NF-κB signalling is inhibited by glucocorticoid receptor and STAT6 via distinct mechanisms

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Summary

NF-κB transcription factors are involved in the cellular response to stress, and are regulated by inhibitor (IκB) proteins, which prevent NF-κB-mediated transcription by maintaining NF-κB in the cytoplasm. Proteins from other pathways are also known to regulate NF-κB negatively, notably the glucocorticoid receptor (GR) and IL-4-responsive STAT6. Both pathways were shown to inhibit NF-κB-mediated transcription, by expressing either STAT6 or GR and activating the respective pathways. Using fluorescent fusion proteins, we show that GR alters the timing of activated p65 NF-κB nuclear occupancy by increasing the export rate of p65 and is independent of whether GR is present as a dimer or monomer. Expression of STAT6 was also shown to alter p65 nuclear occupancy but appeared to affect the import rate and hence the overall maximal level of p65 translocation. Activating STAT6 with IL-4 prior to activating NF-κB significantly increased this inhibition. Investigation of IκBα showed that activated STAT6 inhibited TNFα-mediated IκBα phosphorylation and degradation, whereas GR activation did not alter IκBα kinetics. This demonstrates a clear separation of two distinct mechanisms of inhibition by STAT6 and GR upon the NF-κB pathway.

Key words: NF-κB, STAT6, Glucocorticoid receptor, Signal transduction, Fluorescent protein fusions, Confocal microscopy

Introduction

There is increasing evidence that cross-talk between signalling pathways is essential to modulate the cellular response to external stimuli. Understanding how these pathways mesh together has major implications for cell biology and human disease. Nuclear factor κB (NF-κB) transcription factors consist of homo- or heterodimers assembled from subunits including p65 (RelA), c-Rel, RelB, p100/p52 and p105/p50 (Baeuerle and Baltimore, 1996). NF-κB proteins are bound by inhibitory proteins (IκB proteins) that include IκBα (Thanos and Maniatis, 1995), IκBβ (Thompson et al., 1995) and IκBε (Whiteside et al., 1997). The p65 and IκBα proteins shuttle independently between the cytoplasm and nucleus in resting cells, with the nuclear export sequence (NES) on IκBα maintaining a predominantly cytoplasmic localization of the NF-κB/IκB complex (Schmid et al., 2000). Activated IκB kinases, IKKα and IKKβ, phosphorylate IκB proteins at N-terminal serine residues (Mercurio et al., 1997; Zandi et al., 1997), phosphorylated IκB is then polyubiquitinated and degraded by the 26S proteasome (Yaron et al., 1998; Coux and Goldberg, 1998). The NF-κB/IκB complex is the preferred substrate for IκB phosphorylation/degradation, rather than IκB alone, thereby only degrading bound but not free IκB (Nelson et al., 2002a). This releases NF-κB with an unmasked nuclear localization sequence (NLS), allowing translocation to the nucleus and activation of transcription.

Downregulation of NF-κB-mediated transcription is thought to occur through newly synthesized IκB entering the nucleus and binding NF-κB. The NF-κB/IκB complex is then translocated to the cytoplasm by CRM1-dependent nuclear export (Arenzana-Seisdedos et al., 1995; Rodriquez et al., 1999). Downregulation does not require cytoplasmic re-localization of NF-κB, because transcription can be switched off even when NF-κB is trapped in the nucleus after stimulation (Nelson et al., 2002a).

The glucocorticoid receptor (GR) is activated by binding glucocorticoid (GC) at its C-terminal ligand-binding domain (Hollenberg and Evans, 1988). Activated GR can then dimerize and translocate to the nucleus and recruit co-activator proteins such as GRIP1 and SRC1, to bind GC response elements (GRE) and to activate transcription (Hong et al., 1997; McKenna et al., 1999). GR localization within a cell appears to be dynamic, with nucleocytoplasmic shuttling of both ligand-bound GR and 'resting' GR in hormone-free cells (Madan and DeFranco, 1993; Defranco et al., 1995; Htun et al., 1996). Activated GR rapidly moves to the nucleus because of the presence of two NLSs (Picard and Yamamoto, 1987) using the cytoskeleton and at least one chaperone, heat shock protein 90 (Yang and DeFranco, 1996; Galigniana et al., 1998).

GCs are potent anti-inflammatory agents. The GR binds to other transcription factors, including NF-κB p65 (Ray and Prefontaine, 1994; Caldenhoven et al., 1995) and prevents transactivation of their target genes, but it has been suggested that this does not alter the occupancy of the DNA response

elements (Nissen and Yamamoto, 2000). It has been possible to dissociate repression of NF-κB from transactivation through targeted mutation of GR, indicating a requirement for distinct regions of the protein for these functions (Ray et al., 1999; Heck et al., 1994; Heck et al., 1997; Bledsoe et al., 2002).

Interleukin 4 (IL-4) exerts multiple effects on the immune system via activation of signal transducer and activator of transcription 6 (STAT6), which is involved in immunoglobulin class switching and T-helper cell type-2 differentiation. STAT6 and NF-κB p50 have been shown to bind one another, but it is not known whether this interaction occurs in the cytoplasm or the nucleus (Shen and Stavnezer, 1998). Synergistic effects between the pathways have been demonstrated in the Ig germline ε promoter (Delphin and Stavnezer, 1995). There have been no reports describing an interaction between STAT6 and NF-κB p65, which is the subunit of the p65/p50 heterodimer that contains a transactivating domain (Schmitz and Baeuerle, 1991).

Conversely, IL-4 has been shown to suppress tumour necrosis factor α (TNFα)-induced expression of the E-selectin gene through STAT6 competing for an overlapping consensus sequence within a dual NF-κB enhancer element (Bennett et al., 1997). In addition, IL-4 has been shown to inhibit TNFα-induced transcription from the interferon response factor 1 (IRF-1) gene promoter (Ohmori and Hamilton, 2000) by competition of NF-κB for the co-activator CBP (CREB-binding protein). In cells transfected with a mutant of STAT6 in which its transactivation domain had been deleted, IL-4 was unable to inhibit TNFα-stimulated transcription. More recently, IL-4 has been shown to inhibit osteoclastogenesis through STAT6-dependent inhibition of RANKL-dependent activation of NF-κB (Abu-Amer, 2001; Wei et al., 2002). These studies have shown that STAT6 inhibits IκB degradation, so preventing NF-κB moving to the nucleus. However, this effect on IκB has only been described when IL-4 was applied for between 24 hours and 3 days. This suggested that STAT6 was probably inducing other factors that feed back onto the NF-κB signalling pathway rather than directly binding NF-κB components.

