Silencer Activity of NFATc2 in the Interleukin-12 Receptor β2 Proximal Promoter in Human T Helper Cells*

Received for publication, March 21, 2001, and in revised form, June 27, 2001
Published, JBC Papers in Press, July 3, 2001, DOI 10.1074/jbc.M102536200

Johanna G. I. van Rietschoten§§, Hermelijn H. Smits‡, Diederik van de Wetering‡, Robert Westland‡, Cor L. Verweij, Marcel T. den Hartog§, and Eddy A. Wierenga‡‡‡

From the ‡Academic Medical Center, University of Amsterdam, Department of Cell Biology and Histology, P. O. Box 22700, 1100 DE Amsterdam, §Tanox Pharma B.V., Kruislaan 318, 1098 SM Amsterdam, and ‡Free University Medical Center, Department of Molecular Cell Biology, P. O. Box 7057, 1007 MB Amsterdam, The Netherlands

Interleukin 12 (IL-12) is a potent enhancer of interferon γ production by activated T cells. The high-affinity IL-12 receptor (IL-12R) is a heterodimer of a β1 and a β2 subunit. Expression of the signaling IL-12Rβ2 chain is usually low, as compared with the more abundant β1 chain, and may be rate-limiting for IL-12 sensitivity. Little is known about the mechanisms controlling IL-12Rβ2 gene expression. Reporter gene assays in IL-12Rβ2-expressing Jurkat cells showed that truncation of the region from −151 to −61 abrogated promoter activity. The proximal promoter region does not contain a typical TATA box, suggesting a role for SP-1. Indeed, mutagenesis of the −63 SP-1 consensus site decreased transcription by 50%. Electrophoretic mobility shift experiments confirmed the binding of SP-1 and SP-3 at this site. In contrast, truncation of −252 to −192 increased promoter activity. Likewise, mutagenesis of the consensus nuclear factor of activated T cells site at −206 increased promoter activity by 70%, suggesting silencer activity of this element. Electrophoretic mobility shift experiments with primary Th (T helper) cells showed the formation of a specific, T-cell receptor-inducible complex at this site that is sensitive to cyclosporin A and supershifted with anti-NFATc2 in both Th1 and Th2 cells. Accordingly, cyclosporin A dose-dependently increased IL-12Rβ2 mRNA expression. These first data on IL-12Rβ2 gene regulation indicate a TATA-less promoter, depending on SP-1/SP-3 transcription factors, and a negative regulatory NFAT element at −206. This element may contribute to the overall low level of IL-12Rβ2 expression on Th cells.

t helper (Th) cells can be categorized according to their cytokine expression profiles. The differential generation of Th cells expressing Th1 and/or Th2 cytokines is key to the outcome of both protective and pathologic immune responses (1, 2). Th1 cells secrete high levels of IFNγ and favor cellular immunity to intracellular pathogens, whereas Th2 cells secrete IL-4 and favor humoral immunity to extracellular pathogens (3). The polarization process of naive T cells is directed by cytokines that are present during initiation of the naive T-cell response. In this respect, IL-4 promotes Th2 cell development, whereas the antigen-presenting cell-derived cytokine IL-12 is a potent inducer of IFNγ production and of the generation of Th1 cells (4, 5). For Th cells to respond to these cytokines, they need functional receptors.

A functional high-affinity IL-12R is composed of two protein subunits, the IL-12Rβ1 and IL-12Rβ2 chains. In the human the β1 and β2 subunits contribute equally to IL-12 binding (6). The β2 chain, however, appears to be rate-limiting for IL-12 responsiveness, as it is crucial for signal transduction (7) and, in contrast to the more abundant β1 chain, is expressed to a maximum of only a few hundred molecules per cell (6). We have previously shown that allergen-specific Th2 cell clones generated from atopic patients revealed a complete lack of signaling via the IL-12R, as indicated by their inability to phosphorylate STAT4 (8) and to secrete IFNγ in response to IL-12. Rogge et al. (9) showed that development of naive T cells into Th2 cells is associated with IL-4-mediated suppression of IL-12Rβ2 mRNA and protein expression leading to the loss of IL-12 responsiveness and, consequently, the inability of IL-12 to promote IFNγ production. As IL-12 responsiveness is a major parameter in the regulation of specific immunity, we started to unravel the molecular mechanisms that govern the transcriptional regulation of the IL-12Rβ2 gene in human Th cells.

To this aim, we cloned 0.6 kilobase of the 5′ flanking region and, by serial truncation, tested for promoter activity applying a reporter gene assay in IL-12Rβ2-expressing Jurkat T cells. In this report we provide the first experimental evidence that SP-1 family members are important for basal and inducible activity of the TATA-less core promoter of the IL-12Rβ2 gene and that the inducible transcription factor NFATc2 binding at −206 has a suppressive role in IL-12Rβ2 expression. This suppressive activity does not underlie the loss of IL-12Rβ2 expression in Th2 cells.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 ligase were purchased from Promega (Leiden, The Netherlands). All high pressure liquid chromatography-purified oligonucleotides were purchased from BIOSOURCE (Nivelles, Belgium). BatEII-digested A DNA (New England Biolabs, Beverly, MA) was used as a molecular weight reference.

