An AP-1 site in the promoter of the human IL-5Rα gene is necessary for promoter activity in eosinophilic HL60 cells

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Abstract Interleukin-5 (IL-5) plays a crucial role in the proliferation, differentiation and activation of eosinophils. The IL-5 receptor is composed of an IL-5-specific α subunit, which is expressed by eosinophils and basophils, and a βc-subunit shared with the receptors for IL-3 and GM-CSF. We identified an AP-1 element which is important for IL-5Rα promoter activity in eosinophilic HL60 cells. The AP-1 site and the previously identified EOS1 site cooperate, since single mutation of either of the sites decreased promoter activity. We show that the AP-1 site of the IL-5Rα promoter binds multiple proteins, including cJun, CREB, and CREM.

Key words: Eosinophil; Interleukin-5 receptor; Transcription regulation; AP-1

1. Introduction

Cytokines such as interleukin-3 (IL-3) and GM-CSF are involved in the early differentiation of multiple lineages, whereas IL-5 is selectively controlling the terminal differentiation of eosinophils and basophils [1–3]. In humans the IL-5 receptor (IL-5R) consists of an α and a β chain, both members of the class I cytokine receptor family. The α chain (IL-5Rα) is exclusively expressed on eosinophils and basophils and binds IL-5 with moderate affinity [4–7]. The β chain is shared with the receptors for IL-3 and GM-CSF and is widely expressed in various hematopoietic lineages [8]. The β chain does not bind IL-5 by itself, but associates with the IL-5Rα subunit to form a high affinity receptor [5,9,10].

Expression of the IL-5Rα gene is critical to the entry of multipotential myeloid progenitors into an eosinophil/basophil developmental program. The gene encoding the human IL-5Rα chain is located on chromosome 3. Recently the 5′ upstream region of this gene was isolated, which appears to be highly active in eosinophilic cell lines [11]. The promoter region contains multiple consensus sites for known hematopoietic transcription factors, including GATA, PU1, C/EBP, and AP-1. Sun et al. [12] identified a unique enhancer-like *cis* element (EOS1) that is necessary and sufficient for promoter activity and binds an unknown transcription factor. In this study we have studied the involvement of an AP-1 site in the regulation of the IL-5Rα gene. The AP-1 family is a set of sequence specific transcription factors, including the Jun, Fos, and activating transcription factor (ATF, including CREB and CREM) families, which form a variety of hetero- and homodimers [13,14]. Interestingly, AP-1 family members are known to be involved in the regulation of myeloid differentiation [15–17].

In this study we demonstrate that, besides the EOS1 site, the AP-1 element of the IL-5Rα promoter located between −440 and −432 is also involved in the regulation of IL-5Rα chain expression and acts synergistically with the EOS1 site.

2. Materials and methods

2.1. Cell culture, reagents, and antibodies

Promyelocytic leukemia-HL60 cells were cultured in RPMI 1640 (Life Technologies, Breda, The Netherlands) supplemented with 8% fetal calf serum (FCS, Hyclone), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies) and 50 μM β-mercaptoethanol. HL60 7.7 cells were generated by culturing HL60 cells for two months at pH 7.7 in RPMI containing 25 mmol/l *N*-[2-hydroxyethyl]piperazine-*N*′-3-propane-sulfonic acid (EPPS; Sigma, St. Louis, MO, USA). To further differentiate HL60 7.7 cells butyric acid (0.5 mmol/l; Sigma) was added up to 6 days. Polyclonal rabbit antibody against cJun was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against CREB and CREM were described previously [18].

