Genes: Structure and Regulation:

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Smad-dependent Cooperative Regulation of Interleukin 2 Receptor α Chain Gene Expression by T Cell Receptor and Transforming Growth Factor-β*

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The interleukin 2 receptor α chain (IL-2Rα) is a component of high affinity IL-2 receptors and thus critically regulates T cell growth and other lymphoid functions. Five positive regulatory regions together control lineage-restricted and activation-dependent IL-2Rα induction in response to antigen and IL-2. We now show that TGF-β cooperates with T cell receptor (TCR) signaling to increase IL-2Rα gene expression. Moreover, we identify a sixth positive regulatory region that regulates IL-2Rα expression in cells treated with anti-CD3 + anti-CD28 as well as TGF-β and show that this region contains binding sites for Smad3, AP-1, and cAMP-responsive element-binding protein/ATF proteins. The importance of Smad complexes is indicated by impaired IL-2Rα induction by TGF-β in $CD4^+$ T cells from both $Smad3^{-/-}$ and $Smad4^{-/-}$ mice. Thus, we have identified a novel positive regulatory region in the IL-2Rα gene that mediates TGF-β-dependent induction of the gene. These findings have implications related to IL-2Rα expression on activated T cells and regulatory T cells.

Interleukin 2 (IL-2)[2] critically regulates the magnitude and duration of the T cell immune response following antigen encounter (1), mediates activation-induced cell death (2, 3), regulates the number of regulatory T cells (4), and exerts actions on B and NK cells. The IL-2 receptor α chain (IL-2Rα) (5–7) is a component of high affinity IL-2 receptors (8, 9). Transcription of the IL-2Rα gene (10, 11) is controlled by at least four upstream positive regulatory regions (PRRI, PRRII, PRRIII, and a CD28 response element) and an intronic element (PRRIV) that together contribute to lineage-restricted and activation-dependent IL-2Rα induction (12–26). PRRI, PRRII, PRRIV, and CD28rE are required for mitogenic stimulation of the IL-2Rα gene, whereas PRRIII and PRRIV mediate IL-2 responsiveness (9).

Transforming growth factor-β (TGF-β) proteins influence cell growth and differentiation (27–30). TGF-β1, TGF-β2, and TGF-β3 play important roles in the regulation of immune cells. A loss-of-function mutation in TGF-β1 in mice results in significant embryonic lethality (31, 32), whereas mutation of TGF-β2 or TGF-β3 results in 100% embryonic lethality (33–35), indicating that each TGF-β isoform has critical nonredundant roles *in vivo*, even though they use the same receptors (29, 30) and exhibit indistinguishable effects on immune cells *in vitro* (36, 37).

TGF-βs signal through serine/threonine kinase transmembrane receptors (29, 30). They first bind TGF-β-RII, resulting in the recruitment and the activation of TGF-β-RI, which then phosphorylates the C-terminal region of the Smad family transcription factors, Smad2 and Smad3. Smad2/Smad4 and Smad3/Smad4 complexes form and translocate into the nucleus where they bind to CAGAC motifs and can associate with other DNA-binding proteins or CREB-binding protein/p300 or P/CAF (CREB-binding protein-associated factor) transcriptional coactivators.

TGF-β1 suppresses proliferation of wild type (WT) T cells (38) but not of T cells from mice expressing a dominant-negative (DN) TGF-β-RII transgene (39, 40), or from Smad3 knock-out mice (41, 42). In addition to suppressive effects of TGF-β1 on T cell function (27, 28), TGF-β1 can also have positive regulatory effects, particularly on naive T cells (43). TGF-β1 can synergize with IL-2 to prevent apoptosis and promote effector cell function (44–46), and it promotes the generation of $CD8^+$ T cells that suppress antibody production (47, 48).

We now show that IL-2Rα expression is increased following exposure to TGF-β1. Moreover, we describe a novel positive regulatory region (PRRV) in the IL-2Rα gene that contains binding sites for Smad, AP-1, and CREB/ATF proteins, which mediate PRRV function in response to costimulation via the T cell receptor and TGF-β1. The discovery of PRRV clarifies the basis for TGF-β-mediated IL-2Rα regulation, providing a mechanism by which TGF-β1 can affect IL-2 signaling. Given the importance of IL-2 signaling for the development of regulatory T cells (4), our data suggest a mechanism by which TCR and TGF-β1 augment the development of these cells.

