c-Rel and p65 subunits bind to an upstream NF-κB site in human granulocyte macrophage-colony stimulating factor promoter involved in phorbol ester response in 5637 cells

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Abstract To further clarify the complex transcriptional regulation of the human GM-CSF gene, which was extensively investigated in activated T cells, we have studied the role of an upstream NF-κB like site in the 5637 non-lymphoid cell line, which derives from a bladder carcinoma and constitutively produces GM-CSF. This sequence, named the A element, has an active role on GM-CSF transcription and is responsive to the tumor promoter PMA in transient transfection experiments. We describe here a heterodimeric binding complex of NF-κB subunits (c-Rel and p65) which is identical to the one obtained using the HIV-LTR-κB site as recognition sequence and different from the one (c-Rel and p50) observed with nuclear extracts from Mo T-lymphoid HTLV-II infected cells.

Key words: Nuclear factor κB;
Granulocyte macrophage-colony stimulating factor;
Phorbol 12-myristate 13-acetate; Transcriptional regulation

1. Introduction

GM-CSF is a glycoprotein cytokine involved in hematopoiesis and host defense [1]. With regard to the transcriptional control of GM-CSF gene expression, a distal and a proximal promoter region have been investigated. The first contains an enhancer located approximately 3 kb upstream of the transcriptional start site [2], and the second, spanning at least the first 120 bp from the transcriptional start site, includes recognition sites for the binding of several constitutive and inducible nuclear proteins which contribute to the transcriptional regulation of the GM-CSF gene [3,4]. These proximal elements include two binding sites for the NF-κB/Rel family of transcription factors (the κB element which binds the classical p65-p50 complex and the CK1 element which binds p65-c-Rel) [5–7].

Several reports describe the central role played by the κB element in the response of the GM-CSF promoter to T cell receptor type signals as well as to Tax activation in T cells [4,8] and, up to now, studies on these NF-κB sites have focused on activated T cells.

We previously identified a novel upstream κB element, within a sequence, defined the A element, located between −2002 and −1984 [9], which binds members of the NF-κB family. This sequence is active as a positive transcriptional element in the GM-CSF producing lymphoblastoid cell line Mo, HTLV-II infected and expressing the Tax protein [10]. We also observed a similar positive transcriptional role of the A element, although weaker in quantity, in a cell line of different origin, the 5637 bladder carcinoma cells [9], which constitutively produce GM-CSF [11].

Here, we describe the A element binding complex, which differs for subunit composition from the one previously observed in the human T cell leukemia Mo cell line, in nuclear extracts of 5637 cells, prepared from untreated cells and from stimulated cells with the tumor promoter PMA, the action of which mimics Tax activation by stimulating NF-κB [12,13].

Our data provide evidence that NF-κB family members acting on the GM-CSF promoter associate differently in a tissue specific and activation dependent manner.

2. Materials and methods

2.1. Cell culture and induction conditions

The 5637 cell line, derived from a human bladder carcinoma and constitutively producing GM-CSF, was cultured in DMEM supplemented with 10% fetal calf serum, penicillin and streptomycin in a humidified atmosphere with 5% CO_2. When stimulated, the cells were incubated with 50 ng/ml of PMA for 4, 8 and 24 h prior to harvesting. The Mo cell line, originally obtained from the American Type Culture Collection, was cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, penicillin and streptomycin in a humidified atmosphere with 5% CO_2.

2.2. Plasmids and transfections

Plasmids used for transfections were all prepared in our laboratory: PF2000, containing the first 2010 bp upstream of the transcription start site of the GM-CSF gene, was prepared by subcloning from a phagic clone, and PF20 and PF24, containing respectively the first 1742 and 240 bp of the 5′ flanking sequence upstream of the transcription start site were derived from PF2000, as described [14]. PΔA, deleted of the κB sequence (A element) between −2002 and −1984, was also derived from PF2000, as described [9].

