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## Differential regulation of RANTES and IL-8 expression in lung adenocarcinoma cells

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Running title: RANTES and IL-8 expression in lung adenocarcinoma cells

## Summary

In lung adenocarcinoma, expression of Regulated upon Activation, Normal T cell Expressed and presumably Secreted (RANTES) is a predictor of survival while that of interleukin (IL)-8 is associated with a poor prognosis. In several models, tumorigenesis is abolished by RANTES, while it is facilitated by IL-8. We studied the regulation of RANTES and IL-8 expression in A549 lung adenocarcinoma cells. The effects of tumor necrosis factor (TNF)- $\alpha$  and regulators of protein kinases C (PKC) $\alpha/\beta$  were tested because these have been shown to modulate cancer development and progression. TNF- $\alpha$  stimulated expression of both chemokines, while the PKC $\alpha/\beta$  activator 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induced only expression of IL-8 and inhibited TNF- $\alpha$ -induced RANTES expression. The PKC $\alpha/\beta$  inhibitor Gö 6976 increased TNF- $\alpha$ -induced RANTES production and prevented its down-regulation by TPA. In contrast, it decreased TNF- $\alpha$  or TPA-induced IL-8 release. The differential regulation of RANTES and IL-8 expression was further analyzed. Site-directed mutagenesis indicated that regulation of RANTES promoter activity required two nuclear factor (NF)- $\kappa$ B response elements but not its activator protein (AP)-1 binding sites. An AP-1 and a NF- $\kappa$ B recognition sites were necessary for full induction of IL-8 promoter activity by TNF- $\alpha$  and TPA. Moreover, electrophoretic mobility shift assays demonstrated that NF- $\kappa$ B response elements from the RANTES promoter were of lower affinity than that from the IL-8 promoter. Immunoblotting experiments showed that TPA was more potent than TNF- $\alpha$  to induce in a PKC $\alpha/\beta$  dependent manner the p44/p42 mitogen-activated protein kinases signaling cascade which controls AP-1 activity. Conversely, TPA inhibited TNF- $\alpha$ -induced NF- $\kappa$ B signaling and was a weak activator of this pathway. Thus, TPA did not sufficiently activate NF- $\kappa$ B to increase transcription through the low affinity NF- $\kappa$ B binding sites on RANTES promoter and its inhibitory effect on TNF- $\alpha$ -induced NF- $\kappa$ B signaling resulted in a reduced transcription rate. On IL-8 promoter, increased transcription through the high affinity NF- $\kappa$ B binding site occurred even with poorly activated NF- $\kappa$ B and the functional AP-1 response element compensated any loss of transcription rate. These data provide a mechanistic insight into the differential regulation of IL-8 and RANTES expression by PKC $\alpha/\beta$  in lung adenocarcinoma cells.

**Key Words:** Adenocarcinoma; Bronchiolo-Alveolar; Chemokines; Interleukin-8; NF-kappaB; Protein Kinase C; RANTES; Transcription Factor AP-1.

## Introduction

Lung cancer is the most common cause of cancer-related deaths in industrialized nations and its incidence is on a constant increase [1]. Lung adenocarcinomas represent 30% of all lung cancers [2]. The variability of survival within a given stage and the poor efficiency of current therapies have led to the search for tumor-produced factors that may serve as new prognostic factors or therapeutic targets. In this line, Moran and colleagues have used a global gene expression profiling approach and found that the chemokine Regulated upon Activation, Normal T cell Expressed and presumably Secreted (RANTES) is a predictor of survival in stage I lung adenocarcinoma [3]. RANTES could therefore be used as a prognostic factor to further stratify patients with this disease and is a potential therapeutic target. Indeed, tumor cells that are genetically engineered to secrete RANTES, lose their tumorigenicity in a mouse sarcoma model [4]. Similarly, in a murine lymphoma model, gene transfer of RANTES into established tumors provokes complete tumor regression in 50% of treated mice [5]. These therapeutic effects are the result of a RANTES-induced anti-tumor immune response [4, 5]. In addition, RANTES seems to act in an autocrine manner to decrease tumor growth since abrogation of cell surface expression of the RANTES receptor CCR5 enhances proliferation of human breast tumor cells injected in mice [6]. However, the role of RANTES in breast cancer is debated [6-10].

In contrast to RANTES, interleukin (IL)-8 expression in non-small-cell lung cancer, including adenocarcinoma, is of poor prognosis [11]. This chemokine was found to be produced by cancer cells and to facilitate tumorigenesis, probably through its angiogenic activity. Xenograft experiments in mice have shown that IL-8 increases growth of lung [12], prostate [13] bladder [14] and breast cancer [15], as well as melanoma [16]. One exception is ovarian cancer, in which IL-8 was found in contrary to attenuate tumor growth [17].

We examined herein the effects of tumor necrosis factor (TNF)- $\alpha$  and regulators of protein kinases C (PKC) $\alpha/\beta$  on the expression of RANTES and IL-8 in A549 lung adenocarcinoma cells. TNF- $\alpha$  is a major mediator of inflammation, which can be produced by tumor-infiltrating macrophages [18]. It has paradoxical roles in the evolution and treatment of malignant disease [19]. PKC $\alpha/\beta$  were previously shown to affect tumor progression and malignant phenotype [20, 21]. We show that the PKC $\alpha/\beta$  activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) antagonized TNF- $\alpha$ -induced RANTES expression, while both stimuli increased IL-8 expression. Treatment with the PKC $\alpha/\beta$  inhibitor Gö 6976 prevented TPA from down-regulating RANTES expression. This compound also increased TNF- $\alpha$ -induced RANTES production and diminished TPA or TNF- $\alpha$ -induced IL-8 release. Further analysis showed that the NF- $\kappa$ B signaling pathway is down-regulated through PKC $\alpha/\beta$  and provided insight into the mechanism underlying the differential regulation of RANTES and IL-8 in A549 cells.

