Tumour necrosis factor- α and interferon- γ synergistically activate the RANTES promoter through nuclear factor κ B and interferon regulatory factor 1 (IRF-1) transcription factors

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Inflammatory cytokines such as tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) synergistically activate expression of the *RANTES* (regulated upon activation, normal T-cell expressed and secreted) gene, which plays a crucial role in the chemoattraction of leukocytes during the inflammatory response. To understand at the molecular level the mechanism by which the two cytokines activate *RANTES* gene expression, we determined the requirement of *cis*-acting elements in the *RANTES* promoter and *trans*-acting factors. The murine *RANTES* promoter contained one putative interferon regulatory factor, IRF, and three putative nuclear factor κ B (NF- κ B) binding sites. Specific destruction of the IRF binding site and one of the three NF- κ B binding sites abolished the inducibility of promoter activity by IFN- γ and TNF- α , respectively. In contrast, mutation of the other two putative NF- κ B binding sites did not affect *RANTES*

promoter activity significantly. In addition, the *RANTES* promoter was stimulated by co-transfection of plasmids that expressed either p65, an NF- κ B family protein, or the IRF-1 transcription factor. *RANTES* promoters with mutations in the NF- κ B or IRF binding sites were not stimulated by p65 or IRF-1 expression, respectively. In electrophoretic mobility-shift and immunologic assays, we showed that IRF-1 was induced after cells were treated with IFN- γ and that NF- κ B was activated by TNF- α treatment. These results demonstrate that both NF- κ B and IRF-1 transcription factors mediate the induction of *RANTES* expression via their cognate *cis*-acting elements when cells are stimulated by TNF- α and IFN- γ .

Key words: chemokine, gene expression regulation, NF- κ B, promoter analysis.

INTRODUCTION

RANTES (regulated upon activation, normal T-cell expressed and secreted) is a member of the chemokine family of proteins, which plays an essential role in inflammation by recruiting T cells, macrophages and eosinophils to inflammatory sites [1–3]. Circulating leukocytes are supposed to bind *RANTES* produced in inflamed tissues and eventually migrate into the inflammatory sites through chemotaxis and haptotaxis [4]. *RANTES* has been implicated in a number of diseases characterized by inflammation. These include asthma [5], transplantation rejection [6], atherosclerosis [7], rheumatoid arthritis [8], delayed-type hypersensitivity [9], endometriosis [10], chronic polypus sinusitis [11] and respiratory syncytial virus-related bronchiolitis [12,13]. *RANTES* expression is induced in a variety of cells including fibroblasts [8], epithelial [14–16] and endothelial [9,17] cells, mesangial cells [18], activated T cells [19] and macrophages [8,20]. Cytokines produced during acute inflammation induce *RANTES* expression by fibroblasts and epithelial and endothelial cells [8,14–17]. For example, tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) increase the expression of *RANTES* mRNA by fibroblasts and endothelial cells [8,14,15]. Furthermore, TNF- α and interferon- γ (IFN- γ) were shown to induce *RANTES* production synergistically by epithelial and endothelial cells [16,17].

It has been demonstrated that *RANTES* promoter contains a large number of potential consensus elements for transcription

factors [19,21,21a]. The region R(A/B) of the human *RANTES* promoter, which contains two nuclear factor κ B (NF- κ B)-like sites, was shown to bind not only Rel family members, but also non-Rel factors, which were specifically up-regulated 3–5 days after activation of resting T cells [22]. It was shown that the R(E) site in the human *RANTES* promoter interacted with nuclear factor interleukin 6, and that the R(C) site bound a novel factor that was up-regulated between days 3 and 5 of T-cell activation [23]. Therefore, it was suggested that these transcription factors would participate in the developmentally controlled expression of *RANTES* in T lymphocytes [22,23]. In order to decipher the molecular mechanism by which the *RANTES* gene is activated by TNF- α and IFN- γ , we investigated the requirements for activation of murine *RANTES* gene expression. Upon examination of the murine *RANTES* promoter, we noticed that it contains a single interferon regulatory factor (IRF) and three putative NF- κ B binding sites, each of which is well conserved in the human *RANTES* promoter. Among the three putative NF- κ B binding sites, two were in the region homologous to the R(A/B) site of the human *RANTES* promoter. This raised the possibility that interactions between NF- κ B and IRF and their cognate binding sites are crucial for the induction of *RANTES* promoter activity.