Transfections were performed as described [9] using 10 μg of plasmid PF 2000 and equimolar amounts of the other constructs (for all constructs, at least two different plasmid preparations were used), together with 5 μg of pRSV-bgal as an internal control. Extracts were prepared from unstimulated or PMA stimulated cells for CAT activity assay, which was measured by standard methods [15]. The values of CAT activity were corrected for protein concentration and for β-galactosidase activity to normalize variability in transfection efficiency. Mo cells were electroporated as previously described [9]. Relative CAT activities (mean ± S.E.M.) were calculated from at least three unrelated experiments (performed in duplicate) by using PF2000 plasmid transfected in unstimulated cells as a reference value in all experiments. The magnitude of induction in response to PMA was calculated relative to the unstimulated cells for every construct.

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Abbreviations: GM-CSF, granulocyte macrophage-colony stimulating factor; NF-κB, nuclear factor κB; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyl transferase

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2.3. Preparation of cellular and nuclear extracts

Cells, unstimulated and stimulated with PMA for 4, 8 and 24 h, were lysed in RIPA buffer (1×phosphate buffer saline, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride). After 30 min at 4°C the mixture was centrifuged at 14000 rpm for 15 min and the supernatant was treated as whole cell extract for Western blot.

Nuclear extracts, from unstimulated cells or after stimulation for 8 h with PMA, were prepared according to the method of Dignam [16]. All steps were carried out at +4°C, in the presence of phenylmethylsulfonyl fluoride (0.5 mM). The nuclear extracts were dialyzed against a buffer containing 12.5 mM HEPES (pH 7.9), 100 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 10% glycerol to bring the samples to optimal buffer and salt conditions for binding in EMSA, aliquoted and stored at −80°C.

2.4. Western blot analysis

5637 cellular extracts were subjected to SDS-PAGE (10% polyacrylamide) and Western blot analysis according to a previously described procedure [17]. Chemiluminescent detection was carried out according to the manufacturer's specifications (ECL Western blotting detection system, Amersham), using peroxidase conjugated antirabbit Ig (1:10000). Antibodies, recognizing members of the NF-κB family, were: anti-c-Rel (sc-71x), anti-RelA (sc-109x), anti-p50 (sc-1190x) used at 1:5000 dilution, all purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

2.5. Electrophoretic mobility shift assay (EMSA)

For binding assay, double stranded oligonucleotides corresponding to the A element in the GM-CSF promoter 5′-TCCTCAGCTCTG-GACTTCCCCCT-3′ (upper strand) [9] and to the NF-κB motif present in the HIV-LTR (5′-GTAGGGGACTTTCCGAGCTCGA-GATCCTATG-3′, upper strand) [18] were used as [^{32}P] labeled probes or as unlabeled competitors.

The assays were performed as previously described [9]: nuclear extracts of 5637 cells (3 μg), both unstimulated and stimulated for 8 h with PMA, or 1 μg of Mo nuclear extract, were pre-incubated in a 15 μl incubation mixture containing 12.5 mM HEPES (pH 7.9), 100 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol and 4 μg of poly (dI-dC) as a non-specific competitor for 10 min at room temperature. End-labeled oligonucleotides (5 fmol, about 10000 cpm) were added to each reaction mixture and incubated for an additional 15 min. The bound complexes were separated from free probe by electrophoresis on 5% polyacrylamide gels. Complexes were visualized by autoradiography of the dried gels.

Competition experiments were performed in the presence of a 50-, 100-, or 500-fold molar excess of unlabeled HIV-NF-κB or A oligonucleotide.

For supershift assay, 1 μg of c-Rel, p65 or p50 antibody was included in the pre-incubation mixture prior to the addition of the probe and loading onto polyacrylamide gels, and a lane containing pre-immune serum was always included as control.