## Materials and Methods

### Materials

TPA and human recombinant TNF- $\alpha$  were purchased from Sigma and BD Pharmingen, respectively. Gö 6976 was purchased from Calbiochem.

### Plasmids

Luciferase reporter constructs carrying intact or mutated 1.4 kb human RANTES promoter sequence were obtained from Hiroyuki Moriuchi (Nagasaki University School of Medicine, Nagasaki, Japan). The promoter sequence was point mutated at response elements for NF- $\kappa$ B

and AP-1 [22]. An additional RANTES promoter construct mutated on two NF- $\kappa$ B response elements was purchased from Top Gene Technologies. Promoter of the human IL-8 gene (1.5 kb) was point mutated at AP-1 and NF- $\kappa$ B binding sites as previously described [23]. The cytomegalovirus (CMV) $\beta$ -gal plasmid consists of the CMV early promoter driving the  $\beta$ -galactosidase gene. The plasmid pCCS26 contains a ribosomal protein S26 cDNA and was obtained from Philippe Fort (Centre de Recherches de Biochimie Macromoléculaire, Montpellier, France) [24].

### **Cell maintenance**

The A549 human lung adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC CCL-185) and maintained in Ham's F12/DMEM medium containing 10% heat-inactivated FCS v/v, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM glutamine.

### **Transient transfection and reporter gene assays**

100 000 cells per well were serum deprived overnight, transfected with 60 ng of luciferase construct and 25 ng of CMV $\beta$ -gal, and stimulated as indicated in the figure legends. Transfection, luciferase and  $\beta$ -galactosidase assays were performed as described previously [25]. Luciferase activity was divided by  $\beta$ -galactosidase activity to normalize values for variations in transfection efficiency.

### **Western Blot**

One million cells per well were serum deprived overnight, stimulated as indicated in the figure legends and lysed in 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Triton X-100 v/v, 1mM EDTA, 1 mM EGTA, 20 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, 50 nM okadaic acid, 1 mM dithiothreitol and a protease inhibitor cocktail (Roche). After 5 min on ice, cell extracts were sonicated four times for 5 s, centrifuged at 20 000 g for 10 min at 4°C and analyzed by Western blotting on nitrocellulose membranes. Antibodies used were anti-p44/p42 mitogen-activated protein kinases (MAPK) (9102; Cell signaling Technology), anti-p44/p42 MAPK phosphorylated on Thr202 and Tyr204 (9101; Cell signaling Technology), anti-I $\kappa$ B $\alpha$  (sc-371; Santa Cruz Biotechnology), anti-p65 (sc-109; Santa Cruz Biotechnology), and anti-p65 phosphorylated on Ser536 (#3031; Cell signaling Technology). After incubation with peroxidase-conjugated secondary antibodies, immunoblots were developed in 0.2 mM coumaric acid, 1.25 mM 3-aminophthalhydrazide, and 0.009% hydrogen peroxide mixture and exposed to a cooled charge-coupled device camera (Kodak).

### **RANTES and IL-8 immunoassays**

100 000 cells per well were serum deprived overnight. These were then stimulated as indicated in the figure legends. Concentrations of RANTES and IL-8 in supernatants were determined by ELISA (R&D Systems and Diaclone Elipair, respectively).

### **Northern blot analysis**

Total RNA was isolated with RNA PLUS (Quantum Biotechnologies). Northern Blot was performed according to standard protocols with <sup>32</sup>P-labeled probes. Specific mRNAs were detected with a Storm phosphorimager (Amersham Biosciences). Ribosomal protein S26 mRNA served as an internal control because it is found at an invariable amount in mammalian cell lines [24].

### **Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were prepared as described [26]. Double-stranded DNA was labelled with [ $\alpha$ - $^{32}$ P]CTP (Amersham) using Klenow DNA polymerase (Invitrogen). The following oligonucleotides (MWG Biotech) were used:

1- 5'-GGGGAAACTCCCCTTAGGGGATGCCCT-3'

2- 5'-GGAGGGGCATCCCCTAAGGGGAGTTCC-3'

3- 5'-GGGGAATTTCCCGAGGGGAATTTCCAG-3'

4- 5'-GGCTGGGAAATTCCCCTCGGGAAATTCC-3'

Annealing of oligonucleotides 1-2 and 3-4 formed 2xNF- $\kappa$ BRE RANTES and 2xNF- $\kappa$ BRE IL-8, respectively. Sequences corresponding to the putative NF- $\kappa$ B response elements are underlined. Ten  $\mu$ g of nuclear extract in EMSA buffer (50 mM Tris pH 8, 12 mM MgCl<sub>2</sub>, 2  $\mu$ g poly (dI-dC), 1 mM EDTA, 2 mM dithiothreitol, 20% glycerol v/v, 100  $\mu$ M orthovanadate and 50 nM okadaic acid) containing a protease inhibitor cocktail (Roche) were incubated with labelled probe and competitors for 30 min at 25°C. Samples were subjected to 6% w/v native PAGE and fixed in the gel for 20 min in 10% acetic acid v/v, 45% methanol v/v. The gel was dried before autoradiography with a phosphorimager.

### **Statistical analysis**

Differences between groups were assessed using one-way analysis of variance with appropriate post hoc testing. Statistical significance was set up at  $p < 0.05$ .