We have previously shown, using fluorescent fusion proteins, that the rate of IκBα degradation depends on p65 expression levels and that the kinetics of p65 translocation are altered by IκBα overexpression (Nelson et al., 2002a). Direct interaction between these proteins has been demonstrated through fluorescence resonance energy transfer (FRET) (Schmid et al., 2000). These studies have confirmed the normal function of p65 and IκBα fusion proteins and their usefulness for investigating the principles of the pathway dynamics.

From the literature summarized above, it is clear that STAT6 and GR interact functionally with NF-κB. However, it is still not clear how, where and when these interactions occur. We have used fluorescent protein fusions to components of the three pathways to investigate the interactions between GR and STAT6 with both p65 and IκBα proteins of the NF-κB pathway. We have shown that activating either the GC or the IL-4 pathway resulted in decreased NF-κB-mediated transcription and inhibited the nuclear localization of p65. However, this inhibition appeared to occur through different mechanisms. GR increased the rate of p65 nuclear export after stimulation with TNFα and had no effect upon IκBα, whereas STAT6 appeared to inhibit p65 import after stimulation with TNFα, which appeared to be due to direct inhibition of IκBα phosphorylation and degradation. Both methods of inhibition appear to operate before the formation of transcription initiation complexes and occurred too quickly for their response to be explained by transcriptional regulation.

Materials and Methods
Materials
Human recombinant TNFα and human recombinant IL-4 were supplied by Calbiochem (UK). Tissue culture medium was supplied by Gibco Life Technologies (UK) and foetal calf serum (FCS) from Harlan Seralab (UK). Rabbit anti-phospho-IκBα (Serine 32) and goat anti rabbit IgG conjugated to horseradish peroxidase were purchased from New England Biolabs (UK). All other chemicals were supplied by Sigma (UK) unless stated otherwise.

Plasmids
All plasmids were propagated using *Escherichia coli* DH5α and purified using Qiagen Maxiprep kits (Qiagen, UK). pNF-κB-luc (Stratagene, UK) contains five repeats of an NF-κB-sensitive enhancer element upstream of the TATA box, controlling the expression of luciferase. pTAT3-luc controls the expression of luciferase from a minimal alcohol dehydrogenase promoter, with three copies of the glucocorticoid response element from the tyrosine aminotransferase gene promoter. All fluorescent protein expression plasmids are under the control of the human cytomegalovirus immediate early promoter. pEGFP-N1 expresses enhanced green fluorescent protein (EGFP) without any fusion protein attached (Clontech, UK). p65-EGFP contains a 1.6 kb p65 cDNA cloned into the *Hin*dIII-*Bam*HI site of pEGFP-N1 (kindly donated by M. Rowe, UWCM, Cardiff). p65-dsRed was constructed as described previously (Nelson et al., 2002a). These constructs express a C-terminal p65-fluorescent protein fusion. pIκBα-EGFP (Clontech) contains a fusion of IκBα to EGFP. pEGFP-GR expresses an N-terminal fusion of GR to EGFP (Galigniana et al., 1998). pEGFP-STAT6 expresses an N-terminal fusion of human STAT6 to EGFP (Nelson et al., 2002b).

Cell culture and transfection:
HeLa cells (ECACC No. 93021013) were grown in minimal essential medium with Earle's salts, plus 10% FCS and 1% nonessential amino acids at 37°C, 5% CO_2. For confocal microscopy, cells were plated on 35 mm Mattek dishes (Mattek, USA) at 2×10^4 cells per plate in 2 ml medium. After 24 hours, cells were transfected with appropriate plasmid(s) using Fugene 6 (Boehringer Mannheim/Roche) following the manufacturer's recommendations. The optimized ratio of DNA:Fugene 6 used for such transfections was 1 μg DNA with 2 μl Fugene 6. This DNA concentration was maintained for single and dual transfections with fluorescent protein expression vectors (i.e. 0.5 μg of each plasmid).

For microtitre-plate-based luminescence assays of luciferase expression from the pNF-κB-luc reporter plasmid, 1×10^4 cells were seeded in 1 ml of medium into each well of 24 well plates (Falcon, Becton Dickinson, USA) and grown for 24 hours prior to transfection. Cells were transfected for 24 hours using Fugene 6, at an optimised ratio of 0.25 μg DNA to 0.5 μl Fugene 6 per well.

Fluorescence microscopy
Confocal microscopy was carried out on transfected cells in Mattek dishes in a Zeiss XL humidified CO_2 incubator (37°C, 5% CO_2) using a Zeiss LSM510 Axiovert 200 microscope with a 40× phase contrast oil immersion objective (numerical aperture 1.3). Excitation of EGFP was performed using an argon ion laser at 488 nm. Emitted light was

reflected through a 505-550 nm band-pass filter from a 540 nm dichroic mirror. dsRed fluorescence was excited using a green helium-neon laser (543 nm) and detected through a 560 nm long-pass filter. Data capture and extraction was carried out with LSM510 version 3 software (Zeiss, Germany). Treatment of cells with TNFα (10 ng ml^{-1} final concentration) was carried out immediately prior to microscopy by replacing one tenth of the medium volume in the dish with the appropriate solution. Pre-treatment with dexamethasone, RU486 or IL-4 was carried out in the same manner. Each experiment was carried out at least twice with at least four cells obtained per replicate. For p65-dsRed fusion proteins, mean fluorescence intensities were calculated for each time point for both nuclei and cytoplasm. Nuclear:cytoplasmic fluorescence intensity ratios were determined relative to the initial ratio at 0 minutes. For IκBα-EGFP fusion proteins, mean cellular fluorescence intensities were calculated at each time point per cell and the fluorescence intensity relative to starting fluorescence was determined for each cell.

Reporter luminescence assays

Cells were plated in 24-well microtitre plates as described above. Wells were treated for 6 hours (for NF-κB-luc assays) or 18 hours (for TAT3-luc assays) prior to harvesting the cells in 250 µl lysis buffer (White et al., 1990). Each well was assayed in duplicate by transferring 100 µl of lysate into white 96-well plates (Greiner, UK), with ATP added to a final concentration of 1.25 mM. Luminescence was measured using a BMG Lumistar plate reader fitted with an injector (BMG Labtechnologies, UK), which was used to add 100 µl of 1 mM luciferin (Biosynth, Switzerland) to each well. Experiments were performed in triplicate.

