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Characterization of a Mobile Stat6 Activation Motif in the Human IL-4 Receptor

John J. Ryan,1,2 Lisa J. McReynolds, Hua Huang,3 Keats Nelms, and William E. Paul

The IL-4R induces proliferation and gene expression through the use of conserved tyrosine residues located in growth and gene regulation domains, respectively. We demonstrate that residues surrounding these conserved tyrosines (juxty tyr osine residues) are essential for the proper activation of the signaling molecules IRS-2 and Stat6, as well as for IL-4-induced gene expression. Further, we found that the IL-4R gene regulation domain (amino acids 557–657) contains a tyrosine-based sequence (EAGYKAF) that can convey Stat6 DNA binding and gene expression activities to a minimally active IL-4R mutant, Δ557. Thus, this tyrosine-based sequence can function as a mobile Stat6 activation cassette. However, mutants bearing this sequence induced CD23 expression much less efficiently than did wild-type IL-4R, requiring 150-fold more IL-4 to reach maximal CD23 expression. Our results indicate the importance of juxta tyr osine residues in IL-4R signaling and argue for an essential role of extended domain structure in the recognition and function of juxta tyr osine sequences.

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4 Abbreviations used in this paper: JAK, Janus kinase; aa, amino acid; PTB, phosphotyrosine-binding (proteins); IGF, insulin growth factor; 14R, insulin/IL-4 receptor; IRS, insulin receptor substrate; 125I-IL-4, 125I-labeled IL-4; PI-3K, phosphoinositide-3-kinase; EMRA, electrophoretic mobility shift assay; Ie, germline Ce transcript; WT4R, wild-type IL-4R.
Having confirmed the role of the gene expression domain Y residues in Stat6 activation and gene expression, we were struck by another feature of this domain. Each of the three Ys was equidistantly spaced, 27 aa, from one another. Furthermore, while there was little evolutionary sequence conservation in the domain as a whole, the sequences immediately surrounding each Y residue (the "juxtapatyrosine" sequence) were homologous and were evolutionarily conserved (Fig. 1B). A consensus sequence of GYK/QXF was found surrounding Y2, Y3, and Y4. Interestingly, particularly in view of the weak gene activation activity of the Δ557 truncation mutant, Y1 is embedded in a related sequence, AYRXF. The observed sequence similarities may be critical, as a mutation in the Y2 juxtapatyrosine sequence (polymorphic form of GYREF) has recently been linked to atopic disease (21).

Here, we have examined the capacity of the consensus sequence from the gene regulation domain to induce Stat6-dependent gene expression, by preparing Δ557 mutants in which the sequence surrounding Y1 has been replaced with test sequences. We show that the extended sequence derived from Y3 (EAGYKAF) induces a substantial increase in the capacity of the truncated receptor to induce gene activation. Thus, this sequence can be regarded as a mobile Stat6 activating module. However, the resultant receptor, although displaying such activity, is much less efficient than the wild-type receptor, arguing for an important role of the extended structure of the gene regulation domain in mediating its functions.

Materials and Methods

Cells/reagents

The murine B cell line M12.4.1 was the kind gift of Dr. Richard Asofsky (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and was maintained in RPMI (RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM L-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate, all from Biofluids (Rockville, MD)). Mouse anti-human IL-4Rα was purchased from Genzyme (Cambridge, MA). Horse anti-mouse IL-4Rα was from Upstate Biotechnologies (Lake Placid, NY). Rabbit anti-p85 subunit of phospho-tyrosine-3-kinase (clone B631-3) Ab and rabbit anti-mouse IRS-2 antisera were purchased from Upstate Biotechnologies (Lake Placid, NY). Rabbit anti-mouse Stat6 was the kind gift of Dr. James Ihle (St. Jude’s Research Hospital, Memphis, TN). Mouse IL-4 expressed in the baculovirus system was affinity purified as described previously (15) such that 1 U = 0.5 pg, as determined by proliferation of IL-4-dependent CT-45 cells. Human IL-4 was the kind gift of Dr. Melanie Spriggs (Immunex, Seattle, WA).