MATERIALS AND METHODS

Mice and Cell Culture—C57BL/6 mice were obtained from the Jackson Laboratory. $Smad3^{-/-}$ mice (C57BL/6xSv129) were generated by targeted gene disruption in murine embryonic stem cells by homologous recombination (42). The Smad4 gene was disrupted in T cells of mice by crossing the Smad4-floxed mice (49) with p56lck-Cre (Lck-Cre) transgenic mice. The mice used in these experiments were 8–10 weeks of age. All of the experiments were performed under protocols approved by the National Institutes of Health Animal Use and Care Committee and followed the National Institutes of Health guidelines "Using Animals in Intramural Research." Single-cell suspensions from spleen were prepared, and these splenocytes (1×10^6/ml) were stimulated for 24 h in plates coated with 2 μg/ml each of anti-CD3ε and soluble anti-CD28 monoclonal antibodies (PharMingen, San Diego, CA) in the absence or presence of 2 ng/ml of TGF-β1 (R & D Systems) in RPMI 1640 medium

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[2] The abbreviations used are: IL, interleukin; IL-2Rα, interleukin 2 receptor α chain; TCR, T cell receptor; CREB, cAMP-responsive element-binding protein; TGF, transforming growth factor; WT, wild type; DN, dominant-negative; PRR, positive regulatory region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, phorbol 12-myristate 13-acetate and ionomycin; SBE, Smad2/Smad3 consensus binding element; REA, restriction endonuclease accessibility; KO, knock-out.

containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Splenocytes were stained with phycoerythrin-labeled CD4, allophycocyanin-labeled CD8, and fluorescein isothiocyanate-labeled CD25 (all from PharMingen) and analyzed using a FACSort with CELLQuest software (Becton Dickinson, San Jose, CA). EL4 and PC60 cells were maintained at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

Quantitative Reverse Transcription-PCR—Total RNA was isolated using TRIzol (Invitrogen). First-strand cDNAs were made using the Omniscript reverse transcription kit (Qiagen). Quantitative real time PCR was performed on a 7900H sequence detection system (Applied Biosystems). The sequences of the primers and Taqman probes were as follows: murine IL-2Rα: 5′-TACAAGAACGGCACCATCCTAA-3′, 5′-TTGCTGCTCCAGGAGTTTCC-3′, and 5′-(6-FAM)-TGAATGCAAGAGAGGTTTCCGAAGACTAAAGG-(TAMRA-6-FAM)-3′; murine 18S rRNA: 5′-CCTTTAACGAGGATCCATTGGA-3′, 5′-ACGAGCTTTTTAACTGCAGCAA-3′, and 5′-(6-FAM)-CGCGGTAATTCCAGCTCCAATAGCGTATATT-(TAMRA-6-FAM)-3′.

Plasmid Constructs—To generate the WT murine PRRV luciferase reporter construct, we subcloned the murine −135 to +93 IL-2Rα promoter fragment 5′ to the luciferase gene between the XhoI and HindIII sites in the polylinker of the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). The PCR fragment containing murine PRRV (−7664 to −7566) was then subcloned between the KpnI and SacI sites in the polylinker upstream of the promoter fragment. Site-directed mutagenesis of this PRRV-WT plasmid was performed using a QuikChange kit (Stratagene, La Jolla, CA). Three mutagenic primers, mSBE (5′-GTTACTATAGTTACATACATCTTTACATAAAAGGTGACACAGACATGACTTCACCTTCC-3′), mAP1 (5′-GTTACTATAGTTACATAGACGTCTAGACAAAAGGTGACATGGACATGACTTCACCTTCC-3′), and mCREB (5′-GACACAGACATGACTTTGCCTTCCTCCCCAAAC-3′) were used, respectively, to introduce the underlined 6- or 2-bp changes into the murine PRRV. The dominant-negative Smad3 3S→A plasmid was provided by Anita Roberts, and the CMV-TAM67 plasmid encoding a dominant-negative mutant variant of c-Jun plasmid (50) was a gift from M. J. Birrer.

Transient Transfections and Luciferase Assays—EL4 and PC60 cells were transiently transfected using DEAE-dextran (51). In each case, 5×10^6 cells in logarithmic growth phase were transfected with 10 μg of supercoiled test plasmid and 40 ng of pRL-SV40 as a transfection efficiency control; the cells were then allowed to recover for 24 h at 37 °C. Transfected cells were stimulated for 18 h with either medium alone or 2 ng/ml of TGF-β1 in the absence or presence of 10 ng/ml of phorbol 12-myristate 13-acetate plus 1 μg/ml of ionomycin, and the cells were harvested and analyzed for luciferase activity using a luminometer (Victor^2 1420 Multilabel Counter; PerkinElmer Life Sciences) and a dual luciferase assay system kit (Promega). Transient transfections of normal murine T cells were performed by electroporation, as previously described (22).