Western blotting

HeLa cells were seeded in 90 mm Petri dishes (Falcon, Becton Dickinson) at 1×10^6 per dish. 24 hours later, all plates were pre-treated for 40 minutes with either 10 ng ml^{-1} IL-4 or medium prior to treatment with TNFα (10 ng ml^{-1}). Following treatment, cells were harvested in 750 µl lysis buffer (40 mM Tris-Cl, pH 6.8, 1% w/v SDS, 1% v/v glycerol, 1% v/v β-mercaptoethanol, 0.01% w/v bromophenol blue) at various times and the lysates boiled for 5 minutes. 50 µl of each lysate was run on a 10% SDS-PAGE gel followed by blotting using a Biorad Protean II electrophoresis and blotting apparatus (Biorad, UK), following the manufacturer's instructions. Even loading of the gel was ascertained by Coomassie staining. Detection of serine-32-phosphorylated IκBα with anti-phospho-IκBα antibody was performed following the manufacturer's instructions.

Results

Function of the GFP-GR fusion protein

The functional activity of a fusion of enhanced green fluorescent protein with GR (EGFP-GR) was established by co-transfecting pGFP-GR into HeLa cells together with pTAT3-luc, which contains a GR-responsive promoter directing expression of firefly luciferase. In studies with cells transfected with pGFP-GR, the maximal level of dexamethasone-stimulated pTAT3-luc directed expression was significantly increased, indicating the functional activity of this fusion protein. In addition, the dose response of TAT3-luc to dexamethasone was significantly shifted to give a greater response at lower concentrations of dexamethasone (EC$_{50}$=0.77±0.24 nM; Fig. 1A) than cells transfected with a GFP-expressing control plasmid, pEGFPN1 (EC$_{50}$=2.88±0.27 nM; Fig. 1A). These results demonstrated that GFP-GR was capable of (1) binding to dexamethasone, (2) dimerizing with
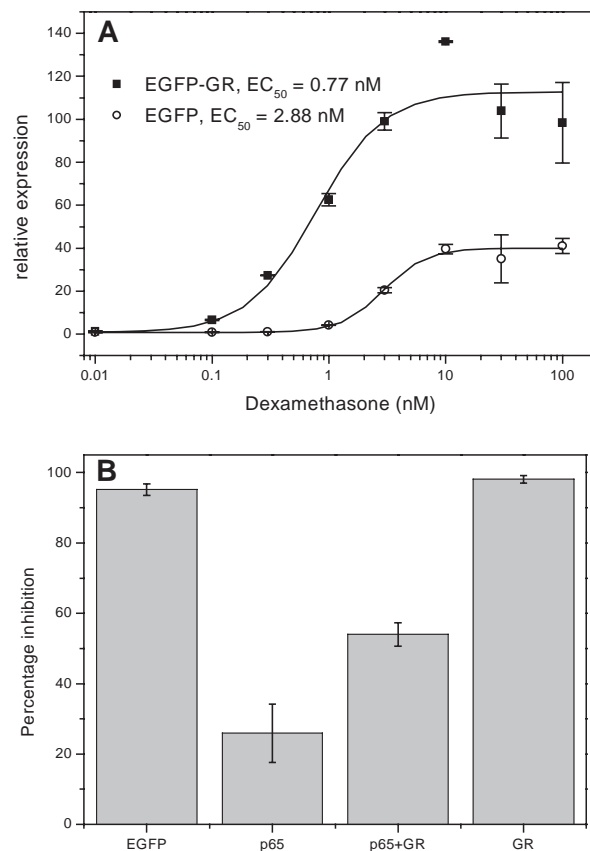

Fig. 1. Functional activity of EGFP-GR in HeLa cells. (A) HeLa cells transiently transfected with pTAT3-luc and either pEGFP-GR or control pEGFP-N1 expression vectors were treated with dexamethasone for 20 hours prior to harvesting. TAT3-directed luciferase expression was measured and plotted relative to the expression for 0 nM dexamethasone-treated cells for each transfection. n=3, error bars±s.e. (B) HeLa cells were transiently transfected with pNF-κB-luc together with various EGFP expression vectors. EGFP expression vectors expressing fusions with p65, GR or both, or a control EGFP were transfected into the cells. Cells were pre-treated for 40 minutes with either medium alone or with 10 nM dexamethasone (in medium) prior to treatment with 10 ng ml^{-1} TNFα for 6 hours. The luciferase activity in cell lysates was determined in pairs of samples either treated with TNFα alone or with dexamethasone and TNFα. The relative luciferase activity obtained between these values reflects the level of inhibition obtained through prior stimulation with dexamethasone. These ratios are plotted as percentage inhibition for each different transfection condition. All transfections were significantly different to one another as calculated by 5% least significant difference. n=3, error bars±s.e.

itself, (or native GR) and (3) binding to its DNA transactivation sequence.

Additionally, we wished to discover whether GFP-GR could also act as a functional transcriptional repressor of the NF-κB signalling pathway (Fig. 1B). Treatment of EGFP-expressing cells with dexamethasone was shown to inhibit the level of TNFα-induced luciferase expression from pNF-κB-luc significantly (95.2±1.7%). In cells expressing GFP-GR, the level of inhibition of pNF-κB-luc expression by dexamethasone was also very significant (98.1±1.04%) and could not be statistically distinguished from that obtained with

the control EGFP-expressing cells. The expression of EGFP-GR together with the p65-EGFP significantly increased dexamethasone inhibition of pNF-κB-luc expression (54.0±3.3%) relative to that obtained with p65-EGFP alone (25.94±8.2%). This showed that expression of the EGFP-GR fusion protein could enhance GC-mediated inhibition of NF-κB-mediated signalling by TNFα.