## Results

### Differential regulation of RANTES and IL-8 expression

Expression of RANTES and IL-8 in A549 lung adenocarcinoma cells was initially analyzed at the RNA level by Northern blotting (Fig. 1A). RANTES mRNA was not detectable in non-stimulated cells and TPA treated cells. TNF- $\alpha$  induced an increase in RANTES mRNA steady state level that was partially inhibited after co-treatment with TPA. In contrast, the amount of IL-8 mRNA was increased by both stimuli. Similar results were obtained by quantifying the amount of the corresponding chemokines in cell supernatants. RANTES release was induced by TNF- $\alpha$  ( $p < 0.001$ ) but not by TPA, and the latter inhibited TNF- $\alpha$ -induced RANTES release by 46% ( $p < 0.001$ ) (Fig. 1B). In contrast, IL-8 production was increased following treatment with either stimulus ( $p < 0.001$ ) (Fig. 1C). To examine the involvement of PKC $\alpha/\beta$  in the regulation of RANTES and IL-8 expression, the cells were pretreated with Gö 6976. This compound prevented the inhibitory effect of TPA on RANTES release ( $p < 0.001$ ) (Fig. 1B). Gö 6976 also increased TNF- $\alpha$ -induced RANTES production ( $p < 0.01$ ) (Fig. 1B). In contrast, this compound inhibited TPA-induced IL-8 production ( $p < 0.05$ ) (Fig. 1C). However, when TNF- $\alpha$  was used alone or in combination with TPA to stimulate IL-8 expression, an inhibitory effect of Gö 6976 was observed but was not statistically significant (Fig. 1C).

### Regulation of RANTES and IL-8 promoter activity after site-directed mutagenesis

To evaluate the role of AP-1 and NF- $\kappa$ B in the regulation of RANTES expression, a series of point mutated RANTES promoter constructs were analyzed by a reporter gene assay (Fig. 2A). The RANTES 1.4 kb promoter region contains four AP-1 binding sites and two NF- $\kappa$ B response elements. TNF- $\alpha$ -induced intact promoter activity was partially repressed by TPA. Mutation of one or the other NF- $\kappa$ B recognition site reduced overall promoter activity, but some regulation by TNF- $\alpha$  and TPA still occurred. The promoter became almost unresponsive when both NF- $\kappa$ B recognition sequences were mutated. In contrast, mutation of the AP-1 response elements did not affect promoter activity in any conditions. The same approach was used to study the involvement of AP-1 and NF- $\kappa$ B in the regulation of IL-8 expression (Fig. 2B). The IL-8 1.5 kb promoter region displays one AP-1 binding site and one NF- $\kappa$ B recognition sequence. Mutation of either the AP-1 or NF- $\kappa$ B response element dramatically reduced induction of IL-8 promoter activity by either TNF- $\alpha$  or TPA. The promoter became unresponsive when both sequences were mutated.

### Regulation of p44/p42 mitogen-activated protein kinases phosphorylation by TNF- $\alpha$ , TPA and Gö 6976

We next analyzed the effect of TNF- $\alpha$ , TPA and Gö 6976 on p44/p42 mitogen-activated protein kinases (MAPK) signaling cascade. PKC activation is known to lead to phosphorylation of p44 and p42 MAPK, which in turn phosphorylate Elk-1 transcription factor, thereby increasing *c-fos* transcription and thus AP-1 activity (reviewed in [27]). Western blot analyses show an increase in phosphorylation status of both MAPK upon stimulation with TPA and, to a lesser extent, with TNF- $\alpha$  (Fig. 3). Basal phosphorylation of p44/p42 MAPK as well as that induced by TNF- $\alpha$  or TPA was inhibited by pretreatment with Gö 6976. However, Gö 6976 did not diminish phosphorylation induced by co-treatment with TNF- $\alpha$  and TPA.

### **Regulation of NF- $\kappa$ B signaling pathway by TNF- $\alpha$ , TPA and Gö 6976**

To monitor activation of NF- $\kappa$ B, the level and phosphorylation status of its p65 subunit and of its inhibitor I $\kappa$ B $\alpha$  were determined by Western blotting. I $\kappa$ B $\alpha$  is phosphorylated by I $\kappa$ B kinases and undergo proteolytic degradation. In addition, these kinases phosphorylate p65, thereby increasing its transactivating capacity [28]. Time-course analyses show that phosphorylation of these proteins was more rapid and complete with TNF- $\alpha$  than with TPA. Upon TNF- $\alpha$  stimulation, phosphorylation of both proteins was clearly apparent at 2 min. The amount of phosphorylated p65 reached a maximum at 5 min and diminished progressively afterwards, but remained higher than in untreated cells for at least 2 h (Fig. 4A and data not shown). At 5 min, phosphorylated I $\kappa$ B $\alpha$  was predominant over I $\kappa$ B $\alpha$  and began to be degraded. Both forms of I $\kappa$ B $\alpha$  disappeared by 10 min, but were later resynthesized. With TPA, the amount of phosphorylated p65 was very low compared to that obtained after TNF- $\alpha$  treatment, while phosphorylation and degradation of I $\kappa$ B $\alpha$  was delayed and incomplete (Fig. 4B). Thus, as compared to TNF- $\alpha$ , TPA is a much weaker activator of NF- $\kappa$ B in A549 cells. Pretreating the cells with TPA for as short as 5 min prevented TNF- $\alpha$  from phosphorylating efficiently p65 and I $\kappa$ B $\alpha$  (Fig. 5A). This inhibitory effect of TPA was clearly weakened in the presence of Gö 6976 (Fig. 5B). The latter compound also slightly increased the amount phosphorylated I $\kappa$ B $\alpha$  after TNF- $\alpha$  treatment (Fig. 5B).