2.2. Synthetic oligonucleotides and plasmid construction

The following oligonucleotides were used in this study (only the upper strands are shown): AP-1 (5′-AGCTTAAATCATGTGTCAGTGTTGA-3′), AP-1 mutant (5′-AGCTTAAATCATGGGCCCGTGTTGA-3′), Col-TRE (5′-GATCTATCTGAGTCAGCAG-3′), *c*-jun TRE (5′-GATCTATCTGACATCAGCAG-3′), and SP1 (5′-AGCTTGGGGCGGGGCT-3′). A human genomic DNA library (λ Fix II, Stratagene) was screened using a random primed IL-5Rα probe (provided by Dr. B. Allet, Glaxo Institute). Secondary and tertiary screening was performed using an oligo corresponding to most of the first exon [6]. Isolated DNA fragments were cloned into the *Kpn*I-*Not*I sites of the pBKS II phagemid (Stratagene). Fragments of the IL-5Rα upstream sequence were generated using the polymerase chain reaction with primers containing *Hin*dIII and *Pst*I sites for sense and antisense sequences, respectively, and ligated into the pCAT basic vector (Promega). Site-directed mutagenesis was performed as described by Kunkel [19].

2.3. Electro mobility shift assay

Nuclear extracts were prepared from the different HL60 sublines following a previously described procedure [20]. Oligonucleotides were labeled by filling in the cohesive with [α-^{32}P]dCTP using Klenow fragment of DNA polymerase I (Life Technologies). Gel retardation assays were carried out according to published procedures with slight modifications [21]. Briefly, nuclear extracts were incubated in a total volume of 20 μl, containing 10 mM HEPES-KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 5 mM DDT, 20

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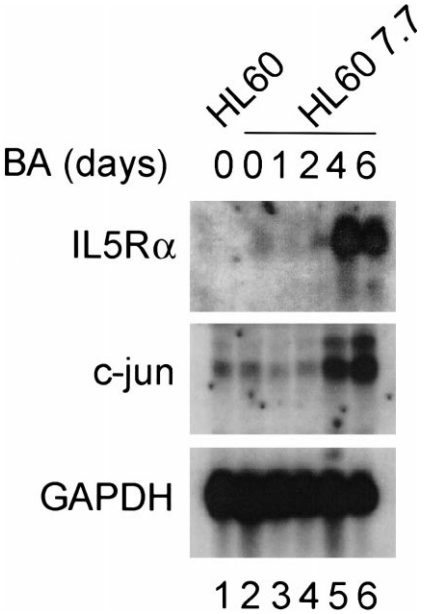

Fig. 1. The expression of the IL-5Rα chain is upregulated during the differentiation of HL60 7.7 cells. RNA was isolated from HL60 7.7 cells treated for various times with butyric acid (BA, 0.5 mM) and analyzed for expression of the IL-5Rα chain gene, *c*-jun and GAPDH. Eosinophilic differentiation of HL60 7.7 cells is clearly accompanied by an increase in expression of the IL-5Rα chain and *c*-jun genes.

μg bovine serum albumin (BSA), 2 μg poly(dI-dC) (Pharmacia Biotech.) and 1 ng of a [α-^{32}P]-labeled AP-1 oligonucleotide for 20 min at room temperature. In competition experiments, indicated molar excess of unlabeled oligonucleotide was added simultaneously with the [α-^{32}P]-labeled oligonucleotide. Antibodies were incubated 30 min on ice prior to the addition of [α-^{32}P]-labeled oligonucleotide. Protein-DNA complexes were then separated on 5% non-denaturing polyacrylamide gels and visualized by autoradiography.

2.4. Transient transfection and CAT assay

HL60 cells were transfected by electroporation (280 V, 960 μF). Transfected cells were harvested for CAT assays after 48 h. Cells were lyzed by repeated freeze thawing in 250 mM Tris, pH 7.4, 25 mM EDTA. Cellular extract was incubated in a total volume of 200 μl 200 mM Tris, pH 7.4, 4% glycerol, 0.5 mg/ml butyryl CoA and 0.05 μCi [^{14}C]chloramphenicol for 2 h at 37°C. Reaction products were then extracted using 400 μl xylene/pristane (1:2), and the percentage of acetylated products was then determined using liquid scintillation counting. A CMV-lacZ construct was co-transfected to correct for differences in transfection efficiency.