Inhibition of the NF-κB pathway by activated GR

In order to investigate the mechanism of GR inhibition of NF-κB-mediated transcription, we used noninvasive single-cell imaging to monitor the localization of a p65-dsRed fusion protein (Nelson et al., 2002a). Stimulation of HeLa cells expressing EGFP and p65-dsRed with TNFα showed a phase of nuclear accumulation of p65 lasting ~40 minutes. Subsequently, the p65-dsRed fluorescent protein was exported back out of the nucleus to the cytoplasm, reflecting a second phase of net nuclear export, which was almost complete after ~80 minutes (Fig. 2B, Fig. 3A). This resulted in a period of nuclear occupancy (defined as half-maximal nuclear import to half-maximal nuclear export) of 40.0±0.6 minutes (Fig. 3C). Pretreatment of EGFP- and p65-dsRed-expressing cells with 10 nM dexamethasone for 40 minutes before TNFα stimulation resulted in a marked alteration in the time course of p65-dsRed nuclear occupation (Fig. 3A). This appeared to be caused by earlier net nuclear export rather than an inhibition of nuclear import, leading to a significantly shorter period of nuclear occupancy (26.4±0.6 minutes, $P<0.05$; Fig. 3C). Overexpression of EGFP-GR (instead of the control EGFP) also resulted in a similarly decreased period of TNFα-induced p65-dsRed nuclear occupation in cells pretreated with dexamethasone (24.4±1.1 minute for cells pretreated with 10 nM dexamethasone compared to 30.3±0.3 minutes for control pretreated cells, $P<0.05$; Fig. 2A, Fig. 3B,C). The expression of EGFP-GR also changed the dynamics of p65-dsRed nuclear occupancy in response to TNFα in cells not pretreated with dexamethasone but this reduction was less than in dexamethasone pretreated cells (Fig. 3B,C). These results suggested that activated GR was directly involved in the mechanism of dexamethasone-stimulated export of p65.

Inhibition of NF-κB signalling by the GR agonist RU486

We wished to determine whether this increased export of p65 required GR-dimerisation, binding to DNA and activation of gene expression. The GR type II agonist RU486 gives rise to nuclear import of GR without stimulating these subsequent events (Beck et al., 1993). Therefore, we pre-treated cells expressing EGFP-GR and p65-dsRed with this agonist in place of dexamethasone. As shown in Figs 3B and 3C, this also led to a decrease in the time of nuclear occupation of p65-dsRed, with very similar kinetics to those shown by cells expressing EGFP-GR activated with dexamethasone. By determining the half time of p65-

dsRed nuclear occupation, we have shown that the time of nuclear occupation is significantly reduced by overexpression of EGFP-GR, by pre-stimulation with dexamethasone/RU486 or by a combination of EGFP-GR expression and dexamethasone/RU486 treatment compared to control cells expressing EGFP (Fig. 3C).

Effect of activation of the STAT6 pathway on the NF-κB pathway

Activated, overexpressed STAT6 has previously been shown to inhibit the TNFα-induced transcription of a synthetic NF-κB promoter (Ohmori and Hamilton, 2000). We therefore wished to establish the nature and mechanism by which STAT6 activation by IL-4 could inhibit activation of the NF-κB pathway. Pretreatment of HeLa cells expressing the control EGFP with IL-4 gave rise to significant inhibition of subsequent TNFα induced NF-κB-directed gene expression (Fig. 4). The expression of EGFP-STAT6 fusion protein gave rise to a significant increase in the IL-4-mediated inhibition of TNFα-stimulated gene expression. The IL-4 mediated
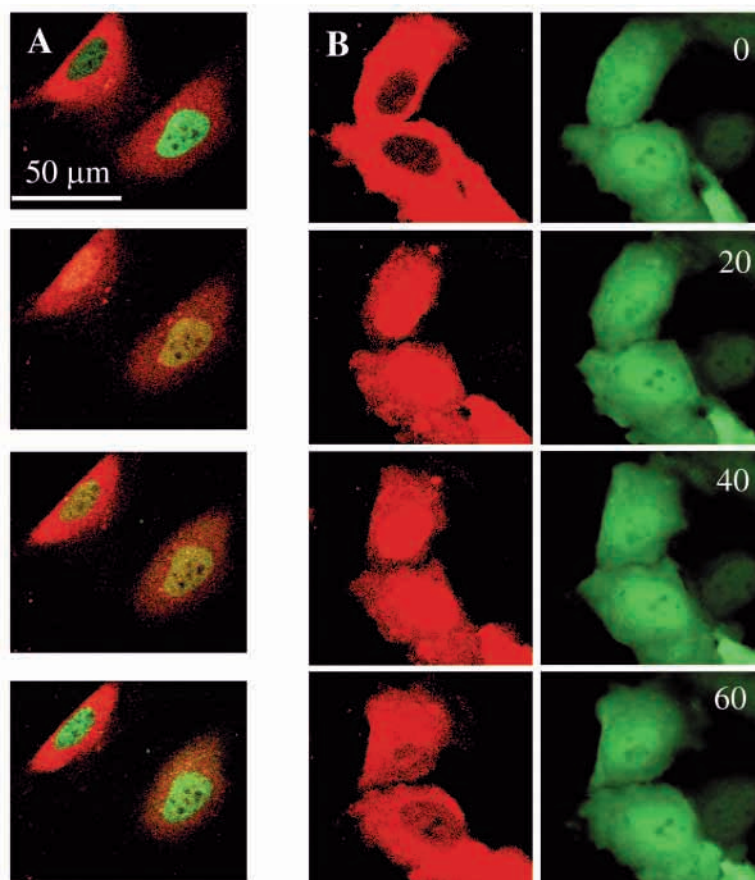