### **Affinity of NF- $\kappa$ B response elements from the RANTES and IL-8 promoters**

As shown in Figure 2B, TPA induced IL-8 promoter activity via the AP-1 binding site but also through the NF- $\kappa$ B response element, even though it is a weak activator of the NF- $\kappa$ B signaling pathway. In contrast, TPA was unable to induce significantly RANTES promoter activity. Analysis of NF- $\kappa$ B recognition sequences reveals that those on RANTES promoter (GGAAACTCC and GGGATGCCC) diverge by one base pair from consensus (GGA<sup>A</sup>/<sub>c</sub>TN<sup>T</sup>/<sub>c</sub>CC) [29], while that on IL-8 promoter (GGAATTTCC) is identical to consensus, suggesting that these may have different binding affinities. To compare their relative affinity, competitive binding followed by EMSA was performed. The two NF- $\kappa$ B binding sites present in tandem on RANTES promoter (2xNF- $\kappa$ BRE RANTES) and a tandem repeat of the NF- $\kappa$ B response element from the IL-8 promoter (2xNF- $\kappa$ BRE IL-8) were used as competitors. Nuclear extract of TNF- $\alpha$ -stimulated cells was incubated with <sup>32</sup>P labelled 2xNF- $\kappa$ BRE IL-8 in the presence of increasing amount of competitor. Data show that 2xNF- $\kappa$ BRE IL-8 is a better competitor, and hence of higher affinity than 2xNF- $\kappa$ BRE RANTES (Fig. 6). Supershift experiments indicated that the complex observed in TNF- $\alpha$ -stimulated cells contained the p65 subunit of NF- $\kappa$ B (data not shown).

## Discussion

Analysis of lung adenocarcinoma clinical series indicate that IL-8 expression is associated with a poor prognosis while that of RANTES is a predictor of survival [3, 11]. In several models using xenografts of tumor cells in mice, it was found that IL-8 facilitates disease progression while RANTES reduces tumor growth (cf. Introduction for details and references). Thus, IL-8 and RANTES are potential therapeutic targets in lung adenocarcinoma. We found herein that RANTES and IL-8 expression is differentially regulated through PKC $\alpha/\beta$  in A549 lung adenocarcinoma cells. The mechanism underlying this differential regulation was investigated.

Firstly, regulation of RANTES promoter activity required intact NF- $\kappa$ B binding sites while the four putative AP-1 recognition sites were dispensable as shown by reporter gene assays (Fig. 2A). Others have shown that these AP-1 response elements contributed modestly to the induction of RANTES promoter activity in T lymphocytes [22]. Thus, these sites appear to be non functional or weakly active, although two of them are identical to the AP-1 consensus recognition sequence [22, 29]. Possibly, lack of responsiveness may be dictated by the surrounding sequences.

Secondly, we show that TPA was a weak activator of the NF- $\kappa$ B signaling pathway and, in fact, inhibited the strong induction of this pathway by TNF- $\alpha$  (Figs. 4 and 5). Conversely, TPA was more potent than TNF- $\alpha$  to induce in a PKC $\alpha/\beta$  dependent manner the p44/p42 MAPK signaling cascade, which is known to control AP-1 activity (Fig. 3). The precise mechanism by which TPA weakly activates NF- $\kappa$ B signaling pathway and simultaneously prevents its strong activation by TNF- $\alpha$  remains to be determined. Nevertheless, our results indicate that the negative effect on NF- $\kappa$ B activation went through PKC $\alpha/\beta$  and targeted directly or indirectly I $\kappa$ B kinases. Indeed, a decreased phosphorylation of I $\kappa$ B $\alpha$  and of p65 at Ser536 is indicative of a down-regulation of these kinases [30].

Thirdly, regulation of IL-8 promoter activity involved strong cooperation between an AP-1 recognition sequence and a high affinity NF- $\kappa$ B binding site while that of RANTES required only two low affinity NF- $\kappa$ B response elements (Figs. 2 and 6).

These data provide an explanation as to why TPA stimulated expression of IL-8 but not that of RANTES. These also explain why TNF- $\alpha$ -induced expression of RANTES was inhibited by TPA and restored by Gö 6976. TPA did not inhibit TNF- $\alpha$ -induced IL-8 expression probably because its inhibitory effect on TNF- $\alpha$ -induced NF- $\kappa$ B activity was compensated by the activation of AP-1. In addition, the high affinity NF- $\kappa$ B binding site on IL-8 promoter conferred a significant response even after weak activation of NF- $\kappa$ B by TPA (Fig. 2B).

Of note, the PKC $\alpha/\beta$  inhibitor Gö 6976 increased TNF- $\alpha$ -induced RANTES production and prevented its down-regulation by TPA, while it diminished TNF- $\alpha$  or TPA induced IL-8 release (Fig. 1). Gö 6976 has been described to be very effective at sensitizing breast cancer cells to toxicity of DNA-damaging agents [31]. It was also found to promote formation of cell junctions and to inhibit invasion of urinary bladder carcinoma cells [32]. Together, these data support the notion that Gö 6976 may be of therapeutic value in the treatment of cancer, including lung adenocarcinoma. Of note, a PKC $\alpha$  antisense oligonucleotide did not enhance survival and other efficacy measures in patients with advanced non-small cell lung cancer [33]. It is tempting to speculate that this approach was not efficient because it did not target PKC $\beta$  as well. Indeed, PKC $\beta$  has apparently similar function to PKC $\alpha$  in cancer cells; it has been shown to account for increased invasion [34, 35] and proliferation rate [36]. In addition, inhibiting PKC $\alpha/\beta$  activity may prove efficacy only at early stage of the disease and/or in a subtype of non-small cell lung cancer, such as lung adenocarcinoma. Finally, we observed that as opposed to the positive effect of Gö 6976 on RANTES release, its inhibitory effect on

IL-8 production was not statistically significant when cells were stimulated with TNF- $\alpha$  alone or concomitantly with TPA. Hence, this compound may reach efficacy only in combination therapy that includes other anti-angiogenics.