3. Results and discussion

3.1. The A element participates in response to PMA stimulation in 5637 cells

PMA has been reported to stimulate GM-CSF promoter activity in Jurkat and MLA144 cell lines in transient transfection assay with a 193 bp promoter fragment immediately upstream of the transcription start site [19]. Since this fragment contains responsive κB recognition sites [5–7], we have investigated the role of the upstream A element, also containing a κB site [9], on PMA mediated regulation in 5637 cells.

The transcriptional effect of PMA on the κB element between −1984 and −2002 was verified by transfecting PF2000, PΔA, PF20 and PF24 plasmids in both uninduced and PMA-induced 5637 cells. Transfections showed a 3.4-fold stimulation of pPF2000 compared with uninduced level, while only a slight increase was obtained with pΔA (1.5-fold), a construct

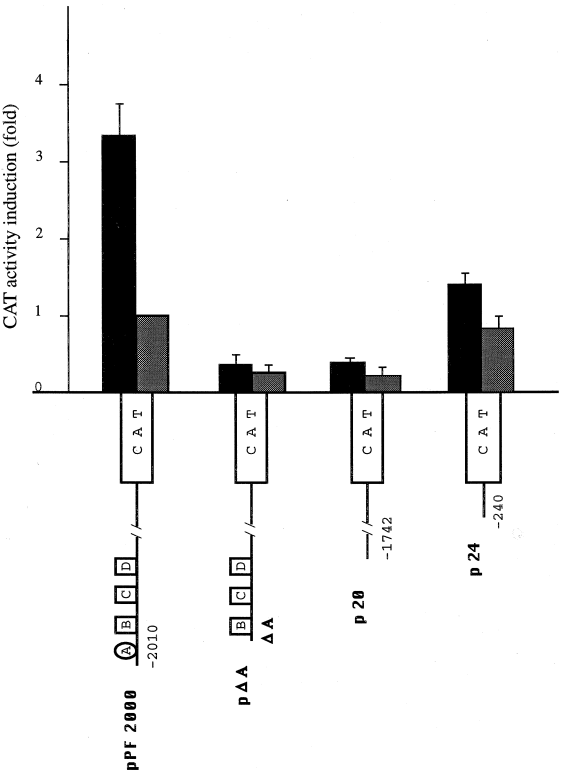

Fig. 1. Activation of the GM-CSF promoter by PMA in 5637 cells. Results of CAT assay (mean ± S.E.M. of four experiments performed in duplicate) are represented with grey bars for cells in basal conditions and dark bars for cells stimulated with PMA. Induction by PMA is expressed as fold stimulation relative to unstimulated cells. CAT activity of PF2000 in unstimulated cells was set to one and all other values were related to it.

deleted of the κB site, and with further deleted promoter constructs (1.9-fold for p20 and 1.6-fold for p24), as described in Fig. 1. In a preliminary subset of experiments we did not observe a significant stimulatory effect of PMA on T-lymphoid Mo cells transfected with all plasmid constructions (data not shown).

Therefore, we started to investigate PMA action on NF-κB family members binding to the A element in 5637 cells.

3.2. NF-κB members binding to the A element are induced by PMA stimulation

Optimal NF-κB subunit induction by phorbol ester was ascertained by a preliminary Western blot analysis of extracts from 5637 cells, both untreated and after stimulation for 4, 8, and 24 h. Treatment with PMA resulted in an increase of the endogenous p65 and c-Rel levels which peaked at 8 h and declined thereafter (Fig. 2). This time was chosen as the optimal time of stimulation, and nuclear extracts of cells treated for 8 h were used for EMSA and supershift analyses.

