation. ii, At tethering nGREs, transcriptional activators, such as NF-κB or AP-1 (X and Y), bind DNA in the context of co-activators such as CBP. GR exerts a repressive effect by further recruiting p160 family members (not shown) and one or more histone deacetylases (HDAC), which deacetylate both the DNA in the promoter region leading to closing of the chromatin structure and GR to promote interaction with NF-κB. Finally, GR-dependent loss of the Pol II C-terminal domain kinase, P-TEFb, reduces phosphorylation of Pol II and reduces transcription of target genes.

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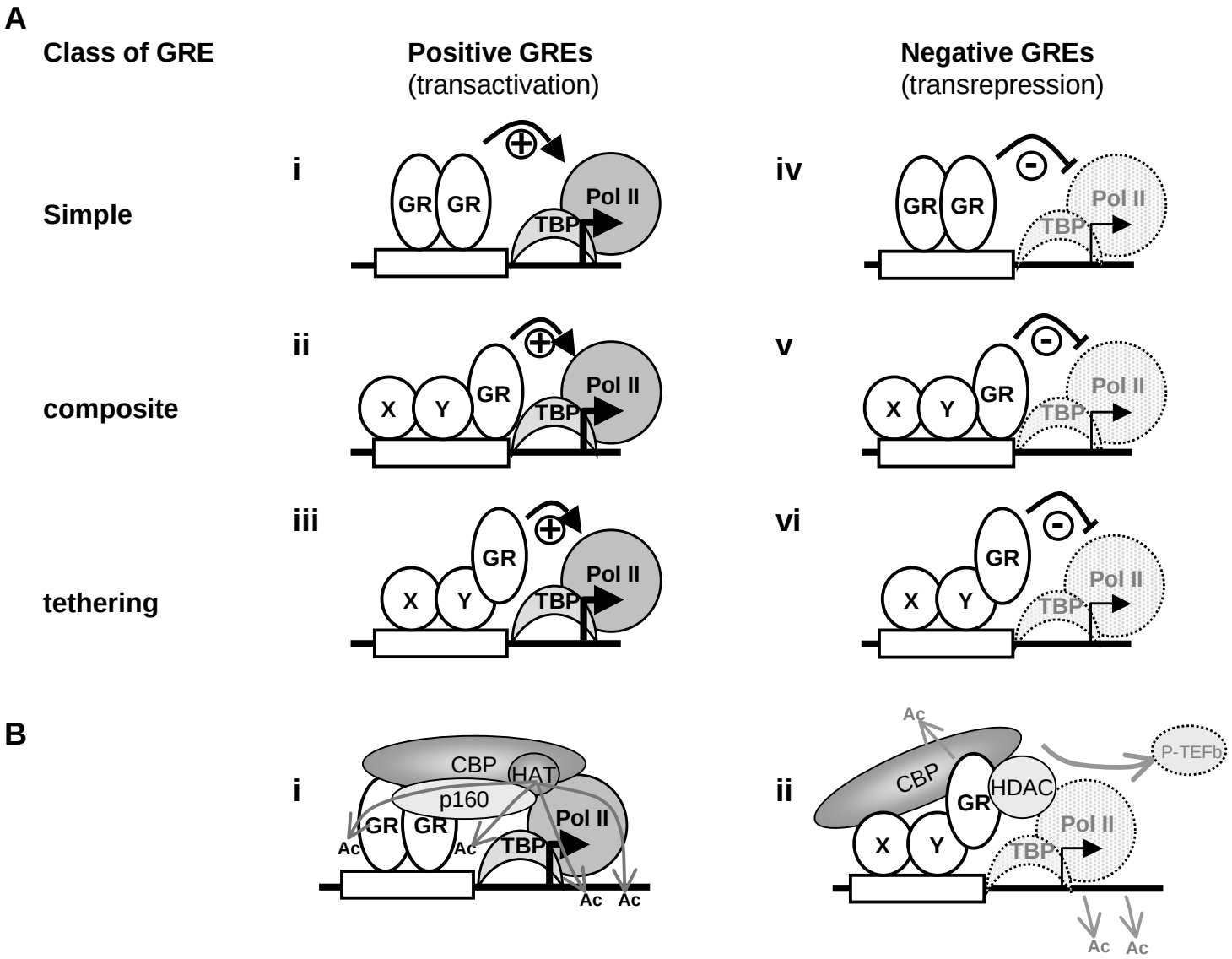
Fig. 2. Global impact of glucocorticoid-inducible genes on inflammatory gene expression. A schematic representation the cascades leading to inflammatory gene expression is depicted with possible targets and sites of action for putative anti-inflammatory glucocorticoid-inducible genes. Activation of a pro-inflammatory cascade, following binding of cytokine to its cognate receptor in the plasma membrane (pm), is shown occurring via a number of kinases (K1-3). The signal crossed the nuclear membrane (nm) and leads to transcription factor (TF) activation and the production of inflammatory gene mRNA. Under the influence of further kinase cascades (here K1-3), the mRNA is stabilized and translated into protein. Finally, many proteins are exported into the extracellular space for function. Sites of action of glucocorticoid-inducible genes are indicated. The glucocorticoid-inducible type II IL-1 receptor (IL-1R) acts as a decoy receptor to prevent activation of the cell by IL-1. Dexras, Src-like adaptor protein (SLAP) and downstream of tyrosine kinase (Dok)-1 all inhibit, through a variety of mechanisms, the activation of signal transduction cascades. A similar role for glucocorticoid-induced leucine zipper (GILZ) protein is suggested. Mitogen activated protein kinase phosphatase (MKP)-1 is an inhibitor of the MAP kinase family and therefore impacts on numerous cellular mechanisms including activation of transcription, mRNA stability and translation. Inhibitor of κB (IκB) α and GILZ inhibit key inflammatory transcription factors (NF-κB and AP-1). Tristetraprolin (TTP) promotes deadenylation and degradation of mRNA. Lipocortin-1 and p11/calpactin binding protein inhibit PLA_2. Finally secretory leucocyte protease inhibitor (SLPI) is a potent inhibitor of serine proteases.