The NF- κ B-family transcription factor is composed of two DNA-binding subunits, one from the p50 family and one from the p65 family of transcription factors [24]. In resting cells, NF- κ B is sequestered in the cytoplasm as a complex with an inhibitory

Abbreviations used: TNF- α , tumour necrosis factor- α ; IL-1 β , interleukin-1 β ; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; *RANTES*, regulated upon activation, normal T-cell expressed and secreted; NF- κ B, nuclear factor κ B; IRF, interferon regulatory factor; κ B, inhibitory κ B; EMSA, electrophoretic mobility-shift assay.

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protein, inhibitory κ B, or $I\kappa$ B. Multiple stimuli, including inflammatory cytokines, induce phosphorylation and degradation of $I\kappa$ B, which allows the release of NF- κ B from the inhibitory complex. NF- κ B then translocates into the nucleus and binds to promoters that contain the GGGRNYYYCC consensus sequence [25]. The IRF family includes a number of transcription factors that share a strong homology in the N-terminal 115 amino acids [26]. This homologous region contains a DNA-binding domain that recognizes the consensus sequence G(A)AAASYGAAASY [27]. IRF-1 was shown to function as a transcriptional activator, whereas IRF-2 antagonizes the function of IRF-1 [28,29]. The IRF-1 gene is induced by a number of stimuli that include IFN- α , IFN- γ , TNF- α and lipopolysaccharide (LPS) [30,31,31a]. Recent findings suggest that virus-induced activation of the IFN- β gene may be mediated by a third IRF family member IRF-3 [32,33].

In this report, we analysed systematically in NIH 3T3 fibroblast cells the *cis*-acting elements in the murine RANTES promoter that are critical for promoter induction by TNF- α and IFN- γ . We also present evidence that both NF- κ B and IRF-1 transcription factors participate in the induction of the RANTES promoter in response to TNF- α and IFN- γ treatment.

EXPERIMENTAL PROCEDURES

Cells and cytokines

NIH 3T3 fibroblastic cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Gibco-BRL, Grand Island, NY, U.S.A.). Recombinant murine TNF- α (1×10^7 units/mg) and IFN- γ (1×10^7 units/mg) were purchased from Endogen (Woburn, MA, U.S.A.) and PBL Biomedical Lab (New Brunswick, NJ, U.S.A.), respectively. Cells were stimulated with TNF- α and IFN- γ at final concentrations of 100 units/ml and 200 units/ml in complete media, respectively.

Plasmid constructs

A fragment of the murine RANTES promoter that extends from -979 to +8 (with the transcription start site being position +1) was amplified using PCR with two primers, -979-S (5'-AAGGATCCTCCTGTGCCACCA-3', with the *Bam*HI restriction site underlined) and +8-A (5'-GGGAAGCTTGCAAGGGGTGCTCTGCA-3', *Hind*III site underlined). The amplified fragment was digested with *Bam*HI and *Hind*III and cloned into the pGL3-Basic vector (Promega, Madison, WI, U.S.A.) between the *Bgl*II and *Hind*III sites to obtain p-979 (this number and those of plasmids described below indicate the distal nucleotide position from the transcription start site of the RANTES promoter). The plasmid p-271 was constructed by digesting p-979 with *Xho*I and *Bgl*II, filling-in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and self-religation. The plasmids p-115, p-74 and p-40 were constructed by inserting RANTES promoter fragments (generated by PCR amplification with the appropriate primer pairs) that extended from each 5' end (-115, -74 and -40) to +8 into the pGL3-Basic vector between the *Bgl*II and *Hind*III, as described for p-979.