FACS analysis

For IL-4Rα analysis, cells were washed once in FACS buffer (PBS/3% FCS/0.1% sodium azide), and resuspended in 10 μl of FACS buffer in 96-well bottom-plates. Cells were incubated with 1 μl of normal mouse serum diluted 1:5 for 10 min at room temperature, followed by mouse anti-human IL-4Rα at a final concentration of 10 μg/ml for 30 min at 0°C. Cells were washed in FACS buffer, resuspended in 10 μl buffer, and incubated 30 min at 0°C with horse anti-mouse Ig at a concentration of 10 μg/ml. Cells were washed in FACS buffer, resuspended in 10 μl buffer, and incubated 30 min at 0°C with FITC-streptavidin at a concentration of 10 μg/ml. Cells were washed, resuspended in 200 μl FACS buffer, and analyzed in the presence of propidium iodide with a FACSScan (Becton Dickinson, San Jose, CA). For assessment of CD23 or I-A^d expression, cells were first incubated with 3 μl of 2.4G2 rat anti-mouse FcγRIII ascites for 10 min at 4°C, followed by 10 μg/ml FITC-conjugated rat anti-mouse CD23 or rat anti-mouse I-A^d for 30 min at 4°C in FACS buffer. Cells were then washed twice and analyzed in the presence of propidium iodide.

To determine the percentage of mouse IL-4 responsiveness in CD23 assays, FACS histograms were analyzed by setting a marker with a left-side boundary to include no more than 6% CD23-positive cells in the unstimulated control population. The fraction of mouse IL-4 responsive cells was then calculated by using the marker to determine the percentage of positive cells in each sample; % human IL-4-stimulated CD23^+ cells - % unstimulated CD23^+ cells/ % mouse IL-4-stimulated CD23^+ cells - % unstimulated CD23^+ cells.

cDNAs/expressional vectors

Wild-type and mutated human IL-4RA cDNAs were cloned into the vector pREP9 (Invitrogen, San Diego, CA).

Site-directed mutagenesis

Mutagenesis of specific residues in the human IL-4Ra was conducted using the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI), according to the manufacturer’s specifications. Oligonucleotides used for mutagenesis are indicated, with mutated nucleotides in italics: Δ557 HIR, 5'-GTG ATC TGC GCA GGC AAC CTC GAA TAC CTC AGC GCC AGC AAC TCC CTG AGC T; Δ557.EYKAF, 5'-ATG CAA GCC GGC GAG GCT GCT TAC CGC AGC T; Δ557.GKYSAF, 5'-ATG CCA GCC AAC CCT GGT TAC AAA GCC TTC AGC AAC TCC CTG AGC TCG; Δ557.EAGYKAF, 5'-CCC TGC TGC TGC ATC GCA GCC GGC GAG GCT GCT TAC AAC GCC TTC AGC AAC TCC ATG; Δ557.5'-GCC TAT CAG GAG TTT GTA TAA CCG GTG GAG GAG YAF; Y3F (Y603 to F), 5'-GCC AGT AGC TTT CAG GAG TTT GTA Y3F (Y603 to F), 5'-GGG GAG GCT GGT TTT AAC GCC TCA TCA; Y4F (Y631 to F), 5'-GGG GAA GAG GGT TTT AAG CCT TGC CAA.

Creation of stable transfectants

The mutants used in this study are shown in Figure 2. M12.4.1 cells (5 × 10^6) were transfected with 20 μg of uncut plasmid DNA with a Gene pulser electroporation device (Bio-Rad, Melville, NY) using 960 μF and 200V. Cells were grown in 10 ml RPMI overnight, then selected for resistance to neomycin (G418; Life Technologies) at 800 μg/ml for 12 to 21 days. Colonies were screened by Ab staining and/or 125I-labeled IL-4 (125I-IL-4) binding.

125I-IL-4 binding assay

To detect surface expression of human IL-4Rα, cells (1 × 10^7) were incubated in HBSS/20% M HEPES/2% FBS with 20 ng/ml 125I-IL-4 (kindly provided by Dr. Jacalyn Pierce, National Cancer Institute, Bethesda, MD) for 60 min at 0°C. Cells (5 × 10^6) were then layered on 250 μl of phlastane oil solution (1% dioctyl phthalate and 1.5% dibutyl phthalate, from Aldrich Chemical, Milwaukee, WI, and Sigma Chemical, St. Louis, MO, respectively) in 0.4 ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA), and centrifuged 10 min at 15,000 rpm. Tube bottoms were cut, and radioactivity in pellets was measured using a gamma counter (Beckman Instruments, Fullerton, CA). Specifically bound 125I-IL-4 and receptor number of cells were determined by subtracting binding to mock transfected cells.

