Identification of Two Novel Regulatory Elements in the *IL2RA* Gene Locus

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Abstract

Regulatory T-cells (T\(_{\text{reg}}\)) express the high-affinity chain of the interleukin 2 (IL-2) receptor, CD25, encoded by the *IL2RA* gene. *IL2RA* is one of the most extensively characterized genes regarding its regulatory regions and their functional links to cell surface receptors and their associated signal transduction cascade in the context of the antigen-dependent activation of mature CD4\(^+\) T lymphocytes. However, converging evidences strongly suggested that they were some missing pieces in this already complex puzzle made of 6 well-characterized regulatory regions. Here, by combining principally in silico genomic footprinting approach and meta-analysis of several ChIP-seq studies, we identified and characterized 2 new putative CD28-responsive elements. We show that a recently-characterized intronic enhancer at +11 kb harbors a functional CREB site. Further, we evidence a repressor element consisting of two GAAA repeats located 5’-most of a previously identified enhancer 4 kb upstream of the *IL2RA* gene. Mass-spectrometry analyses revealed Poly ADP-ribose polymerase 1 (PARP-1) as part of the complexes binding this element. Altogether, our observations extend our understanding of the molecular basis of the multiple options offered by such a complex organization in term of T cell responses.

Keywords: *IL2RA*; Enhancer; Regulatory T-cell; Gene regulation; CREB; PARP-1; EMSA

Introduction

Regulatory T-cells (T\(_{\text{reg}}\)) are immunosuppressive CD4\(^+\) T-cells that constitutively express the high-affinity chain of the interleukin 2 (IL-2) receptor, CD25, and the forkhead P3 (FoxP3) transcription factor (TF) [1,2]. Like other helper T-cells (Th\(_1\), Th\(_2\), Th\(_{17}\)), these cells are thought to originate from T-cells activated by antigen presentation, during which T-cell receptor (TCR), CD3 stimulation and CD28 co-stimulation take place [3]. Conversely, in other inactive helper T-cells, CD25 expression is downregulated, which makes CD25 a specific marker for T\(_{\text{reg}}\). T\(_{\text{reg}}\) ontogeny is specifically promoted by tumor growth factor β (TGFβ) in an autocrine and paracrine manner [4]. TGFβ and interleukin 10 (IL-10) secretion confers T\(_{\text{reg}}\) a prominent role in suppressing autoreactive T-cells and in regulating cell- and humoral-mediated immune responses [4,5]. Due to their immunosuppressive nature, these cells also largely play a role in suppressing allergy and asthma [6,7]. The maintenance of T\(_{\text{reg}}\) populations is thus critical to suppress allergic Th cells via IL-10 and TGFβ secretion [8,9]. However, in autoimmune diseases and severe allergic pathologies, the ratio of Th\(_{\text{reg}}\)/Th\(_{\text{h}}\) cells tend to be biased towards Th2 cells, resulting in reduced immunosuppression [10-12]. In some cases of allergy, this discrepancy even seems to originate neonatally [13].

CD25 is encoded by the *IL2RA* gene and is one of the three subunits of the IL-2 receptor, which constitutes the centerpiece of the IL-2/IL-2R system [14]. The low affinity IL-2 receptor comprises the constitutively-expressed β and γ chains only. The β chain is shared with the receptor for IL-15, while the γ chain is shared with that of IL-4, IL-7, IL-9, IL-15 and IL-21 [15]. The IL-2 receptor acts synergistically with TCR and CD28 (co)-stimulation to induce cell survival and proliferation in T lymphocytes [16]. CD25 is normally expressed in DN2/3 thymocytes [17], basophils [18], activated T-cells [19] and marginally in circulating B-cells [20]. IL-2 binding to the medium-affinity IL-2 receptor triggers oligomerization of the β and γ chains, which initiates transduction of three signaling pathways: (i) JAK-STAT [21], (ii) MAP-kinase [22] and (iii) PI3-kinase [23], thereby inducing the expression of early-response genes, including IL-2 itself and IL2RA. This results in an amplification of high affinity IL-2 receptors at the cell surface. Due to these properties, CD25 is a therapeutic monoclonal antibody target [24]. In fact, inactivation of the high-affinity murine IL-2 receptor causes loss of T\(_{\text{reg}}\) and subsequent invasive auto-immune inflammation of the intestine [25]. In humans, a truncated form of CD25 was associated with reduced peripheral T-Cell count and proliferation but normal B-cell development [26]. CD25 expression is also higher in allergen-specific T-cell clones in cow’s milk allergy [27].

