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Dynamic analysis of STAT6 signalling in living cells

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Abstract Functional activity of N- and C-terminal fluorescent fusion proteins between STAT6 and EGFP was demonstrated through IL-4-dependent transcriptional activation and nuclear translocation. The N-terminal (EGFP–STAT6) fusion protein appeared to be more active than the C-terminal fusion. In HEK-293 cells both fusion proteins formed fluorescent nuclear foci following IL-4 stimulation, but in HeLa cells nuclear accumulation was homogeneous. Stimulation of the NF-κB pathway through TNFα treatment, or expression of p65–EGFP fusion protein, repressed both basal STAT6-dependent transcriptional activity and the extent of activation in response to IL-4. This indicates a novel mechanism of inhibition of STAT6 signalling by NF-κB activation.

Key words: STAT6; NF-κB; Confocal microscopy; Green fluorescent protein

1. Introduction

STAT6, the signal transducer and activator of transcription protein, is a critical component in one of two pathways that mediate signalling from the interleukin 4 (IL-4) receptor [1]. Through STAT6, IL-4 regulates MHC class II, CD23 and IL-4Rα expression [2,3], B cell isotype switching [4], and T helper cell differentiation to the Th2 phenotype [5,6]. Thus, STAT6 plays a pivotal role in allergic conditions such as asthma, rhinitis, and a variety of immune disorders such as type 1 diabetes, arthritis and enterocolitis.

On binding to its receptor, IL-4 activates the IL-4R-associated janus kinases, JAK1 and JAK3. These tyrosine kinases phosphorylate the receptor itself, generating a site to which STAT6 can bind. The JAKs in turn phosphorylate STAT6 [7,8], allowing the STAT6 molecule to be released. The phosphorylated STAT6 molecules form dimers, which then translocate to the nucleus and bind to GAS-like elements containing the consensus sequence $TTCN_4GAA$. Other STAT's recognise the similar consensus sequence $TTCN_3GAA$, but not N_4 [9–11].

We have applied time-lapse fluorescence microscopy [12] and novel GFP–STAT6 fluorescent fusion proteins to track the dynamics of the movement of this transcription factor in single living cells. Both N- and C-terminal fusions retained STAT6 function as determined by protein translocation and transcriptional activation. We also show for the first time that activation of the tumour necrosis factor α (TNFα)/nuclear factor κB (NF-κB) pathway inhibits the IL-4/STAT6 signalling pathway.

2. Materials and methods

2.1. Materials

Human recombinant TNFα and human recombinant IL-4 were from Calbiochem (UK), tissue culture medium was from Gibco Life Technologies (UK) and foetal calf serum (FCS) from Harlan Seralab (UK). Other chemicals were supplied by Sigma (UK).

2.2. Plasmids

The pC/EBP–STAT6luc reporter (obtained from Dr M. Varga and Dr L. Severinsson, AstraZeneca R and D Lund, Sweden) contains four GAS enhancer elements of the sequence $TTCN_4GAA$, recognised by STAT6, and additionally a C/EBP-binding site, all located upstream of the TATA box, controlling expression of firefly luciferase for production of the STAT6 reporter. All expression plasmids are under the control of the human cytomegalovirus immediate early (hCMV-IE) promoter. The construct pEGFP–STAT6, expressing an N-terminal fusion-protein between human STAT6 and enhanced green fluorescent protein (EGFP), was constructed by subcloning the STAT6 cDNA sequence from pCRscript–STAT6 into pEGFP–C2 (Clontech, UK). pCRscript–STAT6 was digested with *Not*I and pEGFP–C2 with *Kpn*I. The linearised vectors were blunt-ended with T4 DNA polymerase and digested with *Eco*RI. This released a 2.6-kb fragment (STAT6 cDNA), which was then ligated into the linearised pEGFP–C2. The plasmid expressing a C-terminal fusion of STAT6 to EGFP (pSTAT6–EGFP) was constructed by PCR-amplifying the STAT6 sequence without the stop codon from pEGFP–STAT6 followed by directional cloning into the *Eco*RI and *Kpn*I sites of pEGFP–N1 (Clontech). p65–EGFP was described previously [12].