p Δ κ B1, p Δ κ B2, p Δ κ B3, p Δ IRF and p Δ κ B2/ Δ IRF were derived from p-271 by site-directed mutagenesis using a PCR-based megaprimer method [34]. Briefly, megaprimers were generated by PCR amplification using +8-A as the 3' primer and the following 5' primers: p Δ κ B1, 5'-GAGTCATACTTCCAAtctTGATTTTCAGTTTTCTT-3' (-175/-142); p Δ κ B2, 5'-GTCTT-TGTGGAAACTagaCAAGTCCTGGGGCT-3' (-97/-65);

p Δ κ B3, 5'-CCTGGGGCTGGGGGGGttaACTTCCTCTGCT-AC-3' (-73/-41); and p Δ IRF, 5'-CAAGGGTGATTTTCAG-gagTCTTTTCCATTTTGT-3' (-163/-131). The numbers in parentheses indicate the starting and finishing nucleotide positions in the murine RANTES promoter with respect to the transcription start site and nucleotides in lower case denote mismatched sequences. A second round of PCR amplification was performed with the RV3 primer (Promega) and each megaprimer described above. Finally, the resulting PCR products were digested with *Xho*I and *Hind*III and then ligated into pGL3-Basic that had been cleaved with the same restriction enzymes.

CMV-p50, CMV-p65 and CMV- $I\kappa$ B were provided by Dr J. Lee (Chonnam University, Kwangjo, South Korea). pIRF-1 (originally designated pCMIRS) [30] was a gift from Dr T. Taniguchi (Tokyo University, Tokyo, Japan).

Transfection and reporter-gene assays

Cells were transfected by a modified calcium phosphate method [35]. Briefly, cells (1×10^5) that had been aliquoted into a 35-mm dish the day before were incubated for 10 h with a transfection cocktail containing 1 μ g each of the reporter and pSV- β -gal plasmids. For the co-transfection experiments, various amounts of plasmids were used as indicated in the Figure legends. After transfection, the cells were washed and then refed with complete media. After 38 h of incubation in complete media, the cells were stimulated with cytokines for 8 h and harvested for the luciferase and β -galactosidase assays. Luciferase assays were performed according to the manufacturer's instructions (Promega). To determine transfection efficiencies, β -galactosidase assays were carried out with the use of a chemiluminescent reporter-gene assay system (Tropix, Bedford, MA, U.S.A.).

Nuclear extracts

Nuclear extracts were prepared from 2×10^6 NIH 3T3 cells, as described previously [36]. Cells were washed twice in ice-cold PBS, harvested by scraping, and resuspended in 400 μ l of ice-cold buffer A (10 mM Hepes/NaOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 μ g/ml leupeptin and 1 μ g/ml aprotinin). After 15 min of incubation on ice, cells were lysed by the addition of Nonidet P40 (25 μ l; final concentration, 0.625%, v/v) and immediate vortexing for 10 s. Nuclei were harvested by centrifugation and resuspended in 50 μ l of ice-cold buffer C (20 mM Hepes/NaOH, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.2 mM PMSF, 1 μ g/ml leupeptin and 1 μ g/ml aprotinin). After a 20-min incubation at 4 °C on a shaking platform, nuclei were clarified by centrifugation at 25000 *g* for 5 min. The resulting nuclear extracts were stored at -80 °C.

Electrophoretic mobility-shift assays (EMSAs)

Double-stranded κ B2 and IRF DNA probes (containing the NF- κ B and IRF binding sites, respectively) were prepared by annealing the following complementary oligonucleotide pairs: κ B2, 5'-TTTGTGGAAACTCCCCAAG-3' and 5'-GGCTTGGGGAGTTTCCACAAA-3'; and IRF, 5'-TGGGTGATTTCA-GTTTTCTTTTCCATTT-3' and 5'-AAATGGAAAAGAAAACTGAAATCA-3'. The annealed probes were end-labelled with [α -³²P]dCTP and the Klenow fragment, and passed through a Sephadex G25 spun column to remove unincorporated nucleotides. The protein-DNA binding reaction mixtures (15 μ l) contained 5 μ g of nuclear protein extract and 20 fmol of labelled probe in a binding buffer [10 mM Tris/HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 50 μ g/ml poly(dI-dC).

poly(dI-dC); Pharmacia, Piscataway, NJ, U.S.A.]. Reactions were carried out at room temperature for 15 min. In some experiments, nuclear extracts were preincubated with antisera for 15 min at room temperature prior to adding the labelled probes. Rabbit polyclonal antibodies against p65 and murine IRF-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Reaction mixtures were electrophoresed through a native 6% polyacrylamide gel in $0.5 \times$ TBE (45 mM Tris/borate/1 mM EDTA). The gels were dried and exposed to X-ray film.