Fig. 2. Confocal microscopy of p65-dsRed translocation. Green fluorescence represents either EGFP-GR (A) or EGFP (B); red fluorescence represents p65-dsRed. Times after TNFα treatment (minutes) are shown. (A) Merged images of p65-dsRed and EGFP-GR. p65-dsRed nuclear localization is very brief in cells expressing EGFP-GR and treated with 10 nM dexamethasone for 40 minutes prior to TNFα (10 ng ml^{-1}) treatment. (B) p65-dsRed and EGFP fluorescence are shown as separate channels for clarification. p65-dsRed (left) shows increased nuclear localization in cells expressing control EGFP (right) and treated with TNFα (10 ng ml^{-1}).
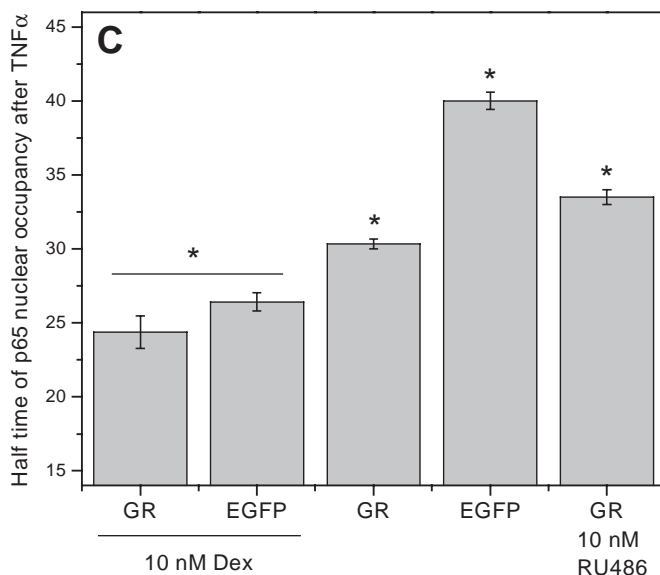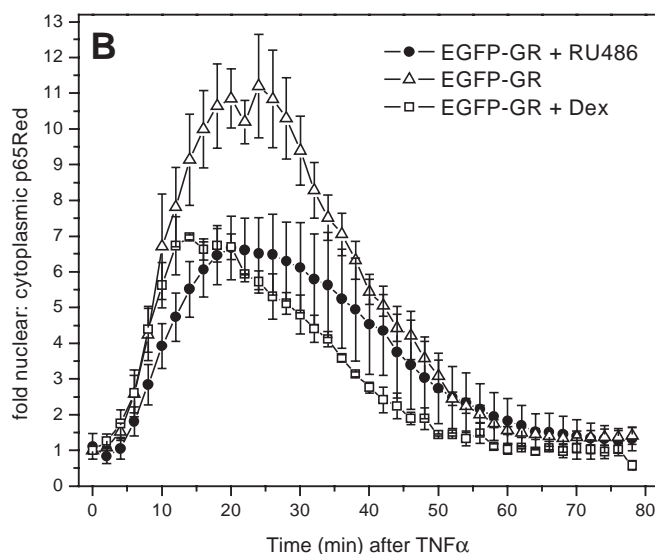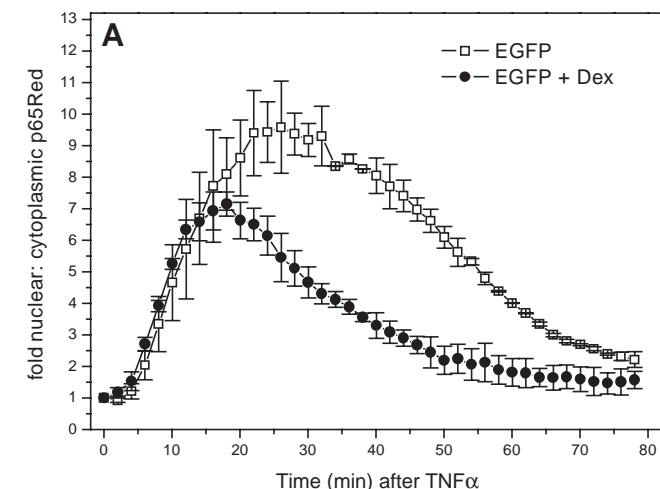
Fig. 3. Modulation of p65-dsRed translocation by glucocorticoids. HeLa cells expressing p65-dsRed and either EGFP (A) or EGFP-GR (B) were pretreated for 40 minutes with medium, 10 nM dexamethasone (Dex) or 10 nM RU486, followed by confocal microscopy (data not shown). Cells were then treated with 10 ng ml^{-1} TNFα and monitored every 2 minutes by confocal microscopy. p65-dsRed subcellular fluorescence was analysed and plotted as a ratio of nuclear:cytoplasmic fluorescence relative to the ratio at 0 minutes. (C) The time of 50% maximal nuclear import to 50% nuclear export of p65-dsRed from the data represented in A and B was determined for each treatment and plotted as the half-life of nuclear occupancy. Calculation of the 5% least significance difference between treatments showed all to be significantly different to one another (*) with the exception of EGFP and EGFP-GR transfected cells pretreated with dexamethasone. Each treatment was performed in triplicate with a minimum of 20 cells per experiment. Data plotted represent mean ratio per cell ± s.d.$_{(n-1)}$ for each experiment.

inhibition of TNFα-stimulated luciferase expression could be overcome by overexpression of p65-EGFP and this was also true in cells co-expressing EGFP-STAT6. Expression of EGFP-STAT6 also lowered the basal transcription level from NF-κB-luc (data not shown). This suggests that EGFP-STAT6 behaves in a similar manner to native STAT6 in its effect on the NF-κB pathway.