Plasmid Construction—Clone pAC188 containing IL-12Rβ2-encoding genomic DNA was selected by screening a human genomic PAC
Transcriptional Regulation of the Human IL-12Rβ2 Gene

**Table I**

Oligonucleotides used in this study

| Description | Location | Purpose | Primer sequences<sup>a</sup> |
|-------------|----------|---------|----------------------------|
| ∼591        | −591 to −572 | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| −404        | −404 to −384 | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| −252        | −252 to −231 | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| −192        | −192 to −171 | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| −151        | −151 to −133 | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| −61         | −61 to −44  | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| −36         | −36 to −17  | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| AS          | +34 to +54  | Cloning | 5′ gcgcagctgcatctaatcttattgttct 3′ |
| NFAT WT     | −224 to −184 | EMSA | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| NFAT Mut    | −224 to −184 | EMSA/MUT | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| SP-1 Mut    | −17 to 151   | MUT | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| SP-1#2 WT   | −81 to −44   | EMSA | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| SP-1#2 Mut  | −75 to −50   | Mut | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| SP-1#2 Mut  | −81 to −44   | EMSA | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| SP-1 HSV    | n.a. | EMSA | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| NF-αB       | n.a. | EMSA | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| IL-12Rβ1 S  | n.a. | RT-PCR | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| IL-12Rβ1 AS | n.a. | RT-PCR | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| IL-12Rβ2 S  | +1146 to +1166 | RT-PCR; LC | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| IL-12Rβ2 AS | +1466 to +1486 | RT-PCR; LC | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| IL-2 S      | n.a. | LC | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| IL-2 AS     | n.a. | LC | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| β2m S       | n.a. | RT-PCR; LC | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |
| β2m AS      | n.a. | RT-PCR; LC | 5′ tgtactctggccatgctttccttgctggagcactcactct 3′ |

<sup>a</sup> S, sense oligonucleotide; AS, anti-sense oligonucleotide.

<sup>b</sup> Location, represents the location of the oligonucleotide in the IL-12Rβ2 gene relative to the reported start of the mRNA (10), n.a., not applicable for these oligonucleotides.

<sup>c</sup> Mut, LC, oligonucleotide used, respectively, for mutagenesis or Light Cycler.

<sup>d</sup> Appropriate transcription factor core-binding elements are underlined; dinucleotide substitutions in the mutant (Mut) relative to the corresponding wildtype (WT) probes are indicated in italics; bold nucleotides represent 5' overhang used to fill in by Klenow fragment the double-stranded oligo with [α-<sup>32P</sup>]dATP and d(C/G/T)TP.

library (Genome Technology Center, Leiden University Medical Center, Leiden, The Netherlands) using IL-12Rβ2 cDNA (+751 to +3229) as a probe. Starting from IL-12Rβ2 exon 1, clone pAC188 was sequenced in the 5' direction. A fragment spanning −591 to +54 (relative to the start of the reported cDNA sequence (10)), designated construct −591, was amplified by PCR using the pAC188 plasmid as a template. For cloning purposes, the 5'-sense primers were designed with an additional SacI restriction site and the 3'-antisense primer with a natural HindIII site, resulting in PCR products spanning through +54. Serial deletion fragments, designated −404 (−404 to +54), −252 (−252 to +54), −192 (−192 to +54), −151 (−151 to +54), −61 (−61 to +54), and −36 (−36 to +54), were generated by varying the 5'-sense primer (Table I). The PCR products were subcloned into the pGEM®-8T Easy plasmid (Promega) following the directions of the manufacturer. All constructs were checked by DNA sequencing using Thermo Sequenase (PerkinElmer Life Sciences) on an ABI Prism 310 Genetic Analyzer (PerkinElmer Life Sciences). For transfection studies, pGL3-enhancer (pGL3e) vector (Promega) was used, which contains the Firefly luciferase gene. The cloned PCR products and the pGL3e vector (Promega) were digested with SacI and HindIII or SacI and Smal, respectively, agarose gel-purified (Qiagen, Hilden, Germany), and ligated with T4 DNA ligase (Promega). pGL3e constructs were checked by sequencing. Plasmid DNA was prepared from bacterial cultures using Qiagen Plasmid Midi Kits.

**Mutagenesis**—Site-directed mutagenesis of the IL-12Rβ2 −591 to +54 promoter construct was carried out using the Altered Sites II in vitro mutagenesis system from Promega. All reactions were carried out according to the manufacturer's instructions. The internal forward primers containing the mutated sites are shown in Table I. Products from this procedure were cloned into pGL3e and sequenced to confirm the introduction of the desired mutations.