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## **Conflict of interest statement**

None declared

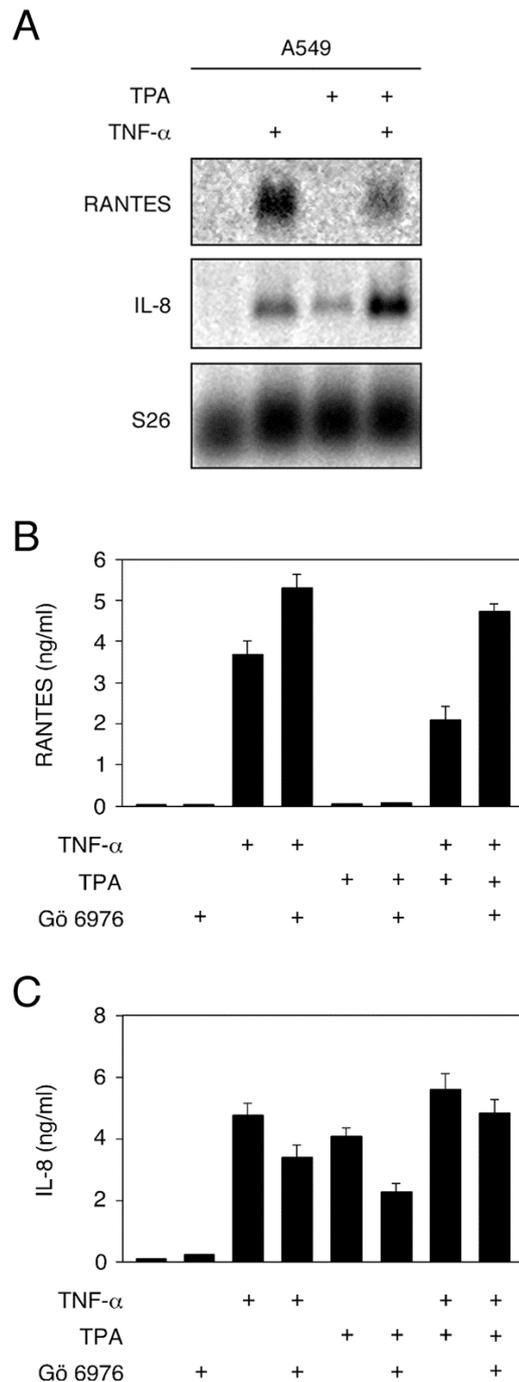
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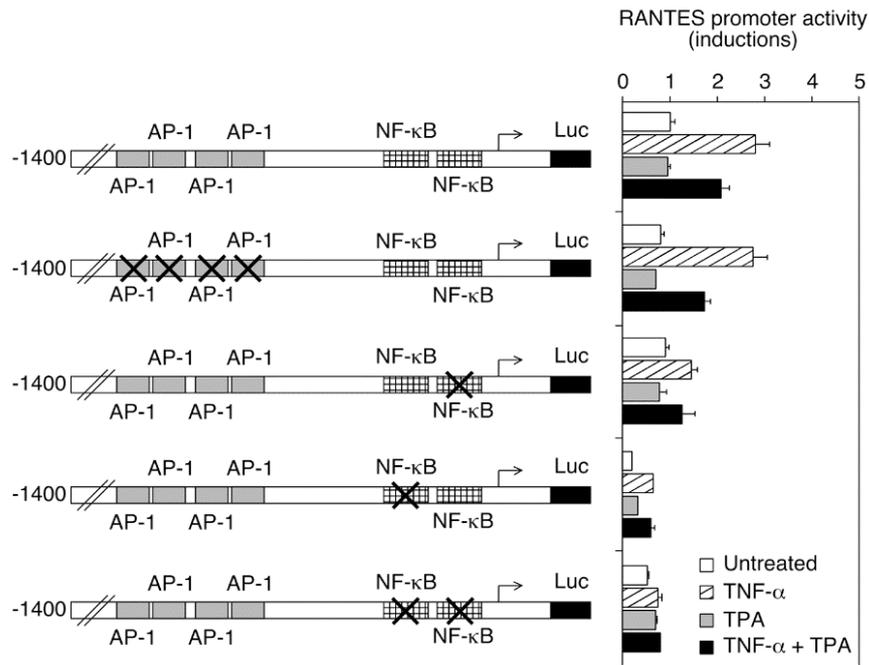
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## Figure legends