We therefore applied imaging of the dynamics of p65 translocation to investigate the mechanism of inhibition of activated STAT6 inhibition of the NF-κB pathway. Cells expressing p65-dsRed and either EGFP-STAT6 or EGFP (control) were treated with TNFα either with or without IL-4 pretreatment. There was marked inhibition of p65-dsRed
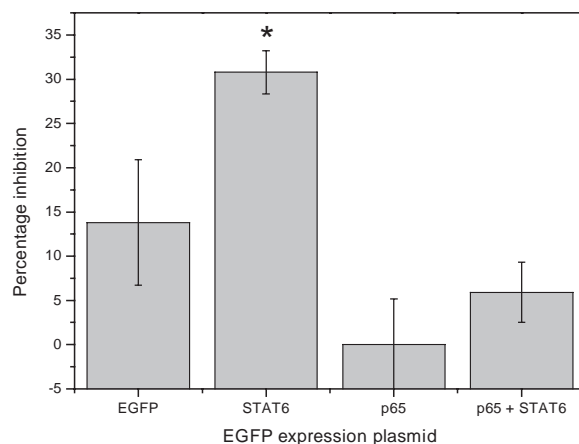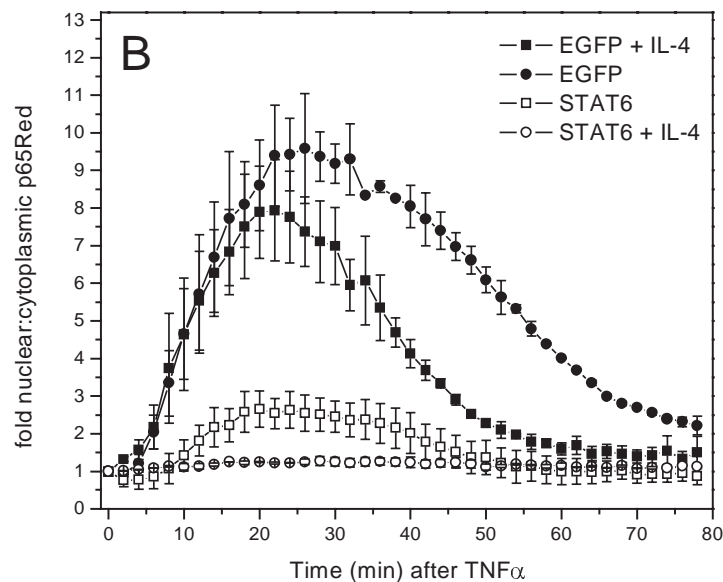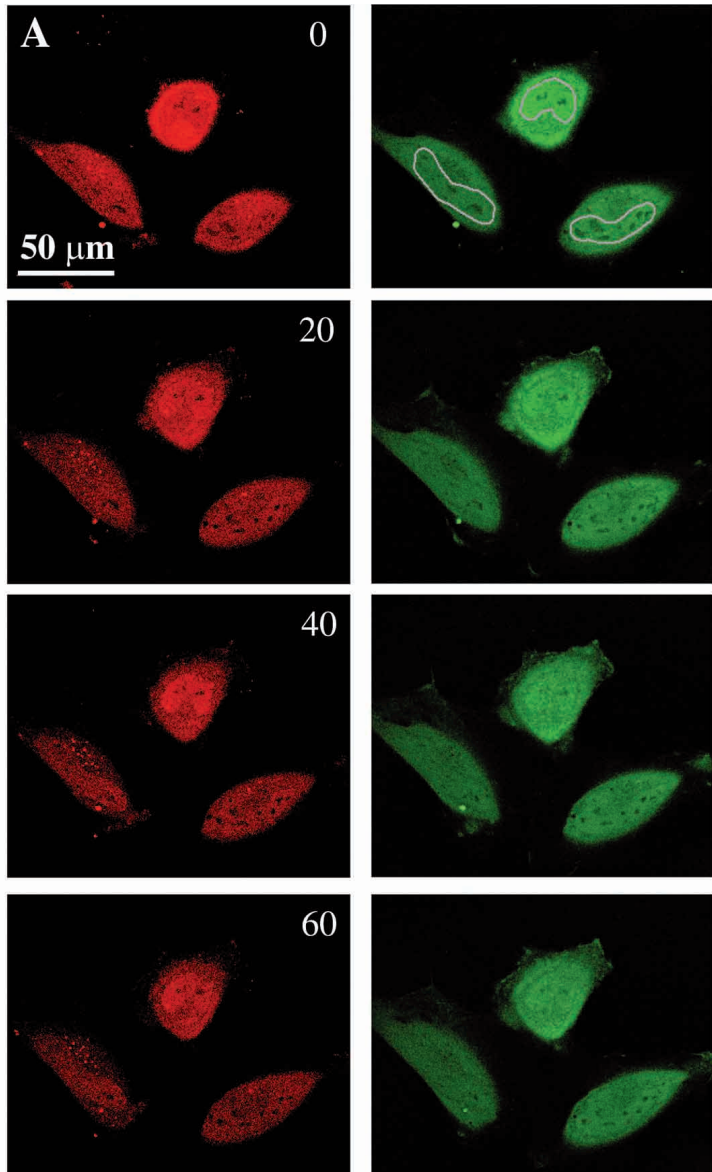

Fig. 4. Inhibition of NF-κB-mediated transcription by STAT6. HeLa cells were transiently transfected with pNF-κB-luc together with various EGFP expression vectors. EGFP expression vectors expressing fusions with p65, STAT6 or both, or a control EGFP were transfected into the cells. Cells were pretreated for 40 minutes with either medium alone or 10 ng ml^{-1} IL-4 (in medium) prior to treatment with 10 ng ml^{-1} TNFα for 6 hours. The luciferase activity in cell lysates was determined in pairs of samples treated either with TNFα alone or with IL-4 and TNFα. The relative luciferase activity obtained between these values reflects the level of inhibition obtained through prior stimulation with IL-4. These ratios are plotted as percentage inhibition for each different transfection condition. IL-4 treatment of EGFP-STAT6-expressing cells significantly inhibited transcription compared with all other transfection conditions (5% least significant difference). *n*=3, error bars±s.e.

Fig. 5. Repression of p65 activation by IL-4/STAT6. HeLa cells transiently transfected with p65-dsRed and either EGFP or EGFP-STAT6 were pretreated with 10 ng ml^{-1} IL-4 or medium for 40 minutes. Subsequent stimulation with 10 ng ml^{-1} TNFα was followed by confocal microscopy for 80 minutes. (A) Fluorescence images of cells expressing p65-dsRed (left; red fluorescence) and EGFP-STAT6 (right; green fluorescence) treated with IL-4 and TNFα, showing marked inhibition of p65-dsRed translocation compared with cells expressing EGFP treated with TNFα (Fig. 2B). Positions of nuclei are highlighted in grey in the first EGFP-STAT6 image. Times after TNFα treatment are shown in minutes. (B) Quantification of p65-dsRed translocation inhibition by activation and expression of EGFP-STAT6. p65-dsRed localization was analysed and quantified as described in Fig. 3. Each treatment was performed in triplicate with a minimum of 16 cells per experiment. Data plotted represent mean nuclear:cytoplasmic ratio per cell±s.d.$_{(n-1)}$ for each experiment.

translocation by expression of EGFP-STAT6 (Fig. 5A,B). Pre-stimulation with IL-4 inhibited p65-dsRed translocation in cells that were overexpressing both EGFP (as a control) and EGFP-STAT6. The effects of IL-4 and EGFP-STAT6 were also cumulative. Statistical analysis (ANOVA) of the kinetics of p65-dsRed translocation under these conditions showed that the maximal nuclear translocation of p65-dsRed was significantly reduced by activation and/or overexpression of EGFP-STAT6 ($P<0.05$).