Restriction Endonuclease Accessibility—Restriction enzyme accessibility assays were based on a published procedure (52, 53) with certain modifications. In brief, splenic T cells (1×10^6/ml) were stimulated for 24 h in plates coated with 2 μg/ml each of anti-CD3ε and soluble anti-CD28 monoclonal antibodies in the absence or presence of 2 ng/ml of TGF-β1. These cells were washed twice in cold phosphate-buffered saline and resuspended in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine) to allow isolation of nuclei. The nuclei were washed once with 200 μl of nuclear buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine), resuspended in 50 μl of 1× digestion buffer (New England buffer 2), and digested with 1 μl of HaeIII (10 units) for 10 min at 37 °C. The reaction was stopped by adding 150 μl of stop buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.4% SDS, 0.6 mg/ml of proteinase K) and incubated at 50 °C for a minimum of 4 h. Following purification by phenol-chloroform extraction and precipitation, the DNA (3 μg) was ligated overnight to an annealed adaptor in a reaction volume of 50 μl containing 60 pmol of adaptor and 800 units of T4 DNA ligase (New England Biolabs). A nested PCR strategy was used to identify the sites of linker ligation. Samples were amplified for 12 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a final 10-min extension step at 72 °C. The primers used in the first step PCR were a gene-specific primer and a common primer (T7 primer) complementary to the long strand of the linker. The reaction product (2 μl) was then used to perform an additional 45 cycles of PCR in a 9700HT Sequence Detection System. The probe and primers used in the real time PCR were a gene-specific probe, the gene-specific primer used in the first PCR reaction, and a nested common primer (nested primer), which is also complementary to the long strand of linker but 3′ of the common T7 primer. The linearity of the assay does depend upon the linearity of the first amplification step. The oligonucleotides used for the adaptor are: 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3 (long strand) and 5′-ACCTGCCCGG-3′ (short strand). The primers and probes used for the PCR are: 5′-CTAATACGACTCACTATAGGGC-3′ (T7 primer); 5′-TCGAGCGGCCGCCCGGGCAGGT-3′ (nested primer); 5′-GAAGCACTAGTCTGTGGGTTTGG-3′ (PRRV primer); 5′-(6-FAM)-AAAAGGTGACACAGACATGACTTCACCTTCCT-(TAMRA-6-FAM)-3′ (PRRV probe); 5′-GACAGCTCAGAGTTTCCACAGTTC-3′ (PRRIV primer); 5′-(6-FAM)-CAGCAGCAGCTAACTGAAGCCACCTTTCTT-(TAMRA-6-FAM)-3′ (PRRIV probe); 5′-AGCCAAACGGGTCATCATCT-3′ (GAPDH primer); and 5′-(6-FAM)-TGCATCCTGCACCACCAACTGCTTAG-(TAMRA-6-FAM)-3′ (GAPDH probe).

Chromatin Immunoprecipitation—Chromatin immunoprecipitation assays were performed essentially as described (22). CD4 splenocytes isolated from C57BL/6 mice were either left unstimulated or stimulated with 2 ng/ml of TGF-β1 in the absence or presence of 2 μg/ml of anti-CD3ε and anti-CD28 monoclonal antibodies (PharMingen) for 18 h at 37 °C, followed by cross-linking with formaldehyde. Formaldehyde-treated nuclear lysates were subjected to immunoprecipitation with antibodies specific for Smad3 (Zymed Laboratories, Inc., South San Francisco, CA).

After treatment with proteinase K to remove protein and reversal of cross-links, the amounts of selected DNA sequences were assessed by real time PCR. The sequences of the primers and Taqman probe for PRRV were 5′-CAATGATGTCAGTCGGAAAGCTA-3′, 5′-GAAGCACTAGTCTGTGGGTTTGG-3′, and 5′-(6-FAM)-AAAAGGTGACACAGACATGACTTCACCTTCCT-(TAMRA-6-FAM)-3′, respectively. The sequences of the primers and Taqman probe for PRRIII are 5′-AGCAAAGGCTCACCTCTAACCTAA-3′, 5′-TTGCTCAACTCTCTTATCAGAAATGTC-3′, and 5′-(6-FAM)-AGGCAATCTGGGTCAGATAGTTGATGCATGATA- (TAMRA-6-FAM)-3′, respectively.

RESULTS

IL-2Rα Protein and mRNA Are Cooperatively Induced in Splenocytes by Costimulation via TCR and TGF-β1—Because TGF-β1 can inhibit IL-2-induced proliferation and has a range of actions in T cells, including inhibition of apoptosis and augmentation of effector cell expansion