Transcriptional regulation of *IL2RA* is a tightly-regulated process that requires activation of the full complement of its cognate pathways for optimal expression. Due to its critical role in immunity, *IL2RA* represents one of models of gene regulation as one of the best-characterized loci to date. Six pathway-specific responsive enhancers or positive regulatory regions (PRRs) have been described: PRRI/II/V/VI are required for initial antigenic and mitogenic stimulation [26,28,30], while PRRII/IV are subsequently activated by IL-2 via JAK-STAT [31-34]. Combinatorial activation of these enhancers is achieved via binding of transcription factors (TFs) responding to specific signaling

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pathways. Thus, PRRI/II possess ETS and NF-κB binding sites, and PRRIII/IV STAT binding sites [14]. Importantly, PRRV and V1 are CD28-responsive elements due to the presence of CREB binding sites in these enhancers [26,30]. Consistent with T_{α} onset, PRRV also harbors a TGFβ response element (SMAD) that favors TCR engagement and promotes T_{α} differentiation [14].

The IL2RA locus also bears further large conserved non-coding sequences (CNSs) in the human and mouse IL2RA locus identified via phylogenetic footprinting [14,35,36], suggesting additional, unidentified regulatory elements. In fact, two additional putative enhancer elements were characterized as demethylated regions using MeDIP-chip [37]. These enhancers are located ≈9kb upstream of the TSS and ≈25kb in the first intron of IL2RA. More recently, further regulatory elements were identified via CRISPR-activation (CRISPRa) screening in the IL2RA locus in a landmark study, also allowing a direct comparison of enhancer activities of previously published enhancers in this locus [38]. This work was largely supported by the recent rise of high-throughput techniques, both for sequencing of transduced gRNA libraries as well as upstream target identification using published ENCODE ChIP-Seq and DNasel-Seq screens [39]. This work uncovered two new regions in the first intron of IL2RA, located at ≈9kb and ≈11kb into the first intron. The element at ≈9kb, called CasRE4, was validated as an enhancer via SNP and indel knock-in assays. Using targeted deletion via CRISPR, both elements (designated IN1a and IN1b) and an additional enhancer at ≈27kb (UPI) were also validated in a subsequent study as STAT5-dependent as well as required for correct CD25 expression [40]. Altogether, although these studies evidenced novel functional regulatory elements in the IL2RA locus, they also call for further characterization of single binding sites within enhancers in this locus.

A 12 base-pair (bp) element upstream of PRRIII IL-2rE was previously identified via genomic footprinting as subject to constitutive and inducible modifications of the chromatin in Kit 225 cells [41]. Furthermore, antigen-mimicking αCD2/αCD28 activation of primary T-Cells caused transitory migration delays of a protein complex located in PRRIII via band-shift assays [33].

In this study, we identify putative CREB, ETS and STAT binding sites via phylogenetic footprinting in the CasR4/IN1b element, which we designated PRRX. We validate the presence of a complex specifically binding the novel CREB binding site. Further, we evidence a novel binding site at the 5’ extremity of PRRIII. We highlight the repressive nature of this element with regards to gene expression. Finally, we identify one of the components of the protein complex binding to the GAA element as Poly ADP-ribose polymerase 1 (PARP-1) enzyme.

**Materials and Methods**

**Cell culture and cell extracts**

Kit 225 cells were cultured in 10% FCS RPMI 1640 medium with 2 mm l-glutamine, 100 μg/ml each of streptomycin and penicillin, and 10% fetal bovine serum at 37°C with 5% CO2 and split every other day. The resulting extract was then dissolved in TM buffer (50 mm Tris-g/l extract) and pelleted by centrifugation as described previously [7]. Nuclear extracts were then subjected to an ammonium sulfate cut (0.33 g/ml extract) and centrifuged as described previously (7). After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, France) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20 to 30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer.

**DNA affinity purification assay (DAPA)**

Nuclear extracts were incubated with 32P-radioactively labeled GADs sequence (5’-TTTCTTCTAAGGAGAGTTCGTTAA-3’) and κB-luciferase plasmid. Cells were grown to 80% confluent in 6 well plates and were transfected on the following day by Lipofectamine™ 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, France) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20 to 30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer.