**Transient Transfection Studies**—Jurkat cells (5 × 10<sup>5</sup>) expressing both IL-12Rβ1 and IL-12Rβ2 were electroporated in the presence of 250 μg of pRL-CMV, an expression vector containing the Renilla luciferase gene under the control of a cytomegalovirus promoter (Promega), and 250 μg of pGL3e vector (Promega), which was added to each sample. To compensate for size differences of the constructs, empty pGL3e vector was added to obtain an equal amount of DNA in each sample. Immediately after transfection, 9.5 ml of complete culture medium was added (Isco/modified Dulbecco's medium, Bio-Whittaker, Walkersville, MD) supplemented with 5% pooled, C-inactivated fetal calf serum (BioWhittaker) and gentamycin (80 μg/ml; Duchefa, Haarlem, The Netherlands). Cells were seeded in 2 wells of a 6-well culture plate (Costar, Cambridge, MA). After 1 day of culture at 37 °C, cells were either left unstimulated or stimulated with 24 h of 1 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) or with mouse mAbs to CD3 (1 μg/ml; CLB-CD3/4E) and CD28 (2 μg/ml; CLB-CD28/1), both obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Cells were harvested 48 h post-transfection. Using the Dual Luciferase reporter gene assay (Promega), cell lysates were prepared and both Firefly luciferase and Renilla luciferase were measured by a dual luminometer (Promega). Luciferase activities were normalized for transfection efficiency using Renilla luciferase activity. Transfections were performed in duplicate, and the results of at least three independent experiments were calculated as the mean ± S.D. values for luciferase activity.

**T-cell Isolation, Culture, and Stimulation**—Peripheral blood mononuclear cells from healthy individuals were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Torshov, Norway). Highly purified CD<sup>+</sup> T cells (normally >98% as assessed by flow cytometry) were obtained from peripheral blood mononuclear cells with anti-CD4-coated Dynabeads (Dynal AS, Oslo, Norway) as described below (12). CD4<sup>+</sup>CD45RA<sup>−</sup>CD45RO<sup>−</sup> naive Th cells were isolated from peripheral blood mononuclear cells through one-step high-affinity negative selection columns (R&D Systems, Abingdon, UK). Naive Th cells were stimulated under Th1 or Th2 driving conditions in IL-12 and IL-4, respectively, as described before (13) to generate highly polarized Th1 and Th2 cells. To test the effect of Ca<sup>2+</sup> on IL-12Rβ2 mRNA expression, naive Th cells were stimulated for 3 days with immobilized CD3 mAb and soluble CD28 mAb (13) in 96-well culture plates (Costar; 10 (5) cells/well) with or without CsA at increasing concentrations (10–1000 ng/ml). All T-cell cultures were formed in complete culture medium with rIL-2 (10 units/ml; Chiron, Emeryville, CA). Proliferative responses were assayed in parallel cultures of 2 × 10<sup>4</sup> cells/well after 3 days as described before (12).

**Preparation of Whole Cell and Nuclear Protein Extracts and Electrophoretic Mobility Shift Assays**—Whole cell protein extracts were prepared from 5 × 10<sup>5</sup> CD<sup>+</sup> T cells. Nuclear protein extracts were prepared from 5 × 10<sup>6</sup> cells CD<sup>+</sup> T cells (Th1 or Th2 cells, which were left unstimulated or were stimulated with anti-CD25/anti-CD28 for 30 min in the presence or absence of cyclosporin A (CsA; 1 μg/ml, Sigma-Aldrich). Cells were washed with ice-cold phosphate-buffered saline. Nuclear and whole cell protein extracts were isolated essentially as described before (8), and the protein concentrations were determined by a Bradford microassay (Bio-Rad) using a calibrated solution of bovine serum albumin (BSA, Bio-Rad).
Bank accession number AF349574. The DNA sequence as deposited under GenBank™ accession number AF349574. The most distal 5′ nucleotide of each oligonucleotide used to construct the different deletion fragments is indicated by a gray triangle. SP-1 family consensus binding elements (SP-1#1 and SP-1#2) and the NFAT consensus binding element are indicated in bold type above the consensus sequence (underlined).