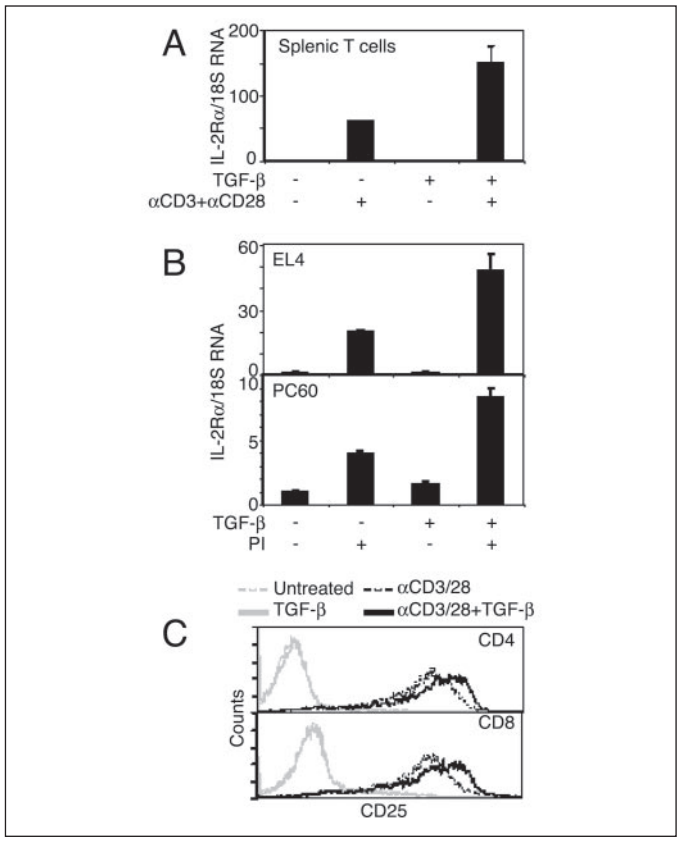

FIGURE 1. **Costimulation of TCR and TGF-β induces IL-2Rα gene expression.** *A*, total cellular RNA was from splenic T cells activated with anti-CD3 + anti-CD28 in the absence or presence of 2 ng/ml TGF-β1 for 14 h, and IL-2Rα mRNA was analyzed by quantitative real time PCR. *B*, total cellular RNAs was isolated from EL4 and PC60 cells stimulated with phorbol 12-myristate 13-acetate/ionomycin in the absence or presence of 2 ng/ml TGF-β1 for 14 h, and IL-2Rα mRNA was analyzed by quantitative real time PCR. *C*, C57BL/6 splenocytes were cultured with anti-CD3/anti-CD28 in the absence or presence of 2 ng/ml TGF-β1 for 24 h. Splenocytes were stained with phycoerythrin-labeled CD4, allophycocyanin-labeled CD8, and fluorescein isothiocyanate-labeled CD25. The histogram was shown for CD25 expression gated for CD4 or CD8 cells.

(43–46), we evaluated the effect of TGF-β1 on IL-2Rα (CD25) expression. TGF-β1 had no effect by itself, but it increased IL-2Rα mRNA levels in C57BL/6 splenic T lymphocytes activated with anti-CD3 + anti-CD28 (Fig. 1A), as well as in EL4 and PC60 cells stimulated with phorbol 12-myristate 13-acetate and ionomycin (PI) (Fig. 1*B*). TGF-β1 also increased IL-2Rα protein levels in both CD4 and CD8 cells activated with anti-CD3 plus anti-CD28 (Fig. 1*C*), suggesting that the IL-2Rα gene might have a TGF-β response element.

Identification of a TGF-β Response Element in the IL-2Rα Gene—Previously it was demonstrated that four PRRs and a CD28 response element are essential for mitogen-induced and/or IL-2-induced activation of IL-2Rα gene expression. PRRI, PRRII, PRRIII, and PRRIV correspond to DNase I-hypersensitive sites and are conserved in the human and murine IL-2Rα genes. We thus searched for conserved noncoding sequences in the human and mouse IL-2Rα gene using the VISTA program (54, 55). One highly conserved region was ~7.6 kb 5′ to the transcription initiation site in both species. Strikingly, this region contains three "AGAC" Smad2/Smad3 consensus binding elements (SBEs), as well as potential binding sites for AP-1 and CREB/ATF (Fig. 2A). Because Smad3 and Smad4 can cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription (56), the juxtapositioning of these sites suggested that this region might be a TGF-β response element. Fragments spanning this region were PCR-amplified, cloned 5′ to the murine IL-2Rα promoter (−135 to +93) in pGL3-Basic, and transfected into EL4 and PC60 cells, and the cells were analyzed for transcriptional activity. TGF-β1 induced ~9.2- and 19.4-fold inducibility in EL4 and PC60 cells, respectively (Fig. 2*B*, *top construct*). Although stimulation with PI had only a modest effect in both cell types, the inducibility was increased when the cells were additionally incubated with TGF-β1. In contrast, the −2543 to +93/luciferase/+93 to +4533 construct, which lacks the putative TGF-β response element, did not respond to TGF-β1, and TGF-β1 actually lowered PI-mediated induction (Fig. 2*B*, middle construct), presumably because of general suppressive action of TGF-β1. Transfection of EL4 cells with a "full-length" −8888 to +93/luciferase/+93 to +4533 construct, showed only modest TGF-β1-mediated induction of promoter activity; however, TGF-β1 greatly increased the inducibility mediated by treatment with PI (Fig. 2*B*, *bottom construct*). Thus, TGF-β1 could increase PI-induced IL-2Rα promoter activity, and this effect was mediated by the PRRV TGF-β response element located ~7.6 kb 5′ to the transcription initiation site in both humans and mice.

TCR and TGF-β-induced Restriction Endonuclease Accessibility at PRRV—To determine whether PRRV exists in an open or closed chromatin conformation, a restriction endonuclease accessibility (REA) assay was used and quantitated by real time PCR. The degree to which HaeIII cut its cognate site was used as a measure of the accessibility of the PRRV and PRRIV region and was measured with a two-step nested PCR strategy (see "Materials and Methods"). A similar analysis of the GAPDH exon was used as a control. The results are expressed as a ratio of cutting at PRRV or PRRIV to cutting at GAPDH.