**Electrophoretic mobility shift assays (EMSAs)**

Probes were designed using the sequences of analyzed regions. For GAA_{μ3}, we used the oligonucleotide sequence CTAGAAAGAATGTTCTTTA. For IX_{μ}, the oligonucleotide sequence was GAGTTAAAGTGTTCACTCTTCTTCGCCCTGGTGCCCCGAGAGTTTCCCGGGAGTTTTGG and the sequence of high-affinity CREα competitor [33] was GATAAAGAGAGTGGTTGTA. The FgR1-GAS [33] competitor sequence was GTATTTCGCCAGAAAAGGAAAC. In Table 1, DMS/LM-PCR genomic footprint analyses were performed as described in [41]. Primer sequences and associated melting temperatures can be found in Table 1.

**Gene reporter assays**

Kit 225 cells were co-transfected with 0.8 μg GAA_{μ3} (CTAGAAAGAATGTTCTTTA) and κB-luciferase plasmid. Cells were grown to 80% confluent in 6 well plates and were transfected on the following day by Lipofectamine™ 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, France) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20 to 30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer.

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revealed using silver nitrate staining. Bands of interest were cut, purified via high-performance liquid chromatography (HPLC) and sequenced using mass spectrometry.

**Phylogenetic footprinting**

Phylogenetic footprinting was performed via ECR browser at dCode [44]. To map conserved motifs, we used the multiTF conserved motif searching facility at dCode, using Homo sapiens, Pan troglodytes, Macaca mulatta, Mus musculus, Rattus norvegicus and Canis familiaris to retrieve synteny blocks. MultiZ alignments were derived from the UCSC genome browser [45]. Phylogenetic analyses of enhancer regions were carried out via ClustalX using bootstrapping [46].

**Results**

**PRRIX (CasRE4/IN1b) contains a functional CRE-binding element**

PRRIX (CasRE4) was recently identified as functional enhancer essential for physiological CD25 expression, containing at least one characterized STAT5 binding site [38,40]. Inspection of the IL2RA locus using publicly available T-cell ENCODE DNAseI-Seq datasets [39] revealed open chromatin at this enhancer and the neighboring one at +49kb (CasRE4/IN1a which we designated PRRVIII), as well as at previously described ones, PRRI, II, III and IV (Figure 1A). Interestingly, PRRIX displayed open chromatin in all types of T-cells, including Th0, as well as in B-cells. The regions characterized at -8kb and +25kb identified by Schmidl et al [37], which we called PRRVII and PRRIX, also showed enrichment in open chromatin. To further characterize potential novel binding sites in PRRIX, we performed phylogenetic footprinting combined with motif discovery in the entire IL2RA locus. Immunity-related genes tending to evolve more quickly than others, we chose closely related mammals as comparison species [10]. We identified seven conserved, motif-rich, regions in the IL2RA locus, as well as in B-cells. Of the selected mammal species (Figure 1B). Inspection of PRRIII also revealed the presence of a RUNX motif. By performing further phylogenetic footprinting, while we observed that PRRIX was well conserved, this was not the case for PRRVI, one of the known CD28-responsive elements (CD28-rE) in IL2RA, was not well conserved (Figures S2A and S2B).

**Figure 1:** (A) UCSC genome browser screenshot of ENCODE DNase-Seq in T-cell populations and CD20+ B-cells at the IL2RA gene locus. Regulatory element annotation is provided underneath, as well as conserved motifs identified via phylogenetic footprinting (see Figure S1 for a full list). Red denotes open chromatin.

(B) MultiZ alignments at PRRIX and PRRIII (top, bottom) in higher mammalian species. Conserved motifs are indicated in different colors.
and S2B). The JAK-STAT responsive PRRIII also showed a high degree of conservation across mammals.

To determine the functionality of putative binding sites in PRRIX, we tested for the presence of specific protein complexes potentially binding this region in vitro via EMSA. To this end, employed nuclear extracts of 1 h IL-2-stimulated Kit 225 IL-2-dependent T-cell lymphoma cells [47] that had been starved of IL-2 for 20 hrs. We used a 32P-labeled probe (B2) corresponding to a region of PRRIX harboring STAT, ETS and CREB motifs, using 1 h IL-2 induced nuclear protein extracts. We observed three specific protein complexes C1, C2 and C3 (Figures 2A and 2B) that disappeared using an unlabeled wild type version of the probe for region IX as a competitor in excess. The addition of a 25x excess of unlabeled, high-affinity competitors carrying the ETS and STAT motifs [33] did not alter complexes 1, 2 and 3, while the same excess of a high-affinity CREB competitor [42] caused the loss of complex 1 (Figures 2A and 2B). We therefore conclude that a complex specifically binds the CREB motif isolated in PRRIX.