2.3. Cell culture and transfection

HEK-293 (ECAAC No. 85120602) were grown in minimum essential medium (MEM) with Earle's salts, 10% FCS, 1% non-essential amino acids (NEAA) and 2 mM L-glutamate at 37°C, 5% CO_2. For confocal microscopy, cells were plated onto 35-mm Mattek dishes (Mattek, USA) at 1.6×10^5 cells in 2 ml of medium. HeLa cells (ECACC No. 93021013) were grown in MEM with Earle's salts, plus 10% FCS, and 1% NEAA at 37°C, 5% CO_2. For confocal microscopy, cells were plated on 35-mm Mattek dishes at 4×10^4 cells in 2 ml medium. After 24 h, cells were transfected using Fugene 6 (Boehringer, Mannheim, Germany). The ratio of DNA:Fugene 6

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Abbreviations: STAT6, signal transducer and activator of transcription protein 6; NF-κB, nuclear factor κB; JAK, janus kinase; IL-4, interleukin 4; TNFα, tumour necrosis factor α; EGFP, enhanced green fluorescent protein

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for all transfections was 1μg DNA:2 μl Fugene 6. For 24-well microtitre plate-based (Sarstedt, UK) luminescence assays from the pC/EBP–STAT6 luciferase reporter plasmid, HEK-293 cells were seeded at 3×10^4 cells per well and HeLa cells at 1×10^4 per well in 1 ml medium/well. Cells were transfected 28 h later using Fugene 6 at 0.25μg DNA plus 0.5μl Fugene 6 per well. In transfections with pEGFP–N1, pEGFP–STAT6 or pSTAT6–EGFP, the vectors were diluted 1:10 with pC/EBP–STAT6 luciferase reporter (i.e. 0.1 μg EGFP vector with 0.9 μg reporter). p65–EGFP plasmid was diluted 1:10 with EGFP–N1 prior to its subsequent 1:10 dilution with the reporter. (This 1:100 dilution was required because higher concentrations led to a high basal activation [12], with a resultant masking of a TNFα-stimulated response.)

2.4. Reporter luminescence assays

Cells were plated in 24-well microtitre plates as described above. Transfected cells were treated for 6 h prior to harvesting with 250 μl lysis buffer per well [13]. Each well was assayed in duplicate by transferring 100 μl of lysate into white 96-well plates (Greiner, UK), and ATP was added to a final concentration of 1.25 mM. Luminescence was measured using a BMG Lumistar platereader fitted with an injector (BMG Labtechnologies, UK), and 100 μl of 1 mM luciferin (Biosynth AG, Switzerland) was injected into each well. Experiments were performed in triplicate.

2.5. Fluorescence microscopy

Confocal microscopy was carried out on transfected cells in Mattek dishes in a Zeiss XL humidified CO_2 incubator (at 37°C, 5% CO_2) using a Zeiss LSM510 Axiovert microscope with a 40× phase contrast oil immersion objective (N.A. = 1.3) when visualising HeLa cells, and a 63× objective (N.A. = 1.4) when visualising HEK-293 cells. Excitation of EGFP was performed using an Argon ion laser at 488 nm. Emitted light was reflected through a 505–550-nm bandpass filter from a 540-nm dichroic mirror. Data capture and extraction was carried out with LSM510 version 3 software (Zeiss, Germany). For the EGFP 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 $t = 0$ min. For visualisation of the EGFP–STAT6 foci in the nuclei of HEK-293 cells (Fig. 2C) a stack of 40 images of 3-μm slices with an interval of 0.2 μm was deconvolved using a calculated point spread function, and then rendered with the transparency function of LSM510 software.