RESULTS AND DISCUSSION

Localization of RANTES promoter elements required for induction by TNF- α and IFN- γ

To determine whether TNF- α and IFN- γ activate the murine RANTES promoter, a fragment of the promoter region extending from -979 to +8 was fused to a luciferase reporter gene (Figure 1A). Basal promoter activity was observed when the resulting p-979 plasmid was transfected alone into NIH 3T3 cells. The activity increased about 3-fold when the transfected cells were treated with either TNF- α or IFN- γ . Treatment of p-979-transfected cells with both TNF- α and IFN- γ resulted in a dramatic increase (24-fold) of luciferase activity, demonstrating that the two cytokines stimulate synergistically the murine RANTES promoter. Deletion of RANTES promoter sequences up to -271 (p-271) from the transcription start site reduced moderately ($\approx$ 2.5-fold) both basal promoter activity and TNF- α - and IFN- γ -induced RANTES promoter activity. Nonetheless, synergistic induction by TNF- α and IFN- γ of p-271 was observed, indicating that essential promoter elements that mediate TNF- α and IFN- γ signals exist within the proximal 271-bp region of the RANTES promoter. A further deletion of the promoter to -115 (p-115) abolished completely induction of the reporter gene by either TNF- α or IFN- γ , or both (Figure 1A).

Within the proximal 271-bp region of the RANTES promoter, we noticed that one IRF and three putative NF- κ B binding sites existed, and probably mediated the TNF- α and IFN- γ induction, respectively, that we observed in the transfection experiments. In order to test this possibility, we altered the putative binding sites individually or in combination by site-directed mutagenesis of p-271, transfected 3T3 cells with the mutant constructs, and tested for their ability to respond to TNF- α and IFN- γ treatment (Figure 1B). Destruction of the putative NF- κ B binding site, designated NF- κ B1 (5'-GGGTGATTTC-3', -160 to -151; p Δ KB1), increased the overall promoter strength ($\approx$ 3-fold), but did not change the stimulation pattern by TNF- α and IFN- γ . Similarly, alteration of NF- κ B3 (5'-GGGCACTTCC-3', -58 to -49; p Δ KB3) had little effect on the synergistic induction pattern of the RANTES promoter by TNF- α and IFN- γ , although the overall promoter activity was reduced slightly ($\approx$ 0.7-fold) compared with the control plasmid (p-271). In strong contrast, destruction of the NF- κ B2 binding site (5'-GGGAGTTTCC-3', -79 to -87; p Δ KB2), which is more similar to well-characterized NF- κ B consensus sequences than are NF- κ B1 and NF- κ B3, abolished completely promoter induction by TNF- α , but not by IFN- γ (Figure 1B). The plasmid p Δ IRF, which carries a mutation in the putative IRF binding site (5'-AGTTTCTTTTCC-3', -150 to -138), displayed stimulation of reporter-gene expression by TNF- α at a level comparable with that observed for the control plasmid (p-271), but was not stimulated by IFN- γ . Plasmid p Δ KB2/IRF, in which both the NF- κ B2 and IRF binding sites were mutated, did not respond to either TNF- α or IFN- γ treatment. Taken together, these results suggest that the NF- κ B2

and IRF binding sites mediate the TNF- α and IFN- γ signal for synergistic activation of the murine RANTES promoter.

NF- κ B and IRF-1 transcription factors activate RANTES promoter activity

To determine whether NF- κ B-family proteins regulate RANTES promoter activity, p-271 was co-transfected with plasmids that contained genes for p50, p65 or I κ B α (a member of the I κ B family of proteins), each alone or in combination (Figure 2A). Interestingly, expression of p65 alone stimulated greatly (> 10 -fold) the RANTES promoter over the vector-alone control (pCDM8). In contrast, p50 did not affect significantly RANTES promoter activity. Stimulation by p65, however, was inhibited when p65 was co-expressed with I κ B α (Figure 2A). In addition, the expression of I κ B α alone reduced the expression level of p-271 below control levels. These results are consistent with reported data that the RANTES promoter was regulated by NF- κ B proteins [22]. We also observed that p Δ KB2, which contained a mutation in the NF- κ B2 site, was not stimulated by p65 (Figure 2B). In contrast, p Δ IRF, which is devoid of the IRF binding site, did not lose the ability to be stimulated by p65 (Figure 2B). These results confirm that NF- κ B-family proteins do indeed regulate RANTES promoter activity.