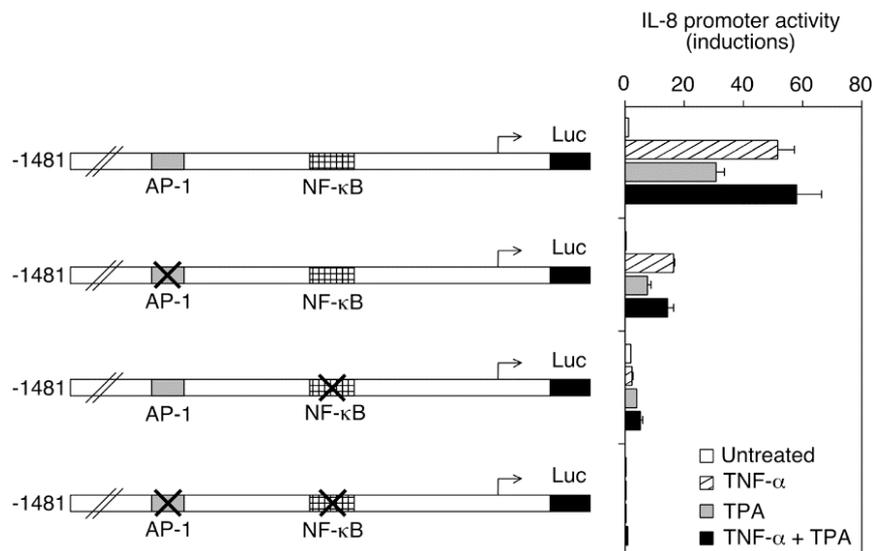


**Fig. 1. Regulation of RANTES and IL-8 expression.** (A) A549 cells were serum deprived overnight and stimulated for 4 h with TNF- $\alpha$  (10 ng/ml) and TPA (100 ng/ml) as indicated. Total RNA was analyzed by Northern blotting. The ribosomal protein S26 mRNA served as loading control. Approximate size of transcript was 1.9 kb for RANTES, 2.2 kb for IL-8, and 0.7 kb for S26. (B and C) A549 cells were pretreated with Gö 6976 (1  $\mu$ M) for 1 h and stimulated with TNF- $\alpha$  (10 ng/ml) and TPA (100 ng/ml) for 20 h as indicated. Concentrations of RANTES (B) and IL-8 (C) in supernatants were determined. Data (n = 6) are plotted as means  $\pm$  SE.