2.5. RNA isolation and Northern blotting

For Northern blotting, RNA (20 μg) was electrophoretically separated on 0.8% agarose gel and transferred to Hybond (Amersham, Arlington Heights, IL, USA). Blots were hybridized with randomly ^{32}P-labeled IL-5Rα, *c*-jun, or GAPDH fragments overnight at 42°C in hybridization buffer, washed, and exposed to film as described previously [22].

3. Results

IL-5Rα expression was previously shown to be upregulated during eosinophilic differentiation of HL60 clone 15 cells [10,23]. To investigate whether in HL60 cells differentiated at pH 7.7 IL-5R expression is also responsive to BA, RNA was isolated from HL60 7.7 cells treated for various periods with BA. Fig. 1 shows an increase in the expression of the IL-5R by BA treatment after 3 days. Reprobing the blot with a GAPDH probe shows that equal amounts of RNA were loaded in each lane (Fig. 1). These results demonstrate that HL60 7.7 cells are suitable for studying the regulation of the IL-5Rα promoter.

Previously it was shown that the EOS1 enhancer located at

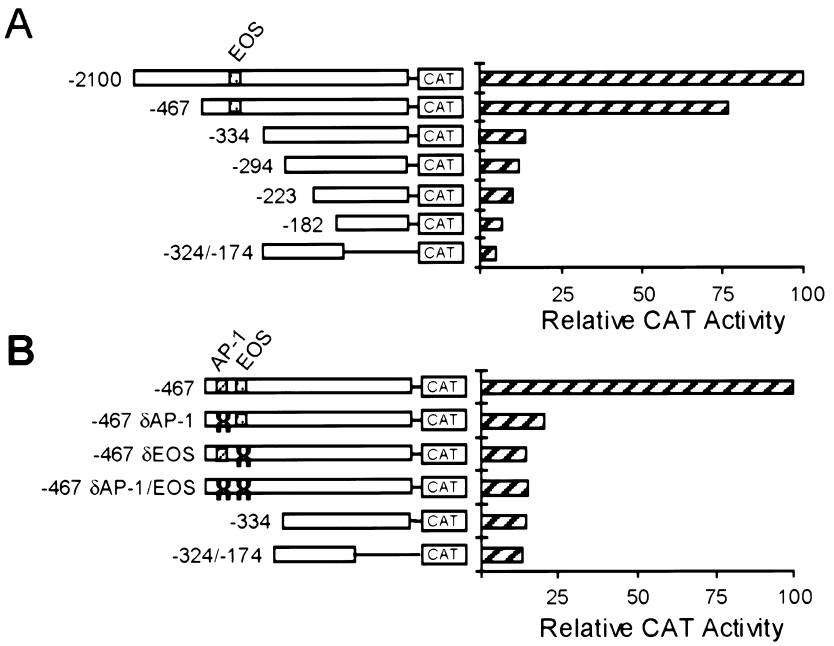

Fig. 2. An AP-1 site in the promoter of IL-5Rα chain is involved in its activity in eosinophilic HL60 cells. A: HL60 7.7 cells treated for three days with BA were transfected with various progressive 5′ deletion constructs of the IL-5Rα chain promoter. Two days after transfection, CAT assays were performed to determine the regions of the IL-5Rα chain promoter involved in its eosinophilic activity. B: IL-5Rα chain promoter constructs with mutations in either the EOS1 site or the AP-1 site were analyzed as described in A. It is clear that both the EOS1 site and the AP-1 site are necessary for full promoter activity.