Mutational analyses showed that a putative IRF site was also required for maximal induction of the murine RANTES promoter. Moreover, it has been shown that IFN- γ induces various members of the IRF family of transcription factors, including IRF-1, IRF-2, p48 and interferon-consensus-sequence-binding protein (ICSBP) [37]. IRF-1 is induced strongly by IFN- γ and after induction participates in the stimulation of many IFN-specific genes [30,31]. To investigate the possibility that IRF-1 stimulates directly the murine RANTES promoter, transient-transfection assays were performed (Figure 3). IRF-1 strongly induced the p-271 promoter only when it contained an intact IRF site. When we used p Δ KB2, expression of the reporter gene was induced markedly in response to IRF-1 expression. However, p Δ IRF, which has a mutated IRF binding site, was hardly induced by the co-transfected IRF-1 gene. These results suggest that the IRF-1 transcription factor participates in the regulation of murine RANTES gene expression.

Because TNF- α and IFN- γ appeared to act synergistically to induce luciferase gene expression driven by the murine RANTES promoter (Figure 3), we investigated further whether p65 and IRF-1 showed synergistic effects on RANTES promoter activity. When p-271 was co-transfected with either a p65 or IRF-1 expression plasmid, the increases in luciferase activity were 12.5- and 22-fold, respectively (Figure 4). When both p65 and IRF-1 expression plasmids were co-transfected with p-271, a nearly 50-fold stimulation was observed. This is in keeping with synergistic stimulation of RANTES promoter activity observed when cells were treated with both TNF- α and IFN- γ . These results strongly support the idea that TNF- α and IFN- γ stimulate RANTES promoter activity through distinct signal-transduction pathways in which p65 and IRF-1 transcription factors are involved in a synergistic fashion.

Inducible nuclear proteins that bind to the NF- κ B2 and IRF DNA-binding sites

On the basis of the transient-transfection assays described above, we proposed that p65 and IRF-1 activate the murine RANTES promoter by binding directly to the NF- κ B2 and IRF binding sites, respectively. To test this hypothesis, we examined whether NF- κ B and IRF-1 are induced by treating cells with TNF- α and IFN- γ . To detect the presence of NF- κ B and IRF-1 in the crude

Figure 1 Transient-transfection analyses of wild-type and mutant RANTES promoter-luciferase constructs

(A) Cells were transfected with the indicated plasmids, which contained RANTES promoter regions with serial deletions from the 5' side, stimulated with various cytokines, and assayed for luciferase activity, as described in the Experimental procedures section. (B) Plasmids with point mutations in putative NF-κB and IRF binding sites were derived from p-271. Two or three bases in the consensus sequences were changed to destroy the binding sites, as described in the Experimental procedures section. For both panels, the values shown are averages of a minimum of three separate experiments with S. D. indicated by error bars; relative luciferase activity represents fold-induction with respect to the value obtained from cells transfected with p-271 without any stimulation.

nuclear extracts prepared from NIH 3T3 cells, we used EMSAs with labelled synthetic DNA probes containing either p65 or IRF-1 binding sites.

A non-specific nucleoprotein complex (Figure 5A, lane 2; indicated by a white arrowhead) was detected in the untreated control extracts. Nuclear extracts prepared from cells treated with TNF-α formed a distinct nucleoprotein complex with the

κB2 DNA probe (Figure 5A, lanes 3 and 6; indicated by the black arrowhead). The amount of complex formed was not increased significantly with prolonged treatment of cells with TNF-α (Figure 5A, lanes 3 and 6). In contrast, IFN-γ treatment did not result in the formation of a specific protein-DNA complex with the κB2 probe (Figure 5A, lanes 4 and 7). Co-treatment of cells with both TNF-α and IFN-γ induced the