3. Results

3.1. Characterisation of the functional activity of N- and C-terminal EGFP–STAT6 fusion proteins in HEK-293 cells

To investigate the dynamics of STAT6 protein translocation, we constructed vectors expressing both C- and N- terminal fusion proteins between STAT6 and EGFP. To characterise the functional activity of the expressed proteins, an IL-4 dose–response curve was obtained from HEK293 cells transfected with each STAT6 construct (or an EGFP–N1 control) together with a STAT6 luciferase reporter construct (Fig. 1). As previously reported [11], these data demonstrate that the HEK-293 cells are STAT6 deficient, as no response to IL-4 was observed when the cells were transfected with EGFP–N1 alone. Both the N- and C-terminal fusion proteins were able to mediate significant IL-4-dependent transcriptional activation of the reporter construct. However, the EC_{50} to IL-4 differed significantly between the cells expressing the two fusion proteins (EGFP–STAT6 20.98 ± 1.79 pg/ml; STAT6–EGFP 12.87 ± 1.43 pg/ml) and the N-terminal EGFP–STAT6 fusion protein had a higher efficacy, with a 7.03 ± 0.14 ($P < 0.001$)-fold induction of the STAT6 reporter compared to a 5.23 ± 0.11-fold induction in cells overexpressing the C-terminal STAT6–EGFP fusion protein.

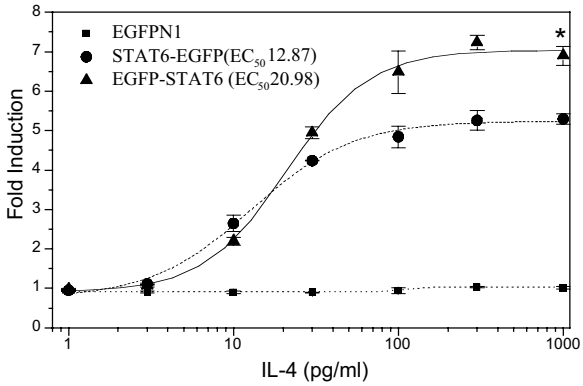

Fig. 1. Dose–response curves for IL-4 in HEK-293 cells expressing STAT6 fluorescent fusion proteins. HEK-293 cells were co-transfected with a C/EBP–STAT6 luciferase reporter, and either the EGFP–N1 control EGFP expression vector, the N- (EGFP–STAT6) or C-terminal (STAT6–EGFP) fusion protein expression vectors. Cells were stimulated with IL-4 24 h following transfection, and assayed 18 h later. (*$P < 0.01$, Student's t-test). Each data point represents mean values ± S.E.M. from triplicate experiments.

3.2. Imaging of the dynamic localisation of EGFP–STAT6 fluorescent proteins in IL-4-stimulated HEK293 cells

The dynamics of protein localisation following IL-4 stimulation were determined by confocal microscopy (Fig. 2). In unstimulated HEK-293 cells, both fluorescent fusion proteins were evenly distributed throughout the cells (Fig. 2A,D). However, upon stimulation with IL-4 (10 ng/ml), both the STAT6 fusion proteins were observed to form foci in the nuclei. Typically, the number of foci increased over time (Fig. 2D–F), stabilising in number at around 60–80 min, with approximately 50 foci per nucleus (Fig. 2C). The formation of foci was evident in the majority of cells and occurred with both fluorescent fusion proteins.

3.3. Characterisation of the function of the STAT6-fluorescent fusion proteins in HeLa cells which express functional endogenous STAT6

Given that the nuclear translocation of the STAT6 constructs in the HEK-293 cells was difficult to quantify (due to the uneven nuclear distribution and the dynamic three-dimensional movement of the foci), we also investigated the activity of the fusion proteins in other cells. Significant homogeneous nuclear translocation of both fusion proteins was observed in HeLa cells in response to IL-4 (Fig. 2G–J). Control transfections with EGFP–N1 revealed no nuclear translocation in response to IL-4 (data not shown).