Effect of activation of the GR or STAT6 signalling pathways on the dynamics of IκB degradation in response to TNFα

IκBα is known to shuttle between the cytoplasm and nucleus, and is believed to act as a chaperone to relocalize NF-κB to the cytoplasm. Therefore, we investigated whether GR or STAT6 affected the timing of IκBα degradation or localization. Transfection of cells with IκBα-EGFP allowed us to investigate whether the increased nuclear export of p65 by activated GR was due to stabilisation of IκBα after treatment with TNFα. For these studies, we used cells expressing both p65-dsRed and IκBα-EGFP, because we have previously shown that the ratio of the two proteins significantly affects the degradation rate of IκBα-EGFP (Nelson et al., 2002a). There was no noticeable effect of pre-incubation with 10 nM dexamethasone on resting cellular IκBα-EGFP localization, and there was no significant effect upon the degradation rate of IκBα-EGFP following treatment with TNFα (Fig. 6A).

We also investigated the effect of IL-4 stimulation of the STAT6 pathway upon IκBα-EGFP. In cells transfected with IκBα-EGFP and p65-dsRed, we saw no effect of pre-incubation of IL-4 upon either p65 or IκBα (data not shown). Subsequent stimulation with TNFα showed a marked reduction in the level of IκBα-EGFP degradation, suggesting that pre-activation with IL-4 inhibited this step in the NF-κB pathway (Fig. 6A). This demonstrated a clear distinction between the mechanisms of inhibition of the NF-κB pathway by STAT6 and GR.

We further analysed the IL-4-mediated inhibition of IκBα by investigating the effect of IL-4 upon phosphorylation of IκBα. Western blotting demonstrated transient IκBα serine-32 phosphorylation after stimulation with 10 ng ml^{-1} TNFα.
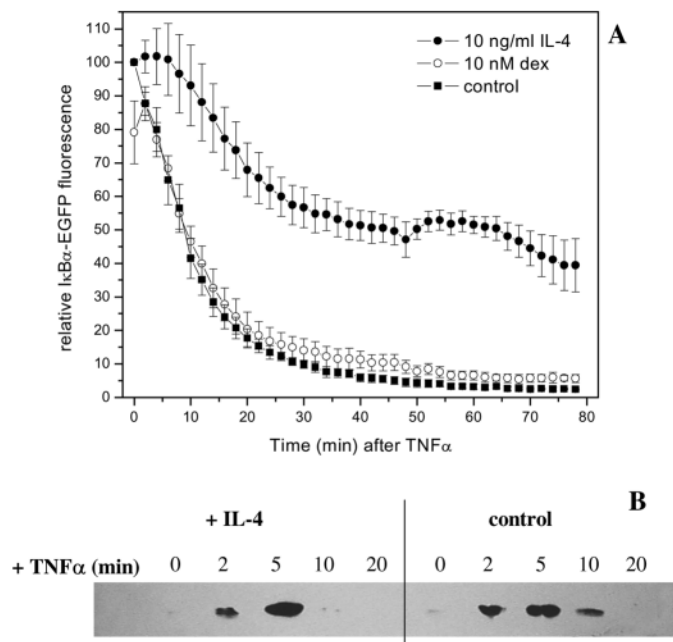
Fig. 6. Modulation of TNFα-induced IκBα degradation. (A) HeLa cells transiently transfected with pIκBα-EGFP and p65-dsRed were pretreated for 40 minutes with 10 ng ml⁻¹ IL-4, 10 nM dexamethasone or medium prior to TNFα (10 ng ml⁻¹) stimulation. Cytoplasmic IκBα-EGFP fluorescence for individual cells was plotted relative to the fluorescence at 0 minutes. Each treatment was performed in triplicate with a minimum of 14 cells per experiment. Error bars±s.d.$_{(n-1)}$. (B) HeLa cells were pretreated with either 10 ng ml⁻¹ IL-4 or medium for 40 minutes. Cells were harvested in lysis buffer at various time points (as shown) following treatment with 10 ng ml⁻¹ TNFα. Samples were blotted with an anti-phospho-IκBα antibody.

Incubating cells with 10 ng ml⁻¹ IL-4 for 40 minutes prior to TNFα treatment caused a decrease in IκBα serine-32 phosphorylation, with very little phosphorylation 10 minutes post TNFα treatment (Fig. 6B).

Discussion

We have investigated the repression of NF-κB-mediated transcription by the GC and STAT6 pathways. Specifically, we have shown that overexpression of EGFP-GR can cause inhibition of NF-κB mediated transcription and that this inhibition can be enhanced by activation of the GR pathway. EGFP-GR repression was overcome by expressing p65, which suggests a mechanism of competitive inhibition either directly through p65/NF-κB or at an upstream stage in the signalling pathway. Investigation of the kinetics of p65 nuclear import and export has demonstrated that GR alters the timing of p65 nuclear occupancy. The rate of nuclear translocation appeared unaltered, as were the maximal levels of nuclear occupancy for each treatment, regardless of whether EGFP-GR or EGFP was overexpressed (Fig. 3). Therefore, these data suggest that the GR pathway is increasing the rate of p65 nuclear export, because the time of nuclear occupancy was decreased by activated GR. Inhibition by simply overexpressing EGFP-GR might be due to serum factors causing basal activation of the GC pathway, which would explain why unstimulated EGFP-GR causes a reduction in p65 nuclear occupancy (resting cells expressing EGFP-GR frequently show a significant nuclear localization of EGFP-GR; Fig. 2A).

RU486 is a type-II GC agonist that allows nuclear translocation of GR and DNA binding but is not an efficient activator of transcription, because it recruits the co-repressor NCoR rather than the co-activator p160 to GR (Schulz et al., 2002). Interestingly, RU486 treatment has been previously shown to inhibit NF-κB directed transcription but is less potent than dexamethasone (Heck et al., 1994; Heck et al., 1997). We show that preincubation with RU486 diminished the time of nuclear occupancy by a similar extent to dexamethasone treatment in cells expressing EGFP-GR (Fig. 3C). This observation therefore suggests that the stimulation of the nuclear export of p65 does not require the GR to adopt a transactivation competent conformation.