Figure 2 The murine RANTES promoter is activated by p65

(A) Each aliquot containing 1×10^5 cells received $0.7 \mu\text{g}$ of p-271, $0.7 \mu\text{g}$ of pSV- β -gal and $0.35 \mu\text{g}$ of the indicated effector plasmids during transfection. The total amount of transfected plasmid was adjusted to $2.1 \mu\text{g}$ in each transfection by adding additional pCDM8. (B) p-271 and its derivatives were co-transfected with either control plasmid (pCDM8) or p65-expressing plasmid (CMV-p65) and pSV- β -gal. The amount of each plasmid used was $0.7 \mu\text{g}$ in each transfection experiment. The values of relative luciferase activity are as described in Figure 1.

Figure 3 IRF-1 activates the murine RANTES promoter

p-271 and its derivatives were co-transfected with either control plasmid (pCDM8) or an expression plasmid (pIRF-1) and pSV- β -gal. The amount of each plasmid used was $0.7 \mu\text{g}$ in each transfection. The values of relative luciferase activity are as described in Figure 1.

formation of a specific protein–DNA complex with similar results to those observed with TNF- α alone, excluding any co-operative effect (Figure 5A, lanes 5 and 8).

Formation of the TNF- α specific complex was highly specific to the sequence contained in the κ B2 probe, because the addition of 10- and 100-fold excesses of unlabelled κ B2 probe as a competitor inhibited complex formation (Figure 5B, lanes 3 and 4). In contrast, the addition of a 10- or 100-fold excess of unlabelled non-specific competitor did not affect the amounts of specific complex formed (Figure 5B, lanes 5 and 6). In order to obtain evidence that p65 is indeed present in the specific complex, we tested whether migration of the complex was affected by antibodies specific to p65. The addition of anti-p65 antiserum, but not control serum, resulted in a decrease in the mobility of the specific complex (Figure 5C), confirming that p65, and most probably a p50/p65 heterodimer, was responsible for the formation of this specific complex.

We performed an identical series of experiments using an IRF probe to examine whether IRF-1 is also induced upon treatment of cells with IFN- γ . We observed a strong non-specific band with

the IRF probe, whose formation was inhibited by the addition of non-specific competitor (Figure 6; indicated by white arrowheads). On the other hand, we were able to detect additional complex formed from nuclear extracts treated with IFN- γ (Figure 6A, lane 4; indicated by a black arrowhead). The induced complex was specific, as it was inhibited by the addition of unlabelled IRF probe to the binding reaction (Figure 6B, lanes 3 and 4), but not by non-specific competitor (Figure 6B, lanes 5 and 6). At the 1-h time point (treatment of cells with TNF- α and IFN- γ for 1 h prior to harvest), TNF- α and IFN- γ activated synergistically the formation of the specific complex (Figure 6A, lane 5). However, the synergistic effect was marginal after prolonged treatment (4 h) of cells with both cytokines (Figure 6A, lane 8). Incubation of the nuclear extracts with anti-IRF-1 antibodies prior to the addition of the labelled IRF probe prevented the formation of the specific protein–DNA complex, indicating that this complex contained IRF-1 (Figure 6C). Taken together, these results indicate that NF- κ B and IRF-1 are induced in response to TNF- α and IFN- γ , respectively, to activate the murine RANTES promoter.

Figure 4 p65 and IRF-1 activate synergistically the murine *RANTES* promoter

Each aliquot containing 1×10^5 cells received 0.7 μ g of p-271, 0.7 μ g of pSV- β -gal and 0.35 μ g of the indicated effector plasmids during transfection. The total amount of transfected plasmid was adjusted to 2.1 μ g in each transfection by adding the appropriate amount of pCDM8 plasmid. The values of relative luciferase activity are as described in Figure 1.