Unlike HEK-293 cells, HeLa cells express functional levels of endogenous STAT6. We therefore compared the IL-4 responses of GFP-expressing (control) cells with those expressing the seemingly more active N-terminal EGFP–STAT6 fusion protein. A dose–response curve to IL-4 (Fig. 3) obtained from cells transfected with the empty expression vector EGFP–N1 revealed an EC_{50} of 73.02 ± 2.63 pg/ml, and a maximal-fold induction of the STAT6 luciferase reporter of 3.58 ± 0.08. In contrast, when EGFP–STAT6 was overexpressed there was an approximate six-fold increase in the response to IL-4-induced transcription, with an EC_{50} of 11.40 ± 2.85, and an accompanying augmentation in transcriptional activity to 4.82 ± 0.17-fold induction of the STAT6 reporter.

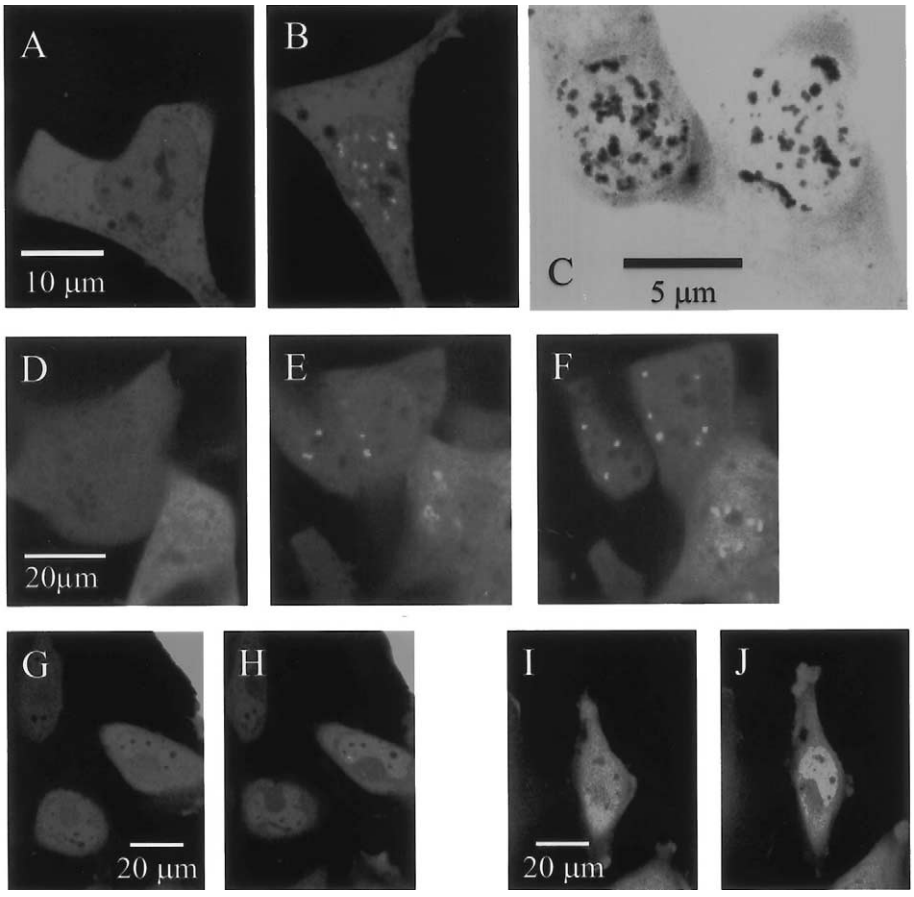

Fig. 2. Nuclear translocation of STAT6 fluorescent fusion proteins in HEK-293 and HeLa cells in response to stimulation with 10 ng/ml IL-4. A–C: HEK-293 cells expressing N- terminal EGFP–STAT6. A: Single confocal image before stimulation with IL-4. B: 80 min after stimulation with IL-4. C: Assembled stack of confocal slices showing typical numbers of nuclear fluorescent foci 80 min after IL-4 stimulation. D–F: Confocal images of HEK 293 cells expressing C-terminal STAT6–EGFP before stimulation (D), 40 min after stimulation (E) and 80 min after stimulation with 10 ng/ml IL-4 (F). G–J: HeLa cells expressing N-terminal EGFP–STAT6 (G,H) or C-terminal STAT6–EGFP (I,J) before (G,I) and 80 min after (H,J) stimulation with IL-4.