A protein complex containing Poly ADP-Ribose Polymerase 1 (PARP-1) binds a transcriptional repressor GAAA element in PRRIII

To determine whether the GAAA sequence identified in PRRIII corresponds to an open chromatin region, we first performed high-resolution genomic in vivo footprinting using dimethyl sulfate/ligation-mediated PCR (DMS/LMPCR, Figure 3A and Table 1). We detected a chromatin-modified element in both purified CD4+ T Cells (Figure 3B) and IL-2 mediated PCR (DMS/LMPCR, Figure 3A and Table 1). We detected a GAAA motif on the antisense strand (GAAA mut123) again resulted the loss of C2a/b and C3. Additionally, this implies that (i) the C2a/b protein complex specifically binds the GAAA element, (ii) that the 3' TGTT sequence is not required for binding of this complex and that (iii) a single GAAA repeat is sufficient for C2a/b binding.

We next wanted to determine whether the GAAA element is transcriptionally active. We performed gene reporter assays in Kit 225 cells stimulated or not by IL-2 [47]. We found that the insertion of GAAA downstream of the κB amplifier strongly decreases IL2RA gene expression regardless of IL-2 stimulation (Figure 4D). This suggests that GAAA is a constitutive repressor of IL2RA expression in this context.

We then proceeded to identifying protein complexes that interact with GAAA. We carried out DNA Affinity Purification Assays (DAPA) using Kit 225 nuclear extracts and thus defined two specific components at 90 and 45 kDa that reacted with the GAAA probe but not the control GAAA or GASd probes (Figure 4E). Interestingly, these complexes...
were obtained regardless of IL-2 activation. We subsequently extracted, purified these bands and performed mass-spectrometry sequencing for identification of bound protein complexes. While we could not identify the complex at 45 kDa, the complex migrating at 90 kDa was identified as Poly ADP-Ribose Polymerase 1 (PARP-1). Consistent with its constitutive repressive activity, this suggests that GAAA is also bound in a constitutive manner by at least PARP-1.

Discussion

In this work, we have expanded the comprehensive repertoire of regulatory elements governing CD25 expression. PRRIX was already described as a JAK-STAT responsive element with a functional STAT5 binding site [40]. The addition of a CREB site further makes it CD28-responsive. This suggests a dual role for this enhancer, likely responsive...
to both low- and high-affinity IL-2 receptors. It would thus be activated during initial antigen presentation whereby CD25 expression is still low, and would play a further role in the amplification of IL2RA expression via JAK-STAT signaling. The addition of a CD28-responsive element to the regulation of IL2RA further highlights the importance of this locus in T<sub>reg</sub> ontogeny. In addition, we observed that PRRVI was not well conserved among mammals, hinting at the need for an ancestral CD28-responsive element. This enhancer might very well be PRRIX, harboring both well conserved JAK-STAT and CD28 responsive elements. Other less conserved enhancers may thus have arisen later in evolution as more specialized elements. Interestingly, we observed that PRRIX was constitutively accessible in all T-cell populations, as well as in B-cells (Figure 1A). Along with its high degree of conservation, this might indicate that this enhancer is used in other cell types expressing CD25 and ancestral. Importantly, we also provide a detailed description of novel, putative transcription factor binding sites that can be directly

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**Figure 4:**

(A) Probe sequences of used in EMSAs around the GAAA element.

(B) EMSAs using the labeled GAAA<sub>wt</sub> probe with nuclear extracts only (leftmost) which reveals three protein complexes (C2a/b and C3) that disappear when using 75X non labeled GAAA<sub>wt</sub> probe competition (second rightmost).

(C) EMSAs using the labeled GAAA<sub>wt</sub> with no competitor (leftmost), with 25X unlabeled competitor excess of GAAA<sub>wt</sub> (second leftmost), GAAA<sub>mu1</sub> (third leftmost) and GAAA<sub>mu123</sub> (rightmost) which do not affect binding since complexes C2a/b and C3 are lost, and GAAA<sub>mu23</sub> (second rightmost) which affects binding of C2a/b and C3 since they are not lost.

(D) Transcriptional effect of a GAAA<sub>wt</sub> trimer on the luciferase reporter gene without (left) and with (right) 1 h IL-2 stimulation in Kit 225 cells shows the in vivo repressive role of GAAA independently of IL-2 stimulation. (E) DAPA reveals two GAAA<sub>wt</sub> specific bands after a 24-hour IL-2 stimulation (left) migrating at 90 KDa. The band was identified in mass spectrometry sequencing as PARP-1. The second band could not be identified.
used as a base for the identification of further regulatory elements in the IL2RA gene locus (Figures S1 and S2).