In this report, we analysed both *cis*- and *trans*-acting elements required for induction of the *RANTES* gene. We have presented evidence that the NF- κ B and IRF-1 transcription factors are induced upon treatment of cells with TNF- α and IFN- γ and activate *RANTES* gene expression through its promoter elements. Treatment of cells with TNF- α and IFN- γ resulted in a significant co-operative induction of IRF-1/DNA-binding activity after 1 h of treatment. Co-operative induction of IRF-1 by TNF- α and IFN- γ has been described previously [31,31a]. Moreover, Hauser and co-workers showed that IRF-1 could activate NF- κ B, which synergized with IRF-1 to induce the *IFN- β* gene [38]. It was also shown that NF- κ B and IRF-1 synergistically induced MHC class I [39], vascular cell-adhesion molecule 1 [40] and inducible nitric oxide synthase genes [41] through physical interaction between the two proteins. The previous results, together with our observation that NF- κ B and IRF-1 activated co-operatively the *RANTES* promoter in co-transfection experiments (Figure 4), suggest that synergistic induction of *RANTES* by TNF- α and IFN- γ is achieved in two separate steps. First, TNF- α and IFN- γ induce IRF-1 in a co-operative manner. Subsequently, both NF- κ B and IRF-1 activate the *RANTES* promoter, probably through forming a complex as demonstrated previously [41]. However, more vigorous biochemical experiments are needed to prove that NF- κ B and IRF-1 interact with each other. These would yield valuable information for the understanding of the precise mechanism by which NF- κ B and IRF-1 synergistically activate the *RANTES* promoter.

Expression of the *RANTES* gene can be induced by a variety of signals, such as inflammatory cytokines [8,14–17], LPS [20], T-cell activation [19] and viral infection [12,13,42]. Interestingly, a recent report demonstrated that IRF-3 plays an essential role in activation of *RANTES* transcription by viral infection [43]. In this case, the promoter element required for interaction with IRF-3 was mapped to the same region that we identified as an IRF-1 binding site. Although IRF-1 is induced by IFN- α , IFN- γ , TNF- α and LPS [30,31], IRF-3 appears to be activated specifically by viral infection [32,33]. This suggests that different stimuli induce different IRF transcription factors that then go on to regulate *RANTES* promoter activity in a similar way. It is not

Figure 5 Specific binding of p65 to the NF- κ B2 site in the murine *RANTES* promoter

(A) EMSAs were performed with a κ B2 DNA probe and nuclear extracts prepared from NIH 3T3 cells either unstimulated (lane 2) or stimulated with TNF- α (lanes 3 and 6), IFN- γ (lanes 4 and 7) or both (lanes 5 and 8) for 1 h (lanes 3–5) or 4 h (lanes 6–8). (B) Specific complex formation was dependent upon the κ B2 binding sequence. Nuclear extracts from cells stimulated with both TNF- α and IFN- γ were used in the binding reactions. Binding reactions were carried out in the absence (lane 2) or presence of a 10- (lanes 3 and 5) or 100-fold (lanes 4 and 6) molar excess of unlabelled κ B2 (lanes 3 and 4) or IRF probe as a non-specific competitor (lanes 5 and 6). (C) p65 is present in the specific complex. Binding reactions were carried out in the presence of anti-p65 (lane 7) or anti-IRF-1 antiserum as a control (lane 8). The super-shifted band is indicated by an arrow. For all panels, specific and non-specific complexes are indicated by black and white arrowheads, respectively.

yet clear why cells, in response to different physiological stimuli, induce distinct transcription factors within a family that act upon the same sequence of the *RANTES* promoter in order to activate the same *RANTES* gene. One possible explanation is that IRF-3 may interact with distinct transcription factors; the IRF-3 pre-initiation complex may be different from that for IRF-1. Maybe IRF-3 is needed for co-ordinated regulation of other

Figure 6 Specific binding of IRF-1 to the IRF site in the murine RANTES promoter

(A) EMSAs were performed as described in Figure 5, except that an IRF probe was used in this experiment. Unstimulated control cells and cells treated with each cytokine are as indicated at the top of the Figure. (B) Competition analysis of IRF binding activity. Binding reactions were carried out in the presence of 10- and 100-fold molar excesses of unlabelled specific (IRF) and non-specific (κ B2) probes as indicated. (C) IRF-1 is present in the complex. Nuclear extracts from cells stimulated with both TNF- α and IFN- γ were used for the binding reaction. Binding reactions were carried out in the presence of anti-IRF-1 or anti-p65 antisera as a control. For all panels, specific and non-specific complexes are denoted by black and white arrowheads, respectively.

related genes that are also activated by viral infection. The physiological reason for this complex mode of regulation remains to be deciphered.

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