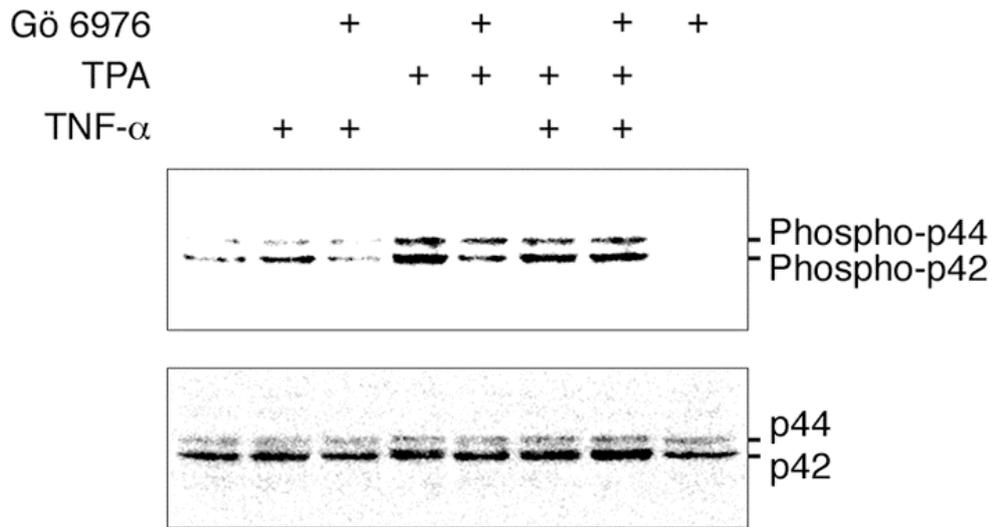
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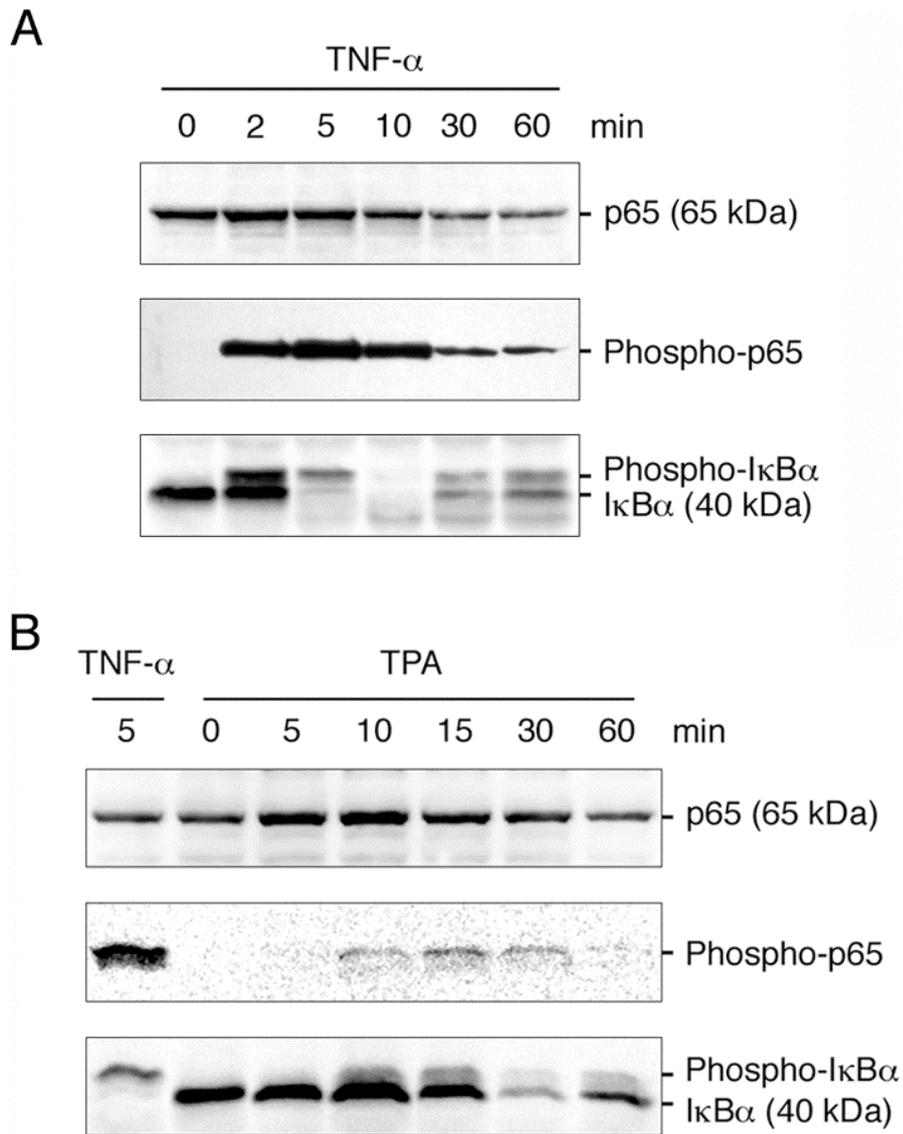
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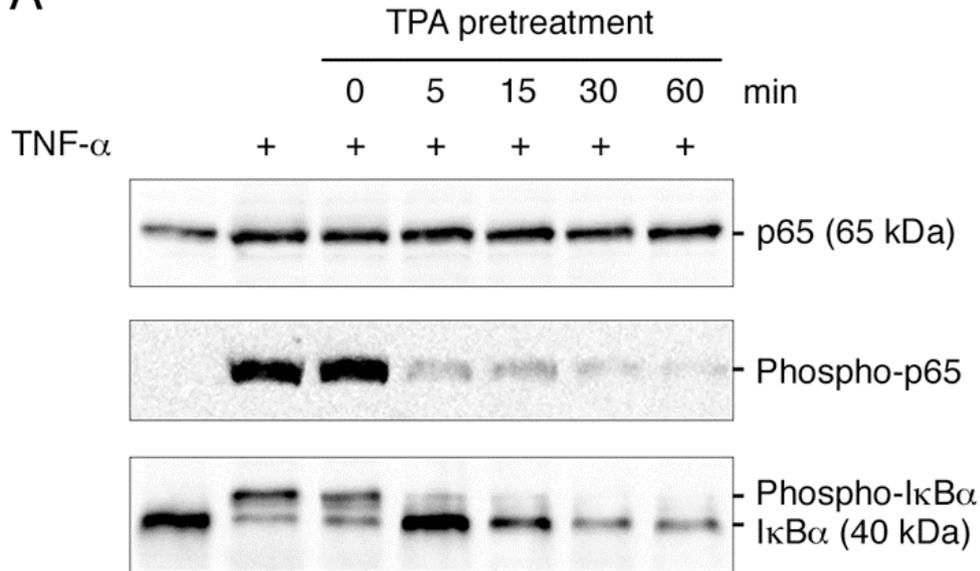
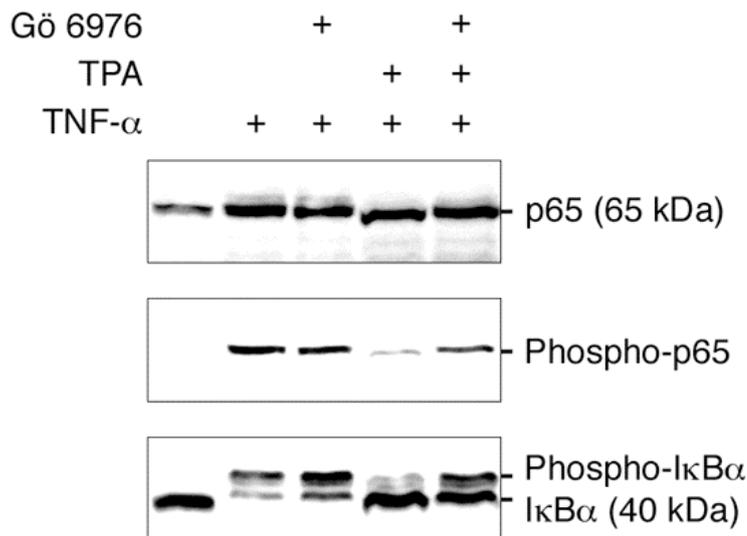
**Fig. 2. Regulation of RANTES and IL-8 promoter activity after site-directed mutagenesis.** (A) A549 cells were transfected with intact or point mutated RANTES promoter-luciferase gene construct as indicated, and CMV $\beta$ -gal. These were treated for 4 h with TNF- $\alpha$  (10 ng/ml) or TPA (100 ng/ml) or the combination of both compounds, and lysed to determine relative luciferase activities after normalization for  $\beta$ -galactosidase activity. Data ( $n = 6$ ) are shown as fold inductions over basal activity and are plotted as means  $\pm$  SE. (B) A549 cells were transfected with intact or mutated IL-8 promoter-luciferase gene construct as indicated, and CMV $\beta$ -gal. These were then treated as in (A). Data ( $n = 6$ ) are shown as fold inductions over basal activity and are plotted as means  $\pm$  SE.