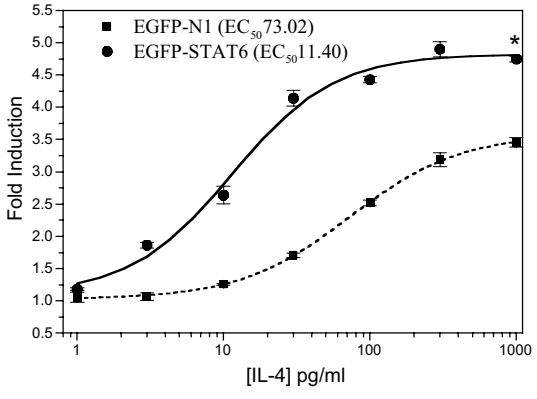

Fig. 3. Dose–response to IL-4 in HeLa cells expressing the EGFP–STAT6 fusion protein. HeLa cells were co-transfected with a C/EBP–STAT6 luciferase reporter, and either the EGFP expression vector, or the N-terminal EGFP–STAT6 expression vector. Cells were stimulated with IL-4 24 h following transfection, and assayed 18 h later. (*$P < 0.01$, Student's t-test). Each data point represents mean values ± S.E.M. from triplicate experiments.

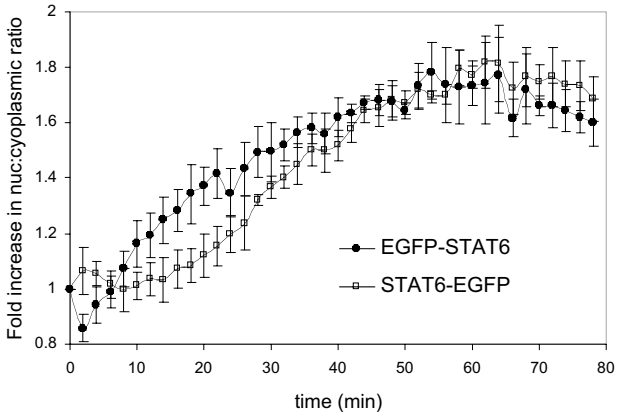

Fig. 4. Rate of nuclear translocation of STAT6 fluorescent fusion proteins in HeLa cells following IL-4 stimulation. HeLa cells expressing either N-terminal EGFP–STAT6 or C-terminal STAT6–EGFP were stimulated with 10 ng/ml IL-4 and imaged by confocal microscopy over an 80 min period. The images were analysed to establish the nuclear:cytoplasmic ratio and its relative change following stimulation is shown. These data represent the mean of three experiments (≥ 4 cells per experiment). $P < 0.05$ (Student's t-test) when comparing 60 min time-point to start for each fusion protein. Bars represent S.E.M.

The extent and timing of translocation in HeLa cells in response to IL-4 was determined. The degree of translocation (at 60 min following stimulation with 10 ng/ml IL-4) was similar for cells expressing both fusion proteins, with a 1.73 ± 0.07-fold increase in the nuclear:cytoplasmic ratio of the N-terminal fusion compared to 1.77 ± 0.05 for the C-terminal fusion (Fig. 4). When comparing the mean of cells taken at 2 min and 60 min following stimulation with IL-4, in both instances the degree of translocation was significant (N-terminal EGFP–STAT6, 2 min vs 60 min, $P < 0.001$; C-terminal STAT6–EGFP, $P < 0.05$). Of the two fusions the N-terminal fusion protein appeared to respond slightly faster following stimulation with IL-4 (10 ng/ml) than the C-terminal fusion, though both constructs reached maximal translocation at approximately the same time.