Immunoprecipitation studies

For immunoprecipitation of the p85 subunit of phosphoinositol-3-kinase (PI-3K), 2 × 10^6 cells were stimulated for 10 min at room temperature with 10,000 U/ml mouse IL-4 or 20 ng/ml human IL-4. Cells were subjected to immunoprecipitation with 5 μl mouse anti-p85 subunit of PI-3K followed by electrophoresis and Western blotting with 1 μg/ml rabbit anti-mouse IRS-2 essentially as described (15) and developed using enhanced chemiluminescence (Rockford, IL). The blot was then stripped by incubation in a solution of 0.1 M 2-ME, 2% SDS, and 0.72 M Tris, pH 6.8, and reprobed with a 1:1000 dilution of mouse anti-p85 subunit of PI-3K.

Induction of CD23 and I-A^d

Cells (1 × 10^6) were incubated for 48 h in the presence of 500 U/ml mouse IL-4 or 1 ng/ml human IL-4, followed by Ab staining and FACS analysis, as described above.

Induction of Ie mRNA

Cells (1 × 10^6) were cultured for 48 h in the presence of LPS (10 μg/ml) (Difco, Detroit, MI) 1000 μ/ml mouse IL-4 or 10 ng/ml human IL-4, as indicated. RNA was harvested using RNAzol (Tel-Test, Friendswood, TX) according to the manufacturer’s specifications. Total RNA (10 μg) was electrophoresed on a 1% formaldehyde agarose gel and transferred to supported nitrocellulose (Schleicher and Schuell, Keene, NH). Blots were probed with a 32P-labeled cDNA containing the C(\text{III}) and C(\text{IV}) elements.
Electrophoretic mobility shift assay (EMSA)

Cells (5–10$\times$ 10$^6$) were stimulated with IL-4 (10,000 U/ml mouse IL-4 or 20 ng/ml human IL-4) for 10 min at room temperature and washed with 10 ml PBS. Cell pellets were resuspended in an equal volume of lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 8.0, 10% glycerol, 100 mM EDTA, pH 8.0, 50 mM NaF, 150 mM NaCl, 100 mM Na$_2$VO$_4$, 1 mM DTT, 400 mM PMSF, and 1 $\mu$g/ml pepstatin A, leupeptin, and aprotinin), and incubated on ice for 60 min. Lysates were cleared by centrifugation at 15,000 rpm for 15 min at 4°C, and supernatants were harvested, resuspended to a final concentration of 5 mg/ml, and stored at –70°C. For assays, 5 to 25 $\mu$g of cell lysate was incubated with 100 ng 32P-labeled oligonucleotide in reaction buffer (40 mM KCl, 1 mM MgCl$_2$, 0.1 mM EDTA, 0.5 mM DTT, 20 mM HEPES, pH 7.9, 6% glycerol, 1 mg/ml BSA, 0.1 mg/ml poly(dI:dC)) for 15 min room temperature.

Reactants were loaded (without loading dye) onto a 6% polyacrylamide, 0.22$\times$ TBE gel, which had been prerun at 200 V for 60 min and electrophoresed at 300 V for 2 h. Gels were dried and exposed directly to film. For these studies, we used a double-stranded oligonucleotide corresponding to a $\gamma$-activated site (GAS)-like element found in the mouse IL-4 promoter at position 2162 to 2152: gatcAAGACC2162TTCACAGGAA2152CTTTAAGAT, which has been shown to bind Stat6 specifically (22), or a GAS-like element found in the human CD23 promoter: 59gatcAAGACC227TTCTAAGAA221CTTTAATCgatc, which binds multiple STAT proteins. For studies using the CD23 oligonucleotide, lysates were confirmed to contain Stat6 by supershift analysis with anti-Stat6 Abs. Oligonucleotides were synthesized with a 5$'\hat{}$ACTG overhang on each end (denoted by lowercase letters) and labeled using Klenow DNA polymerase and [32P]dCTP by standard techniques.

**FIGURE 1.** A, Signaling motifs in the cytosolic region of the human IL-4R$\alpha$. Conserved Y residues are numbered sequentially. Actual aa locations, beginning with first encoded aa: Y1 = 497; Y2 = 575; Y3 = 603; Y4 = 631; Y5 = 713. Also shown are the location of deletion mutants used to delineate the growth and gene expression domains and the location of the conserved I4R motif found in IL-4R$\alpha$, HIR, and IGF-1R. B, JuxtaY residues in the growth and gene expression domains of the human IL-4R$\alpha$, and related Y960 from HIR. Y2, Y3, and Y4 in the gene expression domain share GYK/QXF consensus sequence. Numbering is relative to Y location. – = identity.