3.3. c-Rel and p65 subunits bind to the A element and to the HIV-NF-κB site

Gel shift analysis with the A element used as a labeled probe demonstrated induction of a binding complex showing two major bands, different from the one detected with Mo nuclear extracts (Fig. 3A). This complex showed an increased intensity in the nuclear extracts from PMA stimulated cells

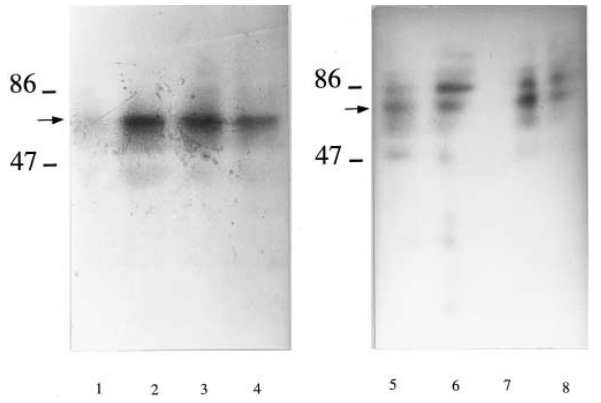

Fig. 2. Western blot analysis of 5637 cellular extracts using anti-p65 (lanes 1–4) and anti-c-Rel (lanes 5–8) antibodies. Extracts were from cells both untreated (lanes 1 and 5) and treated for 4 h (lanes 2 and 6), 8 h (lanes 3 and 7) or 24 h (lanes 4 and 8) with PMA. Molecular weight markers (kDa) are indicated on the left side. The arrows indicate p65 (lanes 1–4) and c-Rel (lanes 5–8).

and was found to be identical also using the κB DNA binding motif present in the HIV-LTR as a labeled probe (Fig. 3C).

Competition experiments (Fig. 3B) showed that the retarded complex observed could be very efficiently competed by an excess of the A oligonucleotide itself as well as by the HIV-NF-κB oligonucleotide.

EMSA analysis in presence of specific antibodies defined c-Rel and p65 as constituents of the complex, since the addition of the antibody to the binding mixture led to disappearance (with anti-c-Rel) or supershift (with anti-p65) of the retarded

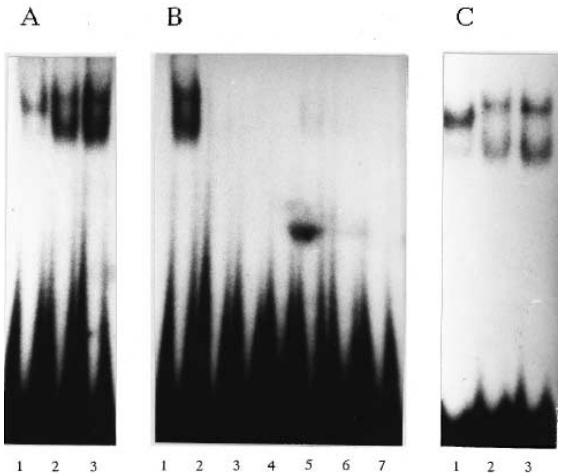

Fig. 3. EMSA assays showing binding of an NF-κB like complex to the A element and to the NF-κB site of HIV. The bands in lanes A1 and C1, A2 and C2, A3 and C3 refer to the same substance obtained with two different labeled oligonucleotides. A: The labeled A oligonucleotide was incubated with 1 μg of Mo nuclear extract (lane 1), 3 μg of 5637 nuclear extract in basal conditions (lane 2), and after stimulation for 8 h with PMA (lane 3). B: EMSA with PMA stimulated 5637 nuclear extract (3 μg) without competitor (lane 1) and in the presence of either a 50-, 100-, and 500-fold molar excess of the unlabeled A oligonucleotide as competitor (lanes 2, 3, 4 respectively) or of a 50-, 100-, and 500-fold molar excess of the HIV-NF-κB competitor (lanes 5, 6, 7 respectively). C: The labeled HIV-NF-κB oligonucleotide was incubated with 1 μg of Mo nuclear extract (lane 1), 3 μg of 5637 nuclear extract in basal conditions (lane 2), and after stimulation for 8 h with PMA (lane 3). The spot in the middle of B4–B5 was due to contamination and has no meaning for the experiment.