3.4. p65/NF-κB inhibits the STAT6 signalling pathway

We investigated the effect of the NF-κB signalling pathway on N-terminal EGFP–STAT6 fusion protein-mediated signalling by IL-4 (Fig. 5). The transcriptional activity of the STAT6 reporter was measured in HEK-293 cells overexpressing EGFP–STAT6 and a control EGFP expression vector. Treatment of cells with 100 pg/ml TNFα decreased the transcriptional activity of the STAT6 reporter compared to untreated cells. The activity of the STAT6 reporter following IL-4 treatment alone resulted in a 4.18 ± 0.07-fold induction of the STAT6 reporter. This was significantly inhibited (to 2.1 ± 0.01-fold induction, $P < 0.05$) by pre-treatment of cells with 100 pg/ml TNFα 20 min prior to treatment with 10 pg/ml IL-4.

In order to investigate whether this effect was NF-κB-mediated, we co-transfected a functional p65–EGFP expressing vector [12], together with EGFP–STAT6, into HEK-293 cells. The expression of p65–EGFP significantly decreased the basal activity of the STAT6 reporter 1.9-fold ($P = 0.014$, $n = 3$) compared to expression of EGFP control (data not shown). Treatment with TNFα alone slightly repressed basal STAT6 reporter activity under the conditions of overexpressed p65 and STAT6 (Fig. 5). In agreement with the TNFα-treated cells without p65–EGFP, we saw significant inhibition of the IL-4-induced reporter activity in cells overexpressing p65, with IL-4 stimulating a 1.81 ± 0.03-fold induction over basal conditions. No significant further attenuation of the IL-4 response was observed in p65 overexpressing cells pre-treated with TNFα, perhaps reflecting that p65 expression already gave maximal NF-κB stimulation (and hence STAT6 inhibition) [12].

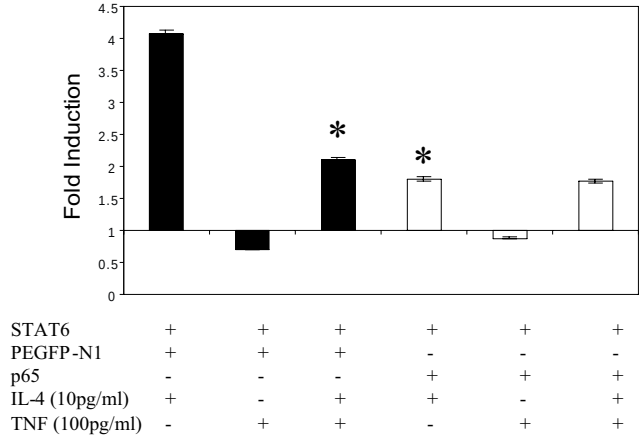

Fig. 5. The effect of NF-κB activation on STAT6 signalling. HEK-293 cells were transfected with pC/EBP-STAT6 luciferase reporter together with pEGFP–STAT6 and pEGFP–N1 (darker bars), or pEGFP–STAT6 and p65–EGFP (clear bars). The cells were treated with 100 pg/ml TNFα and/or 10 ng/ml IL-4, with TNFα treatments (or a control) being performed 20 min prior to stimulation with IL-4 (*$P < 0.05$ (5% LSD) cf. IL-4 stimulation in pEGFP–N1 expressing cells). Each data point represents mean values ± S.E.M. from triplicate experiments.