**FIGURE 2.** Diagrammatic representation of human IL-4R$\alpha$ mutants used in this study, showing location of site-specific mutations and deletions. JuxtaY1 sequences and specific mutations designed to introduce juxtaY3 sequences in the human IL-4R$\alpha$ $\Delta$557 mutant are shown at the bottom, with mutations in bold type.
Yeast two hybrid system

The yeast strain EGY48 (trp1, ura3, his3, leu2; provided by the laboratory of Roger Brent, Harvard Medical School) and yeast two-hybrid system methodology has been previously described (23–25). The insulin receptor β bait containing the kinase domain (pLexA.IR.cyto) and the IRS-1 (pAcid.IRS-1.45-516) yeast expression vectors (provided by Thomas Gustafson, University of Maryland at Baltimore) have also been described (26). A human IL-4R/insulin receptor kinase composite bait containing wild-type or mutant forms of the human IL-4R I4R motif was constructed by inserting a double-stranded oligonucleotide into the EcoRI and internal XhoI site of the pLexA.IR.cyto plasmid (coding strand oligo: 5′-AA TTGAGGCAGCCAGATGGGCCGCTCGAGCCGCTTGTCATCGCAGG CAACCCTGCTT ATCGCAGTTTCTCGAATTCGTTTCCATGCTCTG TGTACGTGCCGGACGAGTGGGAGGTGAGTCGA), replacing the insulin receptor I4R motif and destroying the 5′ EcoRI and 3′ XhoI sites. Underlined bases were those changed to create internal EcoRI and XhoI sites with minimal changes in amino acid sequence outside of the I4R motif. These internal EcoRI and XhoI sites were then used to insert 54-bp double-stranded mutant oligonucleotides, which encoded amino acid changes in the I4R motif. Point mutations were verified by sequence analysis. The effect of amino acid changes in the I4R motif on IRS-1 interaction in the yeast two-hybrid system was determined by assaying for β-galactosidase expression as described (26).

Results

Residues surrounding Y1 are important in IL-4-induced gene activation

Stable transfecants of M12 cells expressing Δ557 weakly induce CD23 and Ie in response to IL-4. Although the single Y in this truncated receptor (Y1) is part of the I4R motif also found in the insulin and IGF-1 receptors, M12.4.1 transfectants expressing insulin or IGF-1 receptors fail to induce either CD23 or Ie in response to insulin or IGF-1 (14). This suggested that the precise sequence surrounding Y1 in the IL-4R α-chain had weak gene activation activity, while the comparable sequence in the insulin or IGF-1 receptors did not. To test this, we prepared a site-directed Δ557 mutant (Δ557.I4R-HIR) (see Fig. 2 for a list of all receptor mutants used in this study) in which the sequence EYLSA from the

FIGURE 3. Role of juxtaY1 sequences in CD23 induction by human IL-4Rα Δ557 mutant. A, Substitution of HIR I4R sequence or Y-to-F mutation decreases CD23 induction by Δ557 mutant, as determined by flow cytometric analysis. Cells were stimulated with mouse or human IL-4 for 2 days and stained with anti-CD23 Abs as described in Materials and Methods. One of three representative experiments. B, Percentage of mouse IL-4-induced CD23 expression for human IL-4Rα Δ557 mutants. Data shown are means and SE from three separate experiments, as calculated in Materials and Methods. p values were calculated using unpaired Student’s t test comparing Δ557.WT to Δ557.I4R or to Δ557.Y1F.
human insulin receptor I4R motif was substituted for the AYRSF of IL-4Rα motif (Figs. 1B and 3).

A stable transfectant of M12 expressing wild-type Δ557 (Δ557.WT) was weakly active, inducing, at 10 ng/ml of human IL-4, CD23 expression on 25% as many cells as did mouse IL-4 acting on the endogenous IL-4R. Not only did human IL-4 cause fewer cells to become CD23-positive, but these cells, on the average, expressed less CD23. Δ557.I4R-HIR receptors were significantly less active in this assay. The importance of Y1 was most clearly demonstrated by mutating Y1 to F, creating a receptor (Δ557.Y1F) that failed to induce CD23.

These data indicate that the minimal gene expression activities exerted by the IL-4Rα truncation mutant Δ557 are completely dependent upon Y1 and that residues surrounding Y1 contribute to gene expression. We reasoned that this site would be valuable for testing the gene expression activity of sequences, derived from other parts of the receptor, that had been inferred to have potent gene activation capacity.