Naive splenic T cells (10^6/ml) were stimulated for 24 h in plates coated with 2 μg/ml each of anti-CD3ε and soluble anti-CD28 monoclonal antibodies in the absence or presence of 2 ng/ml of TGF-β1. An increase in REA of cells treated with αCD3 + αCD28 was observed at PRRV as well as at the PRRIV TCR response element (Fig. 3, *lane 4*), whereas the treatment with TGF-β had no effect on the REA at either site (Fig. 3, *lane 6*). TGF-β1 further increased the REA at PRRV in cells activated with anti-CD3 + anti-CD28 (Fig. 3, *lane 8*), whereas there was no further increase of REA at PRRIV. These data suggested that the chromatin structure of PRRV is converted to an open conformation by both TCR stimulation and TGF-β1, facilitating IL-2Rα transcription.

The Smad, AP-1, and CREB/ATF-binding Sites in PRRV Are Essential for TGF-β1-induced IL-2Rα Gene Expression—To analyze the functional significance and the relative contribution of the Smad-, AP-1-, and CREB/ATF-binding sites, we mutated each site alone and in combination in the context of the −7664 to −7566 murine IL-2Rα-luciferase reporter construct and assayed the TGF-β responsiveness of each construct (Fig. 4A). In EL4 and PC60 cells, selective mutation of the SBE (PRRVm1), AP-1 site (PRRVm2), CREB/ATF site (PRRVm4), or simultaneous mutation of both AP-1- and CREB/ATF-binding sites (PRRVm6) diminished TGF-β inducibility. Simultaneous mutation of SBE- and AP-1-binding sites (PRRVm3), of SBE- and CREB/ATF-binding sites (PRRVm5), or of all sites together (PRRVm7) essentially abrogated TGF-β1-induced IL-2Rα promoter activity (Fig. 4*A*). Although TGF-β1 only induced a 1.8-fold increase in activity of the WT construct in normal mouse T cells *versus* the high inducibility in EL4 and PC60 cells (Fig. 4*A*, *top construct*), selective mutation of components of PRRV reproducibly diminished even this induction, with the SBE mutants having the lease activity (Fig. 4*A*, *constructs PRRVm1 through PRRVm7*). The significance of PRRV for TGF-β1-induced IL-2Rα expression was verified using a long (> 13 kb) reporter construct that contains all of the PRRs (Fig. 4*B*). This construct exhibited lower activity with TGF-β alone than was seen with the PRRV-IL2Rα promoter construct shown in Fig. 4*A*. WT and mutant constructs all exhibited marked PI-induc-

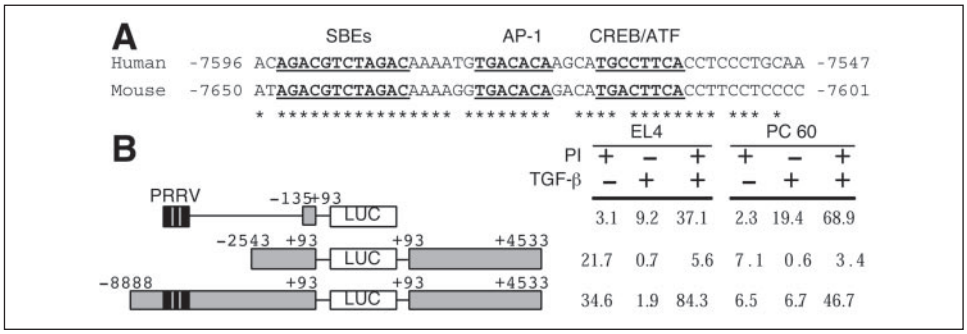

FIGURE 2. **TGF-β responsiveness of PRRV.** *A*, comparison of human and mouse PRRV sequences. The consensus SBEs and potential binding sites for AP-1 and CREB/ATF are indicated. *B*, fragments spanning PRRV (−7664 to −7566) were generated by PCR and cloned 5′ to the mouse IL-2Rα promoter (−135 to +93) in pGL3-Basic. This construct as well as the −2543 to +93/luciferase/+93 to +4533 and −8888 to +93/luciferase/+93 to +4533 murine IL-2Rα reporter constructs were transfected into EL4 and PC60 cells, and cells were then either not stimulated or stimulated with PI in the absence or presence of 2 ng/ml TGF-β1 for 14 h. Shown is fold induction relative to untreated cells.