4. Discussion

The aim of this study was to quantitatively track the regulation of IL-4-mediated gene expression. Expression of both N- and C-terminal STAT6-fluorescent fusion proteins demonstrated their functional activity through IL-4-dependent transcriptional activity and nuclear translocation. There was a significant difference between the EC_{50}s for IL-4 (as determined from transcriptional activity) for the two fusion proteins. The N-terminal EGFP–STAT6 also conferred a higher stimulation by IL-4 compared to the C-terminal STAT6–EGFP. The highly conserved N-terminal of STAT6 has been shown to be responsible for oligomerisation [14], so facilitating co-operative DNA binding on promoters containing multiple potential STAT recognition sites. The more critical domains, for STAT6 activation through dimerisation (SH2 domain) and transcriptional activation, are located at the C-terminal end. The C-terminally located EGFP could therefore be encumbering receptor docking and/or phosphorylated STAT6 dimerisation either through an effect on the SH2 domain, or alternatively through damping of transcription activation. The C-terminal EGFP did not significantly affect the extent of nuclear translocation of the fusion protein in HeLa cells, perhaps supporting the conclusion that the inhibitory affects on transcriptional activation may not be via inhibition of docking and dimerisation. Thus, an effect of the C-terminal fusion on the activation of transcription seems more likely, particularly since the transcription activation domain has the closer proximity to the EGFP.

In HEK-293 cells, IL-4-stimulated fluorescent protein foci formation in the nucleus. This phenomenon was previously demonstrated by immunocytochemistry in bovine aortic endothelial cells, and was also seen to occur with activated STAT1 [15]. One possible explanation is that these foci represent stores for excess transcription factor. The fact that the nuclear foci can occur in certain cell types with either native STAT6 protein or exogenous fluorescent STAT6 protein expression suggests that they may have a functional significance.

When studying the transcriptional activity of the EGFP–STAT6 in HeLa cells, it was observed that expression of the fusion protein resulted in a significant shift in both EC_{50} and efficacy compared to that obtained with untransfected cells. This difference in EC_{50} might be explained by overexpression of STAT6, resulting in a basal activity sufficient to drive the synthesis of new IL-4 receptors [16]. This might increase the number of receptors per cell so that lower doses of IL-4 could signal transcriptional activation. The increase in maximal transcriptional induction through STAT6-expression could occur if the normal signalling pathway did not require acti-

vation of all DNA-binding sites to achieve the required IL-4 response. This leaves the potential for additional activation to occur during cellular events that require enhancement of the IL-4 signalling pathway [17–19]. Increased expression of STAT6 might utilise this extra capacity for activation of transcription.

There is considerable evidence for the interaction of STAT6 and the p50 NF-κB subunit when their respective pathways are activated [18,20–22]. Our results indicate a previously unreported functional interaction between the p65 and STAT6 transcription factors. Pre-stimulation of HEK-293 cells with TNFα attenuated IL-4-induced transcription of the STAT6 luciferase reporter. This observation was supported by the fact that in the absence of IL-4 stimulation TNFα reduced basal transcription in the presence of overexpressed STAT6. This suggests a specific action of the TNFα pathway on STAT6. Expression of the p65 subunit, which is the most common partner to p50 in the activated form of NF-κB [23], significantly inhibited IL-4-induced transcription. Pre-treatment with TNFα was unable to further attenuate the IL-4 response, most likely because the overexpression of p65 saturated the TNF-signalling pathway [12].

It has previously been suggested that the NF-κB subunits p65 and p50 could interact with STAT6 in vitro [18]. However, in vivo only p50 could be co-immunopreciptated with STAT6 in nuclear extracts of cells stimulated with IL-4 [17]. Our observation of an inhibitory effect by the TNFα/p65NF-κB pathway on IL-4 signalling is previously unreported. Most studies have focused on the influence of the IL-4 pathway on the TNFα/NF-κB pathway. These studies suggest that IL-4, through STAT6, inhibits TNFα-stimulated transcription [20,22,24,25]. Those studies in which the TNFα activation was assessed through promoter activity were performed used natural promoters [17] which contained multiple different transcription factor binding sites. Our study has utilised a synthetic STAT6 promoter and therefore indicates that the inhibitory action of p65 on the IL-4-induced STAT6 reporter activity is likely to be through a direct functional interaction with STAT6.

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