serum albumin (Sigma-Aldrich) as a reference. The samples were aliquoted and stored at –80 °C. An electrophoretic mobility shift assay (EMSA) was performed as described before (9) with some minor modifications. The double-stranded DNA probe was [α-32P]dATP-labeled using the Prime-a-gene labeling system (Promega) and purified using Bio-Spin 6 chromatography columns (Bio-Rad). The binding reaction was incubated at 4 °C for 45 min. Cold competitor oligonucleotides were added to the reaction mix prior to the protein extract. The SP-1 consensus sequence, binding SP-1 family members, is derived from human herpesvirus. The NF-κB consensus oligonucleotide was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The nomenclature and sequences of the oligonucleotides used in this study are summarized in Table I. For supershift experiments, 4 μg of IgG, mAb NFATc1 (sc-7294, Santa Cruz), 0.5 μg of IgG, mAb NFATc2 (BD Transduction Laboratories, San Diego, CA), 0.4 μg of polyclonal IgG SP-1 antibody (sc-59-G, Santa Cruz), 4 μg of IgG, mAb SP-3 (sc-644-G, Santa Cruz), or a nonrelevant IgG, isotype control were added to the binding reaction for an additional 45 min at 4 °C. The whole sample was then loaded onto a 4% polyacrylamide gel in 0.5× Tris/borate/EDTA buffer. The gels were prerun for 30 min and then run for 2 h at RT at 150 V. The gels were transferred to Whatman filter paper, dried, and exposed to x-ray film (Kodak XAR5 films, Rochester, NY) at –80 °C.

Reverse Transcriptase (RT)-PCR and Real-time Quantitative RT-PCR Analysis of IL-12Rβ2, IL-12Rβ1, IL-2, and β2m mRNA Expression and IL-13 Measurement—RT-PCR was performed as described before (10) with IL-12Rβ1-, IL-12Rβ2-, and β2m-specific primers (Table I). For quantitative analysis of IL-12Rβ2 mRNA expression, naive Th cells were stimulated as described above and lysed for total RNA extraction at day 3 using a Nucleo Spin RNA isolation kit (Macherey-Nagel, Duren, Germany). First-strand cDNA was synthetized from total RNA using a cDNA synthesis kit (MBI-Fermentas, St. Leon-Rot, Germany). Real-time quantitative PCR was performed in a Light-Cycler (Roche Diagnostics, Almere, The Netherlands) based on specific primers and general fluorescence detection with SYBR green. β2m was used as a control. The primer sequences for IL-12Rβ2 (melting temperature 60 °C), IL-2 (melting temperature 58 °C) and β2m (melting temperature 60 °C) are listed in Table I. Measurement of IL-13 levels in culture supernatants were performed using the PeliKine compact human IL-13 enzyme-linked immunosorbent assay kit obtained from the CLB.

RESULTS

Identification of the IL-12Rβ2 Core Promoter and Regulatory Regions.—To characterize the proximal promoter of the human IL-12Rβ2 gene, we analyzed 0.6 kilobase of 5′ flanking sequence obtained from a genomic pAC clone. This fragment, which is depicted in Fig. 1A, spans the region –591 through +54 relative to the start of the IL-12Rβ2 mRNA (10). In the immediate 5′ region, no typical TATA box or CCAAT box motif was found. To identify core promoter and regulatory elements, the transcriptional activity of the truncated constructs of the IL-12Rβ2 5′ upstream region (Fig. 2A) were assayed. For that purpose, full-length construct –591 and a series of 5′ deletion fragments were subcloned into the pGL3-Enhancer vector, a promoterless luciferase reporter vector with an SV40 enhancer. All constructs were tested for promoter activity in unstimulated and PMA/ionomycin-stimulated conditions in Jurkat T cells, showing constitutive
expression of the IL-12Rβ1 chain and low but inducible expression of the IL-12Rβ2 chain (Fig. 2B). The expression of the IL-12Rβ2 chain in Jurkat cells has been described (15). Promoter activity was up-regulated in stimulated cells transfected with constructs \(591\), \(404\), \(252\), \(192\), and \(151\). Deletion from \(591\) to \(404\) and further to \(252\) reduced the reporter activity stepwise, suggesting multiple positive cis-acting elements between \(591\) and \(252\). Interestingly, deletion from \(252\) to \(192\) led to an increase in promoter activity, suggesting the presence of a suppressor element(s) in this region. Promoter activity was abrogated after truncation to \(61\) and \(36\), indicating that sequences in close upstream proximity of \(61\) are crucial for basal and inducible IL-12Rβ2 promoter activity. Similar results were found upon stimulation with anti-CD3 and anti-CD28 (data not shown).