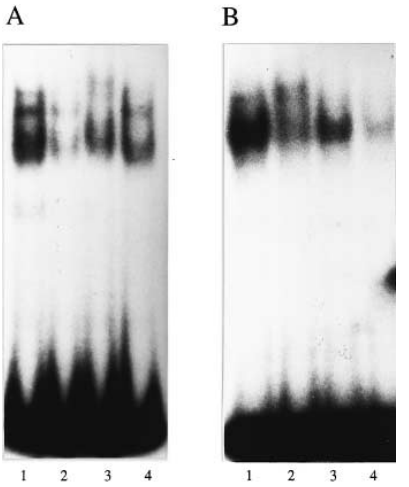

Fig. 4. EMSA assays in the presence of specific antibodies. A: 3 μg of 5637 nuclear extracts were incubated with the A oligonucleotide as a labeled probe in the presence of pre-immune serum (lane 1), or 1 μg of anti-p65 (lane 2), anti-c-Rel (lane 3) and anti-p50 (lane 4). B: 1 μg of Mo nuclear extract was incubated with the A oligonucleotide as a labeled probe in the presence of pre-immune serum (lane 1), or 1 μg of anti-p65 (lane 2), anti-c-Rel (lane 3) and anti-p50 (lane 4).

bands. The anti-p50 antibody caused a much less pronounced effect that might be due to cross reaction (Fig. 4A). A virtually identical pattern was observed when the HIV-LTR-κB site was used as labeled probe (data not shown). Nuclear extracts from Mo cells were also analysed for comparison. The addition of anti-c-Rel to the binding mixture led to supershift of the complex, as we previously described [9], anti-p65 had a minor effect on band intensity and anti-p50 caused a significant disappearance of the band (Fig. 4B). The different effect of anti-c-Rel, which causes supershift in Mo nuclear extracts and band disappearance in 5637, would suggest a different composition and/or heterodimerization of the active NF-κB subunits. In particular, p65 and c-Rel, detected with both the A and the HIV-NF-κB probes, appear as the major components of the retarded complex in 5637, whereas in Mo cells p50 and c-Rel seem to be the major components.

The finding that the active NF-κB complexes appear different in composition in extracts from Mo cells, which express the viral transactivator Tax, if compared with 5637 cells, supports the idea that κB elements may have distinct regulatory properties in different specific contexts and in the presence of differential levels of individual subunits [20].

It has previously been documented [21] that the gene for human GM-CSF is expressed in a tissue specific as well as in an activation dependent manner. Our results suggest that the upstream A element of the GM-CSF promoter, although recognized by a κB factor of different subunit composition depending on cell type, is involved in response to different stimuli acting through a similar activation pathway. In Mo cells the Tax transactivator is likely to be responsible for κB mediated activation of the GM-CSF promoter, while in the 5637 bladder carcinoma cell line, the tumor promoter PMA has the same effect, by acting on the same promoter element through an analogous κB mediated activation, probably via phosphorylation and degradation of the IκB cytoplasmic inhibitor which leads to active NF-κB complexes [12,13].

Treatment of 5637 cells with PMA, as has already been

described, results in an increase in GM-CSF mRNA [22] and, up to now, attention on NF-κB activation by PMA has been focused on the proximal promoter region [5,22]. Furthermore, the downstream NF-κB sites in GM-CSF promoter have been shown to confer strong inducibility upon T cell activation and Tax (in T-lymphoid cells) [5]. Little is known about NF-κB expression and DNA binding activity in non-lymphoid cancers [23]. Our results improve knowledge on the tumor promoter PMA induction in the bladder carcinoma 5637 cells by showing the role of the upstream A element. Moreover, we show that NF-κB mediated activation of GM-CSF transcription is common to lymphoid and non-lymphoid tumor cell lines.

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