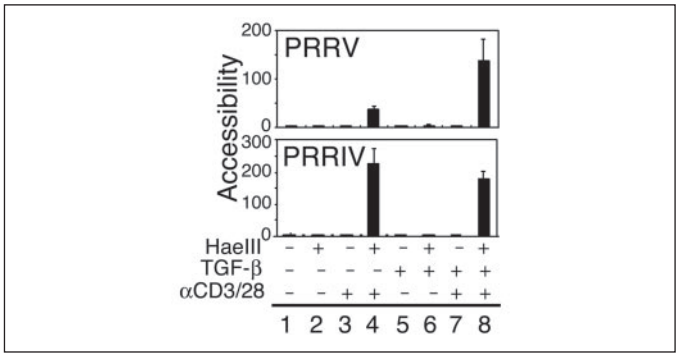

FIGURE 3. **Restriction enzyme accessibility at PRRV.** The cells were treated with TGF-β1 or anti-CD3 + anti-CD28 or all stimuli, and then REA assays were performed for PRRIV and PRRV.

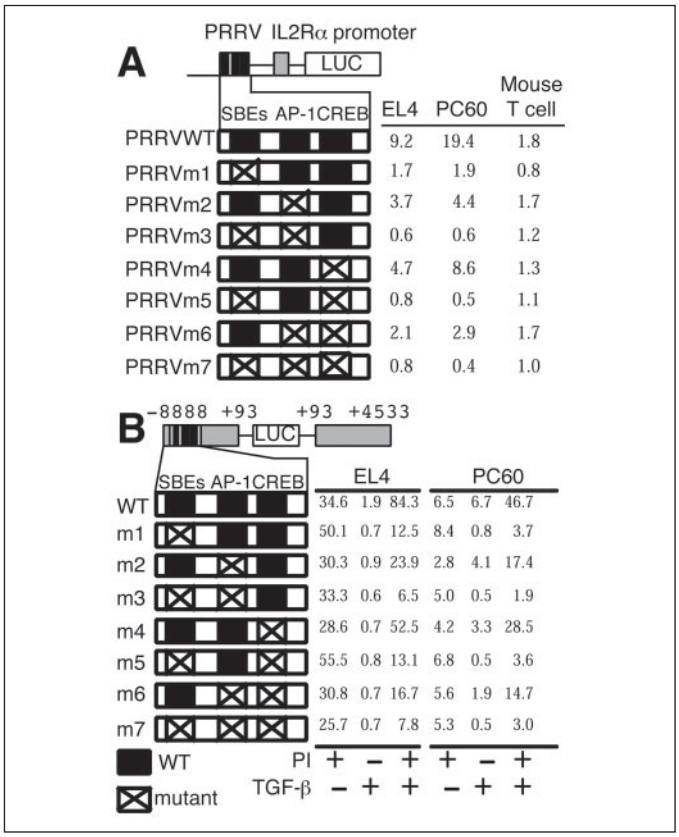

FIGURE 4. **The Smad-, AP-1-, and CREB/ATF-binding sites in PRRV are essential for TGF-β-induced IL-2Rα gene expression.** *A*, mutations in the SBE, AP-1, and/or CREB/ATF sites were made in the context of the −7664/−7566 murine IL-2Rα-luciferase reporter construct (PRRVWT construct). Constructs were transfected into EL4, PC60, and normal murine splenic T cells, followed by no stimulation or stimulation with TGF-β1. *B*, mutations as in *A* were made in the context of the −8888 to +93/luciferase/+93 to +4533 murine IL-2Rα reporter construct. The constructs were transfected into EL4 or PC60 cells, followed by no stimulation or stimulation with PI in the absence or presence of 2 ng/ml TGF-β1 for 14 h. In *A* and *B*, shown are fold induction relative to untreated cells.

ibility in EL4 and PC60 cells. However, TGF-β1-mediated induction was abrogated, and the synergistic effect of PI with TGF-β1 was also markedly decreased in constructs in which the SBEs were mutated (Fig. 4*B*, see *m1*, *m3*, *m5*, and *m7*). Thus, TGF-β specifically acts via the Smad-binding elements.

Essential Role for Smad3/4 and c-Jun Proteins in TGF-β1-induced IL-2Rα Gene Expression—To investigate the importance of Smad3 and AP-1 proteins for PRRV activity in vivo, we next used DN mutants of Smad3 and c-Jun (Smad3 3S→A, in which three C-terminal serines are converted to alanines (57), and TAM67, in which the N-terminal transactivation domain is deleted (50), respectively). In EL4 cells stimulated with PI and TGF-β1, transfection of DN-Smad3 or TAM67 diminished PRRV activity, and the effect was greater when these constructs were combined (Fig. 5*A*).

The essential role of Smad3 and Smad4 in TGF-β1-mediated IL-2Rα induction was next tested using Smad3 knock-out (KO) mice and T cell-specific Smad4 conditional KO mice. The TGF-β1-mediated increase in IL-2Rα expression seen in WT splenocytes treated with anti-CD3 + anti-CD28 (Fig. 5*B*, *top panel*) was eliminated in Smad3 KO or Smad4 KO splenocytes (Fig. 5*B*, *lower two panels*). Together, the studies in Fig. 5 (*A* and *B*) demonstrate the vital role of Smad3/4 and c-Jun proteins in TGF-β1-induced IL-2Rα expression.