IκBα is believed to control the nuclear/cytoplasmic status of NF-κB, by shuttling into the nucleus, where it is thought to bind to the NF-κB complex, masking the NLS so that the NES of IκB predominates, resulting in cytoplasmic NF-κB/IκB. We investigated whether GR might be increasing the rate at which IκB can export NF-κB. This could be through increasing nuclear shuttling of IκB or stabilization of IκB by preventing its phosphorylation by IKK and subsequent degradation. Stimulation of cells expressing IκBα-EGFP and p65-dsRed with dexamethasone showed no detectable alteration in the localization of IκBα-EGFP (data not shown). Subsequent stimulation with TNFα showed no apparent difference in the rate of IκBα-EGFP degradation compared with the control (Fig. 6A), suggesting that the effect of GR upon nuclear export was not directed through IκBα. Newly synthesized IκBα is also unlikely to be the target of GR-mediated p65 export, as the rate of nuclear export after TNFα stimulation is too fast for new protein synthesis.

It therefore seems most likely that transrepression of p65 by GR either occurs through direct interaction with p65 or via alternative proteins. It has recently been demonstrated that other upstream proteins of the NF-κB signalosome, namely IKKα and NIK also shuttle in resting cells (Birbach et al., 2002). It is likely that these contribute to the basal state of NF-κB and so are potential targets for GR. However, because GR can bind p65 (Caldenhoven et al., 1995; Nissen and Yamamoto, 2000), this seems to be a more likely route of inhibition. Exactly how GR increases p65 export remains unclear, however, because EGFP-GR appears to remain in the nucleus when p65-dsRed is exported. GR-bound p65 might be a better target for chaperones mediating CRM-1 dependent nuclear export and hence increasing p65 nuclear export. Another possibility is that GR inhibits another protein interaction with p65 that would otherwise stabilize its accumulation in the nucleus. One such interaction would be with the catalytic subunit of protein kinase A (PKAc), which has been implicated as a member of the NF-κB signalosome complex and shown to phosphorylate p65 (Zhong et al., 1997). Furthermore, PKAc overexpression has been shown to increase NF-κB-mediated transcription, which could be inhibited by also expressing GR (Doucas et al., 2000). Because GR overexpression does not alter p65 import rates, it might alternatively be inhibiting phosphorylation of p65 by PKAc and therefore decreasing its transcription initiation efficacy. This would rely upon p65

nuclear import being independent of the phosphorylation state of p65, which would fit with the findings of Nissen and Yamamoto (Nissen and Yamamoto, 2000), who showed that GR bound p65 but did not affect DNA binding by p65. Instead, GR inhibited the phosphorylation of the C-terminal domain of RNA polymerase (pol) II in the transcription preinitiation complex (PIC), which is required for transcription initiation. It is possible that p65 would have a lower binding affinity for inactive PIC, and this would cause p65 dissociation in the nucleus. Subsequently, the unbound nuclear p65 is more likely to be targeted for nuclear export by IκBα. Both scenarios (GR inhibition of cytoplasmic p65 phosphorylation and nuclear GR inhibition of RNA pol II phosphorylation) can be combined by envisaging that the conformation of phosphorylated p65 encourages the phosphorylation of RNA pol II in the p65-bound PIC. In such a case, GR inhibition of RNA pol II phosphorylation and increased nuclear export of p65 would both be seen as downstream effects of the phosphorylation state of p65.

Overexpression of EGFP-STAT6 had a similar effect to GR on the NF-κB pathway. We have shown inhibition of NF-κB-mediated transcription by IL-4 activated EGFP-STAT6 (Fig. 4). Also, investigation of single living cells expressing EGFP-STAT6 and p65-dsRed showed marked inhibition of p65 translocation by both activating the STAT6 pathway and overexpressing EGFP-STAT6 (Fig. 5). The observed inhibition of p65-dsRed translocation was far greater than the transcriptional inhibition from NF-κB-luc. This is expected because very little transcription factor is required in the nucleus to activate transcription, whereas fluorescence imaging of translocation shows gross properties of the fusion protein. The effects of EGFP-STAT6 and IL-4 upon p65-dsRed translocation were cumulative, suggesting that the inhibition was directed through activated STAT6. However, the half-life of p65 nuclear occupation was not affected to the same extent by activation of EGFP-STAT6 (not significant compared with EGFP; data not shown) compared with activation of EGFP-GR. EGFP-STAT6 slowed the rate of p65 translocation into the nucleus and the overall maximal nuclear translocation was reduced by EGFP-STAT6 (Fig. 6A), whereas this was not the case for EGFP-GR. Therefore, the modes of action appear to be different, with IL-4-mediated inhibition minimizing p65 import and dexamethasone increasing the rate of p65 nuclear export. Because EGFP-STAT6 appeared to be blocking p65 import, we also investigated whether IL-4 had an effect upon IκBα by monitoring IκBα-EGFP degradation in cells pretreated with IL-4. There was significant inhibition of TNFα-mediated IκBα-EGFP degradation (Fig. 6A), which was shown to correlate with a shorter time course of native IκBα phosphorylation at serine 32, suggesting IL-4-mediated inhibition of IκBα phosphorylation rather than ubiquitination (Fig. 6B). This would explain the block in p65 import because, in resting cells, p65 is held inactive by the IκBα. It is possible that cytoplasmic STAT6 is responsible for this inhibition, because activated STAT6 translocates to the nucleus slowly (Nelson et al., 2002b). A recent report has also shown that activated STAT6 inhibits IκBα phosphorylation, supporting the current data (Wei et al., 2002). However, inhibition of IκBα phosphorylation and degradation does not entirely explain why mutation of the STAT6 DNA binding domain knocks out STAT6 suppression of NF-κB-mediated transcription (Ohmori

and Hamilton, 2000). One possibility is that STAT6 interacts directly with p65 and that this interaction both blocks the binding of IκBα with p65 and masks the NLS of p65. IκBα appears to be subject to phosphorylation by only the IKK and/or proteasome-mediated degradation in the context of IκBα/NF-κB dimers (Zandi et al., 1998; Nelson et al., 2002a) and therefore the rate of IκBα degradation would be diminished in this model, whereas p65 nuclear import would also be inhibited by the STAT6 interaction. Therefore, a direct interaction between p65 and activated STAT6 could explain these observations.

We have investigated how GC and STAT6 pathways inhibit NF-κB and have shown them to work through different mechanisms. Both appear to occur independently of up- or downregulation of transcription and also before NF-κB bound the transcription initiation complex. These data suggest complex interactions between signalling pathways affecting the cellular equilibrium of transcription in resting cells and in cells responding to the complex cocktail of cytokines present in vivo.

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