−430 to −422 was necessary for the activity of the IL-5Rα chain promoter in HL60 clone 15 cells [12]. We transfected HL60 7.7BA cells with a number of 5′ deletion constructs to determine the IL-5Rα chain promoter region essential for activity in eosinophilic cells. Fig. 2A shows that deletion of a promoter region between −467 and −408 results in a complete loss of promoter activity in BA-differentiated HL60 7.7 cells. This region contains the previously identified EOS1 enhancer [12]. However, sequence analysis of the promoter of the IL-5Rα chain revealed the presence of a potential AP-1 binding site (5′-ATGTGTCAG-3′) next to the previously identified EOS1 site [12]. Since members of the AP-1 family are known as positive regulators of myeloid differentiation [15–17], we determined whether the AP-1 site plays a role in promoter activity. Therefore the AP-1 site was mutated in the −467 construct and promoter activity was determined in HL60 7.7BA cells. Mutation of this EOS1 site resulted in a dropped promoter activity, comparable to background, as shown previously [12]. Surprisingly, single mutation of the AP-1 site resulted also in a complete loss of promoter activity of the construct (Fig. 2B). The activity of the construct having both the EOS1 and the AP-1 site mutated was comparable with background level (Fig. 2B). These results show that the EOS1 and the AP-1 site are both involved in the regulation of the IL-5Rα gene.

To investigate the nature of the proteins binding to the putative AP-1 binding site in the IL-5Rα gene promoter, gel mobility shift assays with a radio-labeled double stranded oligonucleotide containing this AP-1 site were performed. Fig. 3A shows a complex that binds to the AP-1 element in HL60 7.7BA cells. The specificity of the protein-complex binding to the AP-1 probe was demonstrated by competition with a 10–100-fold molar excess of the unlabeled oligonucleotide (Fig. 3A). By contrast, oligonucleotides containing a mutated AP-1 site or a control SP-1 binding site were not able to compete for binding (Fig. 3A). To study the properties of the AP-1 element, we examined the competition of the formation of this complex with known AP-1 sites. Oligonucleotides containing a TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element (TRE) from the collagenase promoter, or from the *c*-jun promoter were able to block the binding to the AP-1 site of the IL-5Rα promoter, although to somewhat different extents (Fig. 3A). In addition, the binding could also blocked by an oligo containing a cAMP response element (CRE) (data not shown). These results indicate that these sites bind proteins or protein complexes that are also able to bind the AP-1 site of the IL-5Rα promoter.

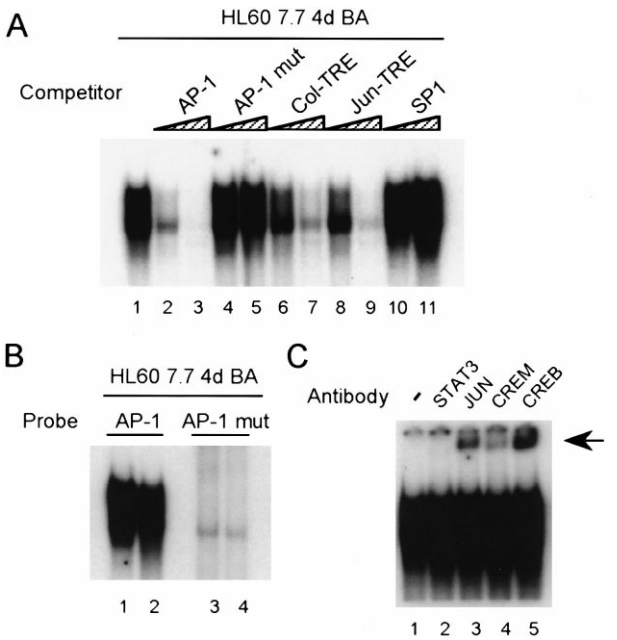

Fig. 3. The AP-1 site in the IL-5Rα chain promoter binds multiple AP-1 family proteins. A: Nuclear extracts isolated from HL60 7.7 cells treated for 4 days with BA were tested for proteins binding to the AP-1 site from the IL-5Rα chain promoter in a gel shift experiment. The specificity of the complex was determined by competition with 10–100-fold molar excess of unlabeled self-oligonucleotide of mutated AP-1 site, SP-1 site, collagenase TRE or *c*-jun TRE. B: Mutation of the AP-1 site completely inhibits protein binding to the oligonucleotide, as shown by gel shift analysis using the wild-type or mutated AP-1 site from the IL-5Rα chain promoter as a probe. C: Supershift analysis with antibodies to *c*-jun, CREB, CREM show the binding of these proteins to the AP-1 site from the IL-5Rα chain promoter in HL60 7.7BA cells. An antibody to STAT3 was used as a negative control.