Smad3 Binding to PRRV in Vivo Requires Both TGF-β and TCR Stimulation—We hypothesized that Smad3 and Smad4 were acting via the SBEs in PRRV. We therefore performed chromatin immunoprecipitation assays to examine Smad binding to PRRV in vivo. As expected, stimulation with anti-CD3 + anti-CD28 did not induce the binding of Smad3 to PRRV (Fig. 6, *lane 12*). Unexpectedly, Smad3 binding *in vivo* was not seen in cells stimulated with TGF-β1 (*lane 14*). Strikingly, however, there was a high level of binding in cells stimulated with the combination of TGF-β1 and anti-CD3 + anti-CD28 (*lane 16*), demonstrating that both signals are required for potent Smad3 recruitment to PRRV. As a negative control for the chromatin immunoprecipitation analysis, we used PRRIII, which contains GAS motifs and Ets-binding sites but lacks an SBE. Although the mean Smad3 binding to PRRIII was slightly increased when anti-CD3 + anti-CD28 + TGF-β1 were combined (Fig. 6, *open bars*, *lanes 15 versus lanes 9*, *11*, and *13*), in two other experiments, there was no increase at all, indicating the specificity of the binding of Smad3 to PRRV (Fig. 6, *closed bars*, *lane 16*).

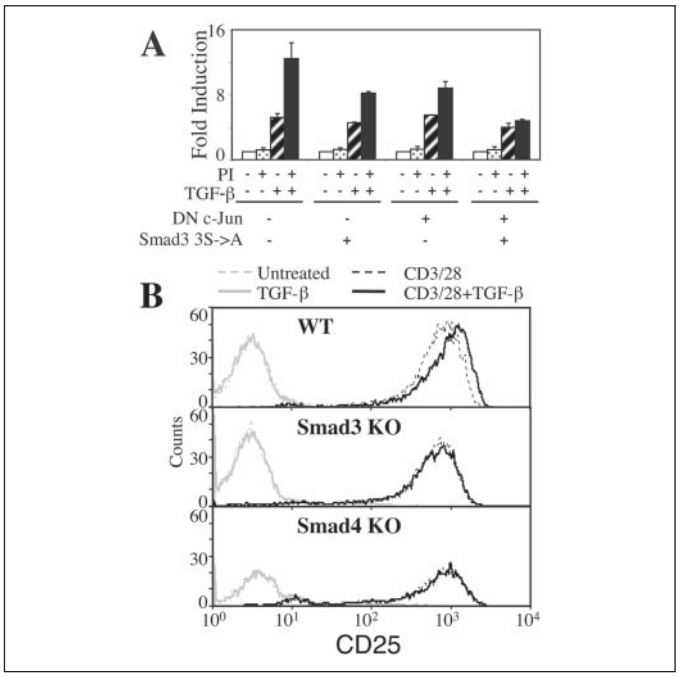

FIGURE 5. **Essential role for c-Jun, Smad3, and Smad4 for TGF-β-induced IL-2Rα gene expression.** *A*, EL4 cells were transfected with the PRRVWT construct plus 15 μg of either empty vector, DN Smad3, and/or DN c-Jun construct. Transfected cells were either not stimulated or stimulated with PI in the absence or presence of 2 ng/ml TGF-β1 for 14 h. *B*, WT, Smad3 KO, or Smad4 KO murine splenocytes were not stimulated or stimulated for 24 h with anti-CD3 + anti-CD28, TGF-β1, or anti-CD3 + anti-CD28 plus TGF-β1. Splenocytes were stained with phycoerythrin-labeled CD4, allophycocyanin-labeled CD8, and fluorescein isothiocyanate-labeled CD25. Shown is CD25 expression on gated CD4 cells.

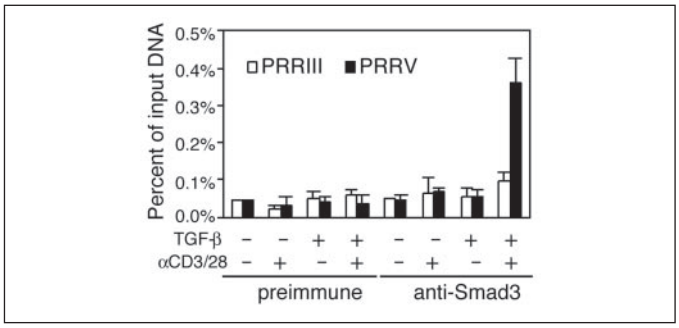

FIGURE 6. **Inducible Smad3 binding to PRRV *in vivo*.** The cells were treated with TGF-β1 or anti-CD3 + anti-CD28 or all stimuli, and chromatin immunoprecipitation assays were performed with preimmune serum or anti-Smad3. PRRIII was a negative control for PRRV.