**Identification of Functional Motifs in the Core Promoter and Negative Regulatory Region**—Two potential binding sites for the SP-1 family of transcription factors (core sequence GGGCGG (16)) are located in the proximal promoter region at \(-3\) and \(-63\) (Fig. 1B). As SP-1 is frequently involved in transcription initiation in the absence of a TATA box (16), we analyzed whether these DNA motifs at \(-3\) (SP-11) and \(-63\) (SP-12) participate in the regulation of IL-12Rβ2 promoter activity. To examine the relative roles of these two SP-1 sites for promoter activity, we mutated the GGGCGG motifs in these elements to GTTCGG in the context of the full-length promoter construct \(591\) to \(54\). We thus generated two IL-12Rβ2 promoter-reporter gene constructs with either one of the SP-1 sites mutated. Promoter activity was tested after transient transfection of Jurkat cells and compared with the wild type full-length IL-12Rβ2 promoter activity. As shown in Fig. 3, mutation of the SP-1 site at \(-3\) does not result in a significant change of activity, whereas mutation of the SP-1 site at \(-63\) results in a reduction of promoter activity by almost 50%, suggesting an important role of this cis-regulatory element. As determined by serial truncation, deletion of the promoter re-
region from −252 to −192 resulted in increased promoter activity. Within this region a reversed NFAT consensus binding site (TTTCC) is located at −591. To examine the functional significance of this NFAT consensus site, we mutated the TTTCC motif into TTTAA in the context of the full-length promoter construct −591 to −206 (TTTCC to TTTAA) as indicated by stars. Cells were stimulated with PMA and ionomycin for 24 h. Corrected luciferase activity was calculated, and promoter activity was expressed as a percentage of wild type promoter activity (top bar, 100%). In addition to deletion fragments, pGL3e (empty vector) is shown as a control.

**FIG. 3.** The −63 SP-1 and −206 NFAT motifs are important for promoter activity. Jurkat cells were transiently transfected with luciferase constructs containing the full-length wild type promoter (construct −591) or the full-length promoter containing the mutated SP-1 motif at −3 or −63 (GGGGCGG to GTTCGG), or the mutated NFAT motif at −206 (TTTCC to TTTAA) as indicated by stars. Cells were stimulated with PMA and ionomycin for 24 h. Corrected luciferase activity was calculated, and promoter activity was expressed as the percentage of wild type promoter activity (top bar, 100%). In addition to deletion fragments, pGL3e (empty vector) is shown as a control.

**FIG. 4.** Identification of −63 binding proteins. The SP-1#/2 wild type (WT) radiolabeled probe was incubated with whole cell extracts from unstimulated CD4⁺ T cells. The binding activities were competed for with excess cold oligonucleotides: either SP-1#/2 WT, SP-1#/2 Mut, SP-1 herpesvirus consensus, or nonrelevant NF-κB consensus. The -fold molar excess of the competitor oligonucleotides is indicated, ranging from 10- to 90-fold. Arrowheads indicate specifically competed complexes (C1 and C2). The addition of anti-SP-1 (lane 11), anti-SP-3 (lane 12), or both (lane 13) resulted in the supershift (S) of complex C1, C2, or both, respectively.

Identification of Nuclear Factors Binding to the −63 SP-1 Motif—To characterize transcription factor binding activities at the SP-1#/2 element at −63, we performed EMSAs using whole cell extracts from CD4⁺ T cells. EMSA with the double-stranded oligonucleotide SP-1#/2 WT containing the intact −63 SP-1 element demonstrated the formation of two DNA-protein complexes C1 and C2 (Fig. 4, lane 1) not formed in the absence of protein extract (data not shown). The formation of these radioactive complexes was dose-dependently inhibited by competition with a 10-, 30-, or 90-fold molar excess of unlabeled SP-1#/2 WT oligonucleotide (lanes 2–4) but was not affected by a 90-fold molar excess of the mutated SP-1#/2 Mut oligonucleotide (lane 5). Similar to the SP-1#/2 WT oligonucleotide, competition with a 10-, 30-, and 90-fold molar excess of a specific SP-1 consensus oligonucleotide dose-dependently competed the bands away (lane 6–8), whereas a 90-fold molar excess of an oligonucleotide containing a nonrelevant NF-κB consensus binding site had no effect (lane 9). SP-1 and SP-3 are known to bind to identical DNA elements (17). Therefore, binding reactions were performed in the presence of anti-SP-1, anti-SP-3, or both antibodies. The addition of anti-SP-1 antibody resulted in a supershift of most of complex C1 (Fig. 4, lane 11), whereas with anti-SP-3 antibody, a complete supershift was observed of the less abundant complex C2 (lane 12). The combination of both anti-SP-1 and anti-SP-3 antibodies did not result in additional shifts. These results suggest the binding of SP-1 and SP-3 at the −63 element.

**Identification of Nuclear Factors Binding to the −206 NFAT Motif**—We next tested whether NFAT could actually interact with the putative binding site at −206 in the IL-12Rβ2 promoter. To do this, we used the −220 to −180 DNA sequence as a double-stranded probe for EMSA. Nuclear extracts from anti-CD3/anti-CD28-stimulated CD4⁺ T cells showed the inducible formation of complex A in addition to the increased intensity of a preexisting complex P (Fig. 5A, lanes 3 and 4). The formation of complexes A and P was abrogated by mutation of the probe (TTTCC to TTTAA; data not shown). Activation of the CD4⁺ T cells in the presence of the immunosuppressant drug CsA, known to inhibit the nuclear translocation of NFAT, inhibited the formation of complex A but not P (Fig. 5B, lane 5). These results indeed suggest the involvement of NFAT in complex formation with the −206 element. Of the growing family of NFAT proteins, NFATc1 (NFATc, NFAT2) and NFATc2 (NFATp, NFAT1) are most prominent in peripheral T cells (18) and bind to the same DNA motif (19, 20). To identify whether NFATc1 or NFATc2 is involved in complex A, binding reactions were performed in the presence of antibodies to NFATc1 or NFATc2. A supershifted...
band (S) was obtained with NFATc2 antibody (Fig. 5A, lane 7) but not with antibody to NFATc1 (Fig. 5A, lane 6) or with the IgG1 isotype control antibody (data not shown), indicating the inducible binding of NFATc2 at −206.