The IL-4R α-chain contains a mobile Stat6 activation sequence

Mutation studies have shown that Y2, Y3, and Y4 are important in gene activation (14). To determine whether the core homologous sequence in which they are embedded (GYK/QXF) was a functional Stat6 activation motif, we inserted the version of this sequence from the Y3 site at Y1 in Δ557. Hou et al. demonstrated that 15-mer peptides derived from Y3 and Y4 but not Y2 were active in their assay of the inhibition of Stat6 dimerization (27). Since human Y3 and Y4 share an E at position 23 (where Y = 0), we tested its importance in Stat6 activation and gene induction. In addition, substituting the sequence EA (=23, =22) from Y3 also results in the replacement of NP at the comparable location in Y1, possibly important in that it should destroy the PTB domain-binding activity of this sequence (28).

Three mutant receptors were created: Δ557, bearing an NP-to-EA mutation (Δ557.EA); Δ557, bearing an AYRSF-to-GYKAF mutation (Δ557.GYKAF); and Δ557, bearing an NPAYRSF-to-EAGYKAF mutation (Δ557.EAGYKAF) (Fig. 2). Each of these

FIGURE 4. Transfer of juxta-Y3 sequence EAGYKAF to Y1 region of human IL-4Rα Δ557 mutant enhances Stat6 activation and Stat6-dependent gene expression. A, EMSA analysis using Stat6-specific 4GL-3 probe, showing augmented Stat6 activation after transfer of juxta-Y3 sequence EAGYKAF to Y1 region. Data shown are representative of four experiments that gave similar results. B, EAGYKAF sequence enhances CD23 induction by human IL-4Rα Δ557 mutant, as determined by flow cytometry. Cells were stimulated with mouse or human IL-4 for 2 days and stained with anti-CD23 Abs as described in Materials and Methods. Data shown are from 1 of 10 representative experiments. C, Induction of MHC class II molecule I-A^d is enhanced by juxta-Y3 sequence EAGYKAF. Flow cytometric analysis of human IL-4Rα mutants; data shown is one of two experiments that gave similar results. D, Transfer of juxta-Y3 sequence EAGYKAF to Δ557 Y1 region only slightly enhances Ie transcription. Northern blot analysis using C1e probe from one of two representative experiments.
receptors was stably transfected into M12 cells and assessed for its ability to activate Stat6 DNA-binding activity in EMSA experiments. In transfectants expressing Δ557.WT, human IL-4 elicited Stat6 DNA-binding activity that was either undetectable (Fig. 4A) or very weak. Transfectants expressing Δ557.EA and Δ557.GYKAF were no more active than was Δ557.WT. By contrast, the Δ557.EAGYKAF mutant was clearly active in eliciting a Stat6 response, although much less so than WT4R. These data indicate that the sequence EAGYKAF can direct Stat6 activation when moved to a new location, arguing that the sequence surrounding Y3 is a mobile Stat6 activation module. Whether EAGYKAF constitutes the essential Y3 Stat6 activation sequence or its superiority over the core GYKXF is accounted for because it replaces the NP in the wild-type receptor remains to be determined. However, the lack of sequence conservation at positions −3 and −2 (Fig. 1B) supports the latter possibility.

**EAGYKAF can enhance IL-4-induced gene expression**

To assess the ability of the Y3 juxtagrösino residues to enhance IL-4-induced gene expression events, we tested each Δ557 mutant for activity in the CD23 expression assay (Fig. 4B), using 10 ng/ml of human IL-4. Δ557.EA and Δ557.GYKAF were no more active in this assay than Δ557.WT. Δ557.EAGYKAF caused a substantially greater degree of CD23 expression than did Δ557.WT. This result indicated that the juxtaY3 sequence could not only direct Stat6 DNA-binding activity, but also could induce expression of an IL-4-responsive gene.

In addition to inducing CD23, M12 cells also up-regulate MHC class II expression in response to IL-4. We assessed Δ557.EAGYKAF transfectants for their IL-4-induced expression of I-A^d and obtained similar findings to those observed for CD23 induction. While Δ557.WT induces little or no increase in expression of I-A^d on M12.4.1 transfectants, Δ557.EAGYKAF receptors increased I-A^d levels in response to human IL-4 to an extent similar to WT4R (Fig. 4C). However, because of the relatively narrow “window” of induction, we did not test the other mutants for I-A^d up-regulation since it would be unlikely that partial activation could be detected.