DISCUSSION

Transcriptional regulation of the IL-2Rα gene has been extensively studied related to its induction in response to TCR and IL-2. Previously, four upstream and one intronic positive regulatory regions (PRRI, PRRII, PRRIII, PRRIV, and CD28rE) have been characterized, and the relevant DNA-binding proteins have been determined (9). Moreover, the nucleosomal organization has been partially analyzed (58). These studies have collectively elucidated the basis for TCR-mediated and IL-2-mediated activation of the gene. We now demonstrate potent cooperative induction of the IL-2Rα gene in response to TGF-β and TCR signals. By searching for additional sequences conserved between humans and mice, we now have discovered PRRV, an element that contains a Smad3-binding element as well as motifs for AP-1 and CREB/ATF. We show that PRRV is required for TGF-β1-mediated induction of the IL-2Rα gene and that Smad3 binds to PRRV *in vivo* in a TGF-β1-inducible fashion when cells are also stimulated with anti-CD3 + anti-CD28. Moreover, analysis of Smad3 and c-Jun DN constructs as well as Smad3 and Smad4 KO mice reveal the critical role of Smad3, Smad4, and c-Jun in TGF-β-mediated IL-2Rα expression.

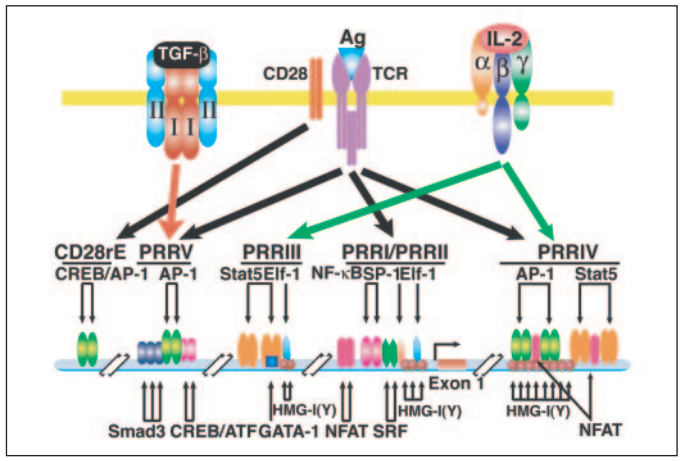

FIGURE 7. **Schematic of six positive regulatory elements in the IL-2Rα gene.** Shown are regulatory elements and binding factors that mediate IL-2Rα regulation through TCR activation, IL-2, and TGFβ.

Smad3 and Smad4 bind to DNA in a sequence-specific manner. The minimal SBE contains only four base pairs, 5′-AGAC-3′ (59–61), although most naturally occurring SBEs are 5′-CAGAC-3′ motifs. Several copies of the SBE are required for TGF-β activation of a minimal promoter (61). This requirement may reflect the relatively low affinity of single SBEs for Smad proteins. We now demonstrate that PRRV contains three copies of the consensus SBE as well as adjacent AP-1 and CREB/ATF sites. The fact that most Smad-responsive enhancers contain only one copy of the SBE and that Smad proteins must cooperate with other Smad proteins and with other DNA-binding proteins such as AP-1 or CREB/ATF to elicit specific transcriptional responses (29) strongly suggested that PRRV be an efficient TGF-β response element.

Both SMAD3 binding to PRRV by chromatin immunoprecipitation and enhanced restriction endonuclease accessibility at PRRV *in vivo* in response to TGF-β were observed only in the context of TCR stimulation. Because chromatin structure is known to be altered by T cell activation (62–64), we hypothesize that such alteration is needed for the effects of TGF-β at PRRV.

TGF-β is a critical factor in regulating T cell-mediated immune responses and in the induction of immune tolerance (27). Abrogation of TGF-β signaling in T cells in mice expressing a DN TGF-βR transgene results in dysregulated T cell proliferation and inflammatory and autoimmune-like diseases (39, 40). Although TGF-β regulation of immune responsiveness has been demonstrated *in vitro* and *in vivo*, the range of the actions of TGF-β and how it mediates these effects is not fully understood. In humans, after culturing naive $CD4^+$ T cells with alloantigen in the presence of TGF-β, $CD4^+CD25^+$ regulatory T cells are induced and potently suppress the development of $CD8^+$ cytotoxic T lymphocytes (65), indicating the cooperation of TGF-β and TCR signals. It is conceivable that the TGF-β response element in PRRV might exhibit differential specificity for IL-2Rα regulation in different T cell subsets, although support for such a model does not yet exist. Additional studies in this area may be informative. Recently, it was reported that murine $CD4^+CD25^+$ anergic/suppressor T cells can be induced and may be derived from peripheral $CD4^+CD25^-$ naive responder T cells through costimulation via the TCR and TGF-β (66). Importantly, our findings provide a molecular mechanism as to how TGF-β affects CD25 expression in a fashion that requires costimulation with the TCR.

The integration of TCR, cytokine, and TGF-β response elements distributed over discrete positive regulatory regions spanning more than 10 kb provides a basis for the compartmentalized yet coordinated complex regulation of the IL-2Rα gene as summarized in Fig. 7. These findings have important implications for the regulation of the IL-2Rα gene not only in activated T cells but also in regulatory T cells and other lymphoid lineages.

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