To further characterize the protein complex binding to the AP-1 binding site within the IL-5Rα promoter, nuclear extracts of HL60 7.7BA cells were incubated with antibodies against different AP-1 family members and additional transcription factors prior to detection of DNA-protein interactions by gel mobility shift assay. The antibodies against CREB, CREM and cJun induced a supershift of the labeled DNA probe (Fig. 3C), indicating that their respective antigens participate in the formation of the complex bound to the AP-1 site of the IL-5Rα promoter. In contrast, there was no supershift with antibodies against JunD, *c*-fos, and ATF3 (data not shown).

While CREB and CREM are ubiquitously expressed proteins, the expression of cJun is tightly regulated. Therefore, we analyzed the expression of cJun mRNA in HL60 7.7 cells after addition of BA which coincides with the increase in expression of the IL-5Rα gene (Fig. 1). There is a remarkable increase in the expression of cJun mRNA after three to four days of addition of BA. Treatment of HL60 7.7 cells with retinoic acid, an inducer of neutrophilic HL60 differentiation, did not induce cJun mRNA expression (data not shown). These results indicate that cJun expression is upregulated during eosinophilic differentiation of the HL60 7.7 cell line, and is likely to play an important role in regulating IL-5Rα expression.

4. Discussion

It has been previously reported that the activity of the IL-5Rα promoter is regulated by the EOS-1 element which binds an unknown myeloid specific factor [12]. As shown in this study, expression of the IL-5Rα gene is also regulated by the AP-1 element of the promoter. Since single mutation of one of the sites inhibited promoter activity, these results indicate that the proteins binding to the AP-1 site and EOS1 site cooperate. This suggests that an eosinophil specific transcription factor cooperates with a general transcription factor and together regulate the expression of the IL-5Rα chain.

Gel retardation assays showed the binding of a nuclear protein complex to the AP-1 oligonucleotide. This binding could be inhibited with the unlabeled self-oligonucleotide and also with other known AP-1 elements, such as a CRE

site and TRE sites from the collagenase or the *c*-jun promoter. These results suggest that the AP-1 site of the IL-5Rα promoter is most related to the CRE which can bind dimers of the ATF family and TRE sites which binds cJun. We showed with supershift analysis the presence of the proteins cJun, CREB, and CREM in the shifted complex. There was not a supershift with antibodies against other AP-1 family members such as JunD, *c*-Fos, and ATF3. This suggests a role for cJun, CREB, and CREM in the binding complexes of the AP-1 site. The expression of cJun mRNA is increased during differentiation of HL60 cells, indicating that cJun might play a role in eosinophil specific gene transcription. It may be that during eosinophilic differentiation the expression of the IL-5Rα chain gene is regulated by different protein complexes of AP-1 family members. Interestingly, others also found the involvement of AP-1 family members in the differentiation of myeloid cells [15–17].

Recently, a second promoter region for the IL-5Rα gene was identified [24], located within the first intron. This second promoter is only active in eosinophilic HL60-C15 cells and the nuclear factor binding to this promoter is also only present in these cells. The existence of another promoter site of the IL-5Rα chain suggests that the transcription of IL-5Rα is under the control of multiple promoters, like other genes such as insulin-like growth factor and colony stimulating factor 1 [25,26]. It is possible that, during eosinophil maturation, an alternative use of the promoters may determine stage-specific expression of the IL-5Rα gene.

The production of eosinophils from bone marrow progenitors as well as the priming, activation, and enhanced survival of mature eosinophils is regulated by IL-5 [1–4]. Understanding the regulation of the IL-5Rα gene orders an opportunity in controlling the development, differentiation, activation, and prolonged survival of the mature eosinophils. We have shown the contribution of the AP-1 site to this regulation. Further experiments are needed to resolve the precise roles of the EOS1 and AP-1 elements and binding proteins.

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