We also assessed induction of Ig class switching in response to IL-4 using the Δ557 mutants. M12 cells induce the expression of Ie transcripts in response to stimulation with LPS and IL-4, a process that has been demonstrated to be Stat6 dependent (18–20). Δ557.EAGYKAF mutants increased Ie mRNA expression over that observed with Δ557.WT, but only modestly (Fig. 4D). Since switching to IgE expression requires substantially higher concentrations of IL-4 than does either CD23 or class II induction (29), the relatively modest enhancement in Ie mRNA expression by transfectants expressing Δ557.EAGYKAF was not unexpected.

**Concentration dependence of IL-4 induction of CD23**

The CD23 induction assay in M12 cells offers a sensitive measure of Stat6-dependent gene expression. We conducted a concentration-response analysis to more precisely characterize the signaling properties of the various mutant receptors. This analysis revealed a more complex picture of gene expression than was shown by the single high dose assay.

We tested concentrations of human IL-4 between 0.003 and 10 ng/ml (Fig. 5). In selected experiments, concentrations as high as 250 ng/ml were used. WT4R induced detectable CD23 expression at 0.016 ng/ml of human IL-4. A peak response, comparable to that induced by 0.5 ng/ml of mouse IL-4 (hereafter referred to as “control response”), was stimulated by −2 ng/ml of human IL-4. The concentration required for 50% maximal induction was 0.06 ng/ml. Transfectants expressing Δ557.EAGYKAF receptors failed to induce maximal responses at any concentration of human IL-4 tested, up to 250 ng/ml (data not shown). They required 10 ng/ml of human IL-4 to induce 50% of control CD23 expression levels, an amount ~150 times that required by transfectants expressing WT4R.

Transfectants expressing Δ557.WT, Δ557.EA, and Δ557.GYKAF failed to induce responses that reached 50% of control levels (Fig. 5). No further increases in induction of CD23 expression were observed with any of the transfectants tested at 250 ng/ml of human IL-4 (data not shown). Their concentration-response curves were essentially identical, indicating that no advantage is conferred by substituting either the core Stat6 sequence alone or by simply replacing the NP from the juxtaY1 sequence.

We have previously shown that maintaining a single Y in the gene expression domain (i.e., preparing the double mutants Y2,3F; Y2,4F; and Y3,4F) resulted in receptors that could induce maximal amounts of CD23 when 10 ng/ml of human IL-4 was used to stimulate transfectants (14). We had observed that such maximal induction required the expression of 2500 receptors/cell or more, while the wild-type receptor induced maximal CD23 induction when expressed at <1000 receptors/cell. To compare mutants in which a Stat6 module was left in its normal location with one in which it had been moved to the Y1 site, we conducted a careful analysis of CD23 induction in transfectants expressing Y2,3F, Y3,4F, and Y2,4F. Each of the double mutants induced CD23 levels close to control, at 10 ng/ml (Fig. 5). The concentrations of human IL-4 required for 50% induction ranged from 0.15 to 0.45 ng/ml, indicating that these receptors were considerably more active than Δ557.EAGYKAF but less active than the WT4R.

**Truncation of the gene expression domain inhibits Stat6 activation and CD23 expression**

The lack of full CD23 or Ie induction by the Δ557.EAGYKAF mutant suggested that the possession of a single functional Stat6-activation sequence was insufficient for maximal IL-4-induced gene expression. The much greater activity expressed by Y2,3F,
Y3,4F, and Y2,4F transfectants might be because each of these possessed a “strong” Stat6 site and a “weak” Stat6 site (i.e., the juxtaY1 sequence). Alternatively, these mutants might retain an overall gene regulation domain structure that was lost when the module was moved to a new site. To examine this possibility, we created a mutant of the IL-4R α-chain truncated C terminally to the

![Image](http://www.jimmunol.org/)
conserved portion of the juxtaY2 sequence (Δ579). This mutant should possess a strong and a weak Stat6 site but has a disrupted gene regulation domain.

Transfectants expressing Δ579 exhibited diminished IL-4-induced Stat6 DNA-binding activity as compared with WT4R, Y3,4F, or Δ657 (Fig. 6A). As noted above, Δ657 possesses each of the three strong Stat6 sites and has full gene activation capacity. Δ579 transfectants induced only partial CD23 expression upon stimulation with 10 ng/ml of human IL-4 (Fig. 6B), similar to that induced in Δ557.EAGYKAF mutants and substantially less than M12 lines expressing either WT4R, Y3,4F, or Δ657 (Fig. 6C). We conclude that the presence of a strong (Y2) and a weak (Y1) Stat6 site in the IL-4