Because expression of the *IL-12Rβ2* gene is suppressed in Th2 cells and NFATc2 plays a role in the suppression of Th2-type cytokines (21, 22), we next tested for differential binding activity of NFATc2 to the −206 element comparing nuclear extracts from Th1 and Th2 cells. However, neither in unstimulated nor in anti-CD3/anti-CD28 stimulated cells any difference was observed between Th1 and Th2 cell extracts (Fig. 5B). Preexisting complex P showed a similar increased intensity in Th1 and Th2 cells after TCR stimulation. Also, complex A was induced to the same extent in TCR-stimulated Th1 cells (lanes 1 and 2) and Th2 cells (lanes 5 and 6). Both in Th1 and Th2 extracts, complex A was supershifted with anti-NFATc2 (lanes 4 and 5 and 7 and 8). The isotype control is shown in lane 9.

**IL-12Rβ2 mRNA Expression Is Up-regulated by CsA**—The data so far suggested a general suppressive role of NFATc2 in the regulation of *IL-12Rβ2* expression, not discriminating between Th1 and Th2 cells. To test the role of NFAT in a more physiological system, naive T cells were stimulated for 3 days with anti-CD3/anti-CD28 in the presence of increasing concentrations of CsA. The mRNA expression of *IL-12Rβ2*, IL-2, and β2m was measured by real-time PCR. The levels of *IL-12Rβ2* and IL-2 mRNA were normalized based on β2m mRNA levels in the same samples. Both IL-2 mRNA expression level, known to be inhibited by CsA (23), and IL-13 protein secretion, known to be enhanced by CsA (24), were used as controls. As expected from the EMSA data, CsA dose-dependently increased *IL-12Rβ2* mRNA expression (Fig. 6A), confirming the suppressive role of NFAT herein. Furthermore, the IL-2 mRNA expression was decreased (Fig. 6A), and the IL-13 protein secretion was increased (Fig. 6B) in the presence of increasing doses of CsA.

**DISCUSSION**

In this report, we have described the first data on the transcriptional regulation of the human *IL-12Rβ2* gene. The proximal promoter region was cloned and functionally characterized. The data indicate a TATA-less promoter, dependent on SP-1 family protein binding at −63, and a silenced NFAT element at −206, which binds NFATc2 and is involved in suppressing TCR-induced *IL-12Rβ2* expression. The pGL3e vector in which the promoter fragments were cloned contains an SV-40 enhancer located upstream of the luciferase gene. The enhancer element normally increases reporter gene expression provided that the promoter is active. Deletion from −151 to −61 resulted in fully abrogated transcription, even in the presence of the enhancer, underlining the critical role of this region in transcription initiation.

The core promoter, which drives TCR-induced transcription (construct −151), does not contain a TATA or CAAT box. In the absence of a TATA box, SP-1 binding motifs are frequently involved in alternative initiation of transcription (25, 26). This seems to apply for the *IL-12Rβ2* gene as well, as it contains a functional SP-1/3 binding motif in its core promoter. Genes with TATA-less promoters, including many so-called “housekeeping” and receptor genes, are generally expressed at low levels (27). Indeed, even fully IL-12-responsive Th1 cells were shown to express only a few hundred IL-12Rβ2 molecules on their membrane (6). The *IL-12Rβ2* gene contains a GC-rich (±75%) 5′ noncoding region (10), which may, at least in part, explain the low rate of expression of these molecules, as GC-rich 5′ noncoding regions are known to hamper translation.