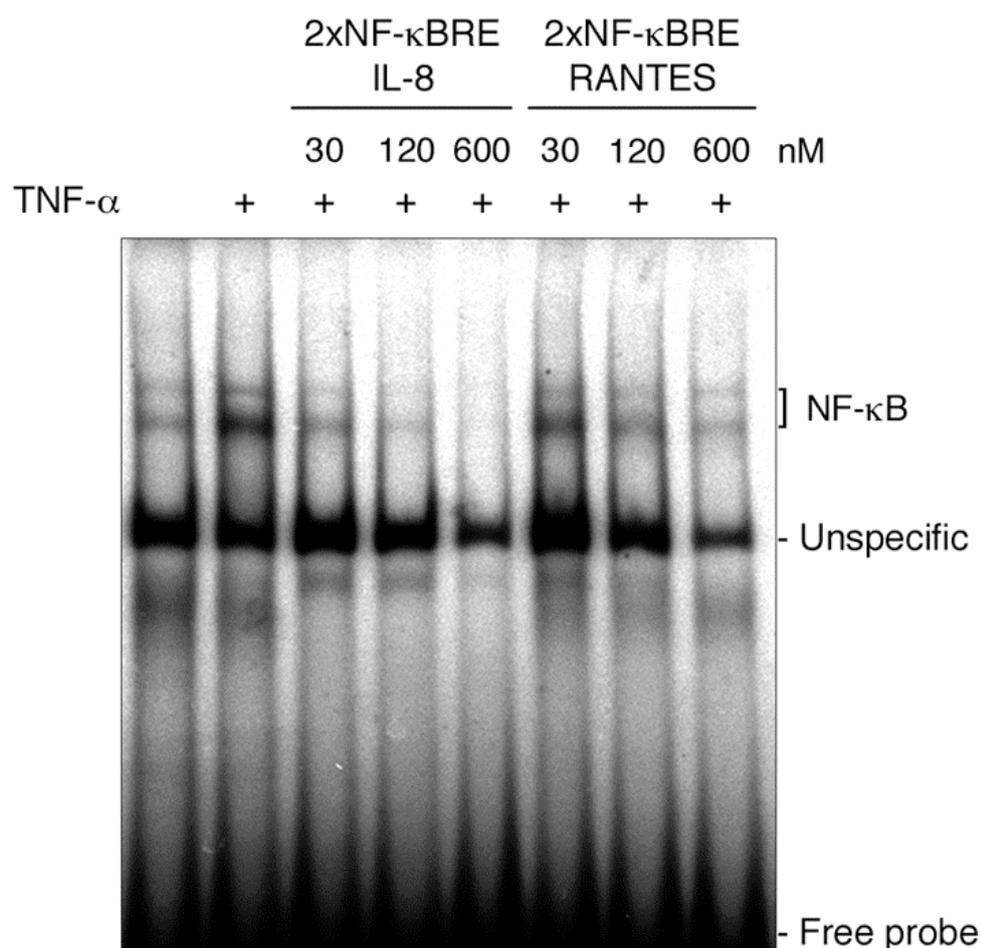
**Fig. 3. Regulation of p44/42 MAPK signaling.** A549 cells were pretreated with Gö 6976 (1  $\mu$ M) for 60 min and exposed to TNF- $\alpha$  (10 ng/ml) and TPA (100 ng/ml) for 10 min as indicated. Cell extracts were analyzed by Western blotting for their content in total p44/42 and phosphorylated p44/42 (phospho-p44/42).



**Fig. 4. Time-course analyses of NF- $\kappa$ B signaling.** (A) A549 cells were exposed to TNF- $\alpha$  (10 ng/ml) for the indicated time. Cell extracts were analyzed by Western blotting for their content in p65, phosphorylated p65 (phospho-p65), I $\kappa$ B $\alpha$  and phosphorylated I $\kappa$ B $\alpha$  (phospho-I $\kappa$ B $\alpha$ ). (B) A549 cells were exposed to TNF- $\alpha$  (10 ng/ml) or TPA (100 ng/ml) for the indicated time. Cell extracts were analyzed as in (A).

**A****B**

**Fig. 5. Regulation of TNF- $\alpha$ -induced NF- $\kappa$ B signaling by PKC $\alpha$ / $\beta$ .** (A) A549 cells were pretreated with TPA (100 ng/ml) for the indicated time and further exposed for 5 min to TNF- $\alpha$  (10 ng/ml). Cell extracts were analyzed by Western blotting for their content in p65, phosphorylated p65 (phospho-p65), I $\kappa$ B $\alpha$  and phosphorylated I $\kappa$ B $\alpha$  (phospho-I $\kappa$ B $\alpha$ ). (B) A549 cells were pretreated with Gö 6976 (1  $\mu$ M) and TPA (100 ng/ml) for 60 min and 10 min, respectively. These were then treated with TNF- $\alpha$  (10 ng/ml) for 5 min. Cell extracts were analyzed as in (A).



**Fig. 6. Relative affinity of NF- $\kappa$ B response elements from the IL-8 and RANTES promoters.** Nuclear proteins were extracted from A549 cells treated for 1 h with medium alone (first lane) or 10 ng/ml of TNF- $\alpha$  (+). EMSA was performed using as labelled probe a tandem repeat of the NF- $\kappa$ B response element from the IL-8 promoter (2xNF- $\kappa$ BRE IL-8; 15 nM) and as competitors 2xNF- $\kappa$ BRE IL-8 or the two NF- $\kappa$ B binding sites present in tandem on RANTES promoter (2xNF- $\kappa$ BRE RANTES) at increasing concentrations.