We further identified a repressor element as the GAAA sequence near the 5' end of PRRIII. The importance of this identification leans more particularly on the fact that so far only negative regulatory elements, NRE-1 and NRE-2, have been identified in the IL2RA locus [48]. The GAAA element might thus regulate the trans-activity of PRRIII and negatively influence the feed-forward mechanism conferred by PRRIII and IV via JAK-STAT signaling. Thus, GAAA may act as a brake on top of NRE-1/2 and SOCS proteins on CD25 amplification. Interestingly, we also identified PARP-1 as part of the complex that binds GAAA. Albeit originally identified as a single and double strand DNA break sensor and repair protein, PARP-1 was shown to play an important role in transcription [49]. PARP-1 was also shown to promote apoptosis, consistent with the repression of CD25 and its activated cell survival and activated genes [50]. PARP-1 contains a central protein-protein interaction domain and an amino-terminal domain capable of interaction with DNA through two zinc fingers and a helix-turn-helix motif. PARP-1 was also described as a transcriptional repressor that acts downstream of CD28 signaling [51], which may indicate a repressor activity promoted by CD28-dependent recruitment of PARP-1 to this region. This finding would add PRRIII to the list of CD28-responsive elements. However, PRRIII is not a constitutively open enhancer, as attested by DNaseI-seq profilings shown in Figure 1A. Its accessibility is however maintained in Th1, Th2, and Treg, similarly to our previous observation that the presence of stable preassembled protein-DNA complexes, in contrast with the bare IL2RA locus in non-T cells, contributes precommitment of T cells to activation [52]. Interestingly, PARP-1 was also shown to facilitate chromatin remodeling at pioneered protein interaction domain and an amino-terminal domain capable of interaction with DNA through two zinc fingers and a helix-turn-helix motif. PARP-1 was also described as a transcriptional repressor that acts downstream of CD28 signaling [51], which may indicate a repressor activity promoted by CD28-dependent recruitment of PARP-1 to this region. This finding would add PRRIII to the list of CD28-responsive elements. However, PRRIII is not a constitutively open enhancer, as attested by DNaseI-seq profilings shown in Figure 1A. Its accessibility is however maintained in Th1, Th2, and Treg, similarly to our previous observation that the presence of stable preassembled protein-DNA complexes, in contrast with the bare IL2RA locus in non-T cells, contributes precommitment of T cells to activation [52]. Interestingly, PARP-1 was also shown to facilitate chromatin remodeling at pioneered enhancers independently of its poly(ADP-ribose) transferase activity [53], hinting at a possible dual role for PARP-1 in this context. This enhancer may thus play a role in the acquisition of epigenetic memory and primed for immediate CD25 expression upon re-stimulation in Th1/Th2 cells [54]. Further, as we observed protein complexes binding to GAAA in both the absence and presence of IL-2, this suggests that this element is a constitutive repressor. The lack of interaction with the GASS probe also effectively rules out STAT5 as binding to GAAA. The molecular weight (45 kDa) of the unidentified protein that was pulled down via DAPA also seems to prohibit the presence of STAT proteins. Other possible candidates could be IRF1 and IRF2 [55-59], which both migrate at 45 kDa and whose binding motif is GAAA. While IRF1 was largely described as a transcriptional activator and IRF2 as a transcriptional repressor [60], IRF1 was however shown to down-regulate CD4+CD25+ regulatory T-Cell differentiation through Fox3 down-regulation [61]. Thus additional repression of IL2RA could be used in this case, as well as suppressing Th1, therefore promoting that of Th2 upon activation through interferon γ (IFNγ) production [62].

Recent studies have also established that the main role of the IL-2/IL-2R system consists in controlling lymphocyte homeostasis [63]. Dysfunctions of this system can prove devastating since regulatory T-Cells normally temper humoral and cell-mediated immune responses [64]; and also normally suppress autoreactive T-cells that have escaped negative selection [65]. At least two reports have also shown that abnormal T-Cell suppression by regulatory T-Cells can lead to evasive mechanisms that may profit to cancer development [66, 67]. This alone justifies anti CD25 monoclonal treatment in these pathologies. On the other hand, loss of Treg populations leading to increased allergic and autoimmune reactions [10-13], one natural approach would entail treating such patients with IL-2. Importantly, the recent finding that low-dose IL-2 administration tends to suppress autoimmune disease and inflammation supports the requirement for fine-tuning of IL2RA transcriptional regulation [68]. Overall, we present two novel CD2-CD28 responsive regulatory features of the human IL2RA gene that are part of fine-tuned, time-dependent regulatory mechanisms that govern the expression of this gene and control Treg ontogeny.

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