**Fig. 5. Identification of −206-binding proteins.** The radiolabeled probe NFAT −206 wild type was incubated with nuclear extracts from freshly isolated CD4+ T cells (A) or from polarized Th1 or Th2 cell lines (B) that were left unstimulated or stimulated with anti-CD3/anti-CD28 for 30 min. Specific complexes (P, A, and S) are indicated by arrowheads. The binding reactions were carried out in the absence or presence of specific antibody to NFATc1 or NFATc2 or in the presence of IgG1 isotype control antibody as indicated. A, nuclear extracts from CD4+ T cells. Preexisting complex P showed increased binding activity upon T-cell stimulation. Complex A was induced after stimulation (lane 4) and was CsA-sensitive (lane 5). The addition of anti-NFATc2 but not anti-NFATc1 results in the supershift (S) of complex A but not complex P. As a control both antibodies are shown to be absent of nuclear extract (lanes 1 and 2). B, nuclear extracts from Th1 or Th2 cells showed no differences in the formation of complex P or A after TCR stimulation (lanes 1, 2 and 5, 6). Anti-NFATc2, but not anti-NFATc1, supershifts complex A equally well in Th1 and Th2 cells (lanes 3, 4 and 7, 8). The IgG1 isotype control is shown in lane 9.
(27). However, data on the translational regulation of the IL-12Rβ2 mRNA are not available yet.

We show here that IL-12Rβ2 expression is inhibited at the transcriptional level. The NFAT element at −206 specifically binds NFATc2 and seems to be important for a general down-regulation of TCR-inducible IL-12Rβ2 gene expression. The availability and suppressive activity of NFATc2 does not discriminate between Th1 and Th2 cells and thus does not explain the loss of expression of the IL-12Rβ2 chain in Th2 cells. The relative importance of the negative regulatory role of this element is underlined by the observation that CsA increases IL-12Rβ2 mRNA expression in stimulated naïve Th cells, i.e. in the context of fully intact regulatory regions, instead of the cloned proximal 591 base pairs of the promoter. The absence of further NFAT sites in the region, at least up to −1.2 kilobases (data not shown), further indicates this particular site as the mediator of the CsA-sensitive suppressive effect.

Similar negative regulatory effects of NFATc2 have been implicated in the regulation of several Th2 type cytokine genes in the mouse (21, 22). Indirect data on the regulation of the human IL-13 gene points in the same direction (24). The present data are the first to suggest a suppressor function of NFATc2 in the regulation of a gene associated with Th1-type responses. Whether or not this site contributes to the polarization process of Th cells under Th1 or Th2 driving conditions is unclear thus far, as sites directly involved in the Th2-specific suppression of the IL-12Rβ2 gene have not been identified yet. Our present data give no indication for differential NFATc2 activity at −206 in Th1 and Th2 cells. Instead, NFATc2 may play a role in the general low expression rate (6) or the kinetics of IL-12Rβ2 gene expression, in particular in the shut-down of expression at later time points after TCR triggering as has been suggested in the regulation of IL-4 gene expression (28).

It is to be expected that several Th1- or Th2-specific transcription factors do play a role in IL-12Rβ2 gene transcription either in a direct or an indirect way. For example, IL-12 strongly up-regulates IL-12Rβ2 expression through phosphorylation of STAT4 (6). Therefore, STAT4 binding sites are expected to be located in the enhancer region further upstream as has been suggested before (29). Another transcription factor which may be directly involved in IL-12Rβ2 gene regulation is the recently identified, Th1-specific T-box transcription factor, T-bet (30). T-bet is up-regulated by IL-12 and accounts for the Th1-specific expression of IFN-γ and repression of the opposing Th2 programs, at least in the mouse. In contrast, GATA-3, a Th2-specific and IL-4-induced transcription factor (31), may be involved in the direct suppression of the IL-12Rβ2 gene. Indeed, Ouyang et al. (32) demonstrated a decrease in IL-12Rβ2 mRNA expression after ectopic expression of GATA-3 into differentiated murine Th1 cells. We are currently investigating the role of Th1- and Th2-specific transcription factors in human IL-12Rβ2 gene expression.

Acknowledgements—We thank Ing. J. Wormmeester for technical support and Des. J. L. M. Schoneveld (Academic Medical Center, Dept. of Anatomy and Embryology, Amsterdam) and Dr. M. F. A. Bierhuizen (University Medical Center Utrecht, Dept. of Medical Physiology, Utrecht, the Netherlands) for helpful discussions.