Table 1. Examples of genes showing post-transcriptional regulation by glucocorticoids

Common name (Gene symbol)	Mode of repression by glucocorticoids	Cell type	Stimulus	Sensitivity to transcriptional or translational blockade [a]	Reference
bradykinin B1 receptor (BDKRB1)	mRNA stability	human lung fibroblast (HEL299)	TNFα	ND	Haddad et al. 2000
bradykinin B2 receptor (BDKRB2)	mRNA stability	human lung fibroblast (HEL299)	TNFα	ND	Haddad et al. 2000
COX-2 (PTGS2)	mRNA stability	human lung fibroblast (IMR-90)	IL-1β	Y	Ristimaki et al. 1996
	transcriptional / mRNA stability	human pulmonary type II (A549)	IL-1β	Y	Newton et al. 1998
	mRNA stability p38 MAPK	human fibroblast (Hela)	MKK6	Y	Lasa et al. 2001
cyclin D3	mRNA stability	murine T lymphoma (P1798)	Cell cycle	Y	Reisman and Thompson 1995
IFN β (Ifnb1)	mRNA stability	murine fibroblast (L929)	-	N	Peppel et al. 1991
GM-CSF (CSF1)	mRNA stability	human lung fibroblast (WI38)	TNFα	Y	Tobler et al. 1992
IL-4Rα (IL4R)	mRNA stability	human lymphocytes	PMA	ND	Mozo et al. 1998
IL-1β (IL1B)	translational / post-translational	human PBMC	LPS	ND	Kern et al. 1988
	post-transcriptional	human monocytic (U937)	LPS	ND	Knudsen et al. 1987
	transcriptional / mRNA stability	human monocytic (U937)	PMA / LPS	Y	Lee et al. 1988
IL-6 (IL6)	mRNA stability	human lung fibroblast (WI38)	TNFα	Y	Tobler et al. 1992

IL-8 (CXCL8)	mRNA stability	human lung fibroblast (WI38)	TNFα	Y	Tobler et al. 1992
	transcriptional / mRNA stability	human pulmonary type II (A549)	IL-1β	Y	Chivers et al. 2006
IL-11 (CCL27)	transcriptional / mRNA stability	human pulmonary type II (A549)	TGFβ	ND	Wang et al. 1999
iNOS (NOS2)	mRNA stability	murine macrophage (J774)	LPS	Y	Korhonen et al. 2002
MCP-1 (CCL2)	mRNA stability	rat arterial smooth muscle cells	PDGF	N	Poon et al. 1999

Selected examples of genes that are inhibited by glucocorticoids are listed. Details of the mode of repression, cell type and the inducing stimulus are provided. [a] The ability of either transcriptional or translational inhibitors, when added after the glucocorticoid, to block the repressive effects of glucocorticoids is indicated (Y = yes, N = no, ND = No determined). References are either cited within the main body of the text or are may be found in Newton (2000) or Stellato (2004). Abbreviations not found in the main text are: IFN, interferon; MKK, MAPK kinase; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate.



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