REFERENCES
1. Salgame, P., Abrams, J. S., Clayberger, C., Golstein, H., Convit, J., Modlin, R. L., and Bloom, B. R. (1991) Science 254, 279–282.
2. Romagnani, S. (1991) Immunol. Today 12, 256–257.
3. O’Garra, A. (1998) Immunol. 6, 275–283.
4. Manetti, R., Gerosa, F., Giudizi, M. G., Biagetti, R., Perronechi, P., Piccinini, M. P., Sampognaro, S., Maggi, E., Romagnani, S., and Trinchieri, G. (1994) J. Exp. Med. 179, 1273–1283.
5. Maggi, E., Perronechi, P., Manetti, R., Simonelli, C., Piccinini, M. P., Rugia, F. S., De Carli, M., Ricci, M., and Sinigaglia, F. (1992) J. Immunol. 148, 2142–2147.
6. Rogge, L., Barberis-Maino, L., Biffi, M., Passini, N., Presky, D. H., Gubler, U., and Sinigaglia, F. (1997) J. Exp. Med. 185, 825–831.
7. Chua, A. O., Chizzonite, R., Desai, B. B., Truitt, T. P., Nunes, P., Minetti, L. J., Warriner, R. R., Presky, D. H., Levine, J. P., and Gately, M. K. (1994) J. Immunol. 153, 128–136.
8. Hilken, C. M., Messer, G., Tesselaar, K., van Rietschoten, A. G., Kapsenberg, M. L., and Wierenga, E. A. (1996) J. Immunol. 157, 4316–4321.
9. Rogge, L., Papai, P., Presky, D. H., Biffi, M., Minetti, L. J., Musto, Agostini, C., Semenzato, G., Fahbri, L. M., and Sinigaglia, F. (1999) J. Immunol. 162, 3926–3932.
10. Presky, D. H., Yang, H., Minetti, L. J., Chua, A. O., Nabavi, N., Wu, C. Y., Gately, M. K., and Gubler, U. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14002–14007.
11. van den Hoff, M. J. B., Christoffels, V. M., Labruyere, W. T., Moorman, A. F. M., and Lamers, W. H. (1995) Meth. Mol. Biol. 48, 185–197.
12. Vieira, P. L., Kalinski, P., Wierenga, E. A., Kapsenberg, M. L., and de Jong, E. C. (1998) J. Immunol. 161, 5245–5251.
13. Smits, H. H., van Rietschoten, J. G. I., Hilken, C. M., Sayilir, R., Steikeeva, F. E., Kapsenberg, M. L., and Wierenga, E. A. (2001) Eur. J. Immunol. 31, 1055–1065.
14. van Rietschoten, J. G. I., Smits, H. H., Westland, R., Verweij, C. L., den Bart, M. T., and Wierenga, E. A. (2000) Immuno Genetics 31, 30–36.
15. Yokoe, T., Suzuki, N., Nomoguchi, K., Adachi, M., and Sakane, T. (2001) Cell. Immunol. 208, 34–42.
16. Suske, P. (1999) Gene 238, 291–300.
17. Lanin, L., Majello, B., and De Luca, P. (1997) Int. J. Bioch. Cell Biol. 29, 1313–1323.
18. Kuo, C. T., and Leiden, J. M. (1999) Ann. Rev. Immunol. 17, 149–187.
19. Hoei, T., Sun, Y. L., Williamson, K., and Xu, W. (1995) Immunol. 2, 461–472.
20. Rao, A., Luo, C., and Hogan, P. G. (1997) Ann. Rev. Immunol. 15, 707–747.
21. Xanthoudakis, S., Viola, J. P. B., Shaw, K. T. Y., Luo, C., Wallace, L. D., Bozza, P. T., Curran, T., and Rao, A. (1996) Science 272, 892–895.
22. Hodge, M. R., Ranger, A. M., de la Brousse, F. C., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1996) Immunity 4, 397–405
23. Manger, B., Hardy, K. J., Weese, A., and Stobo, J. D. (1986) J. Clin. Invest. 77, 1501–1506
24. Van der Pouw Kraan, T. C., Kraan, T. C., Boeije, L. C., Troon, J. T., Rutschmann, S. K., Wijdenes, and Aarden, L. A. (1996) J. Immunol. 156, 1818–1823
25. Azizkhan, J. C., Jensen, D. E., Pierce, A. J., and Wade, M. (1993) Crit. Rev. Eukar. Gene Expr. 3, 229–254
26. Dynan, W. S., and Tian, B. (1983) Cell 35, 79–87
27. Kozak, M. (1991) J. Cell Biol. 115, 887–903
28. Ranger, A. M., Oukka, M., Rengarajan, J., and Glimcher, L. H. (1998) Immunity 9, 627–635
29. Sinigaglia, F., D’Ambrosio, D., Panina-Bordignon, P., and Rogge, L. (1999) Immunol. Rev. 170, 65–72
30. Szabo, S. J., Kim S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000) Cell 100, 655–669
31. Zheng, W. P., and Flavell, R. A. (1997) Cell 89, 587–596
32. Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C., and Murphy, K. M. (1998) Immunity 9, 745–755
33. Yamamoto, K., Kobayashi, H., Miura, O., Hiroswa, S., and Miyasaka, N. (1997) Cytogenet. Cell Genet. 77, 257–258
Silencer Activity of NFATc2 in the Interleukin-12 Receptor β2 Proximal Promoter in Human T Helper Cells

Johanna G. I. van Rietschoten, Hermelijn H. Smits, Diederik van de Wetering, Robert Westland, Cor L. Verweij, Marcel T. den Hartog and Eddy A. Wierenga

J. Biol. Chem. 2001, 276:34509-34516.
doi: 10.1074/jbc.M102536200 originally published online July 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102536200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 12 of which can be accessed free at http://www.jbc.org/content/276/37/34509.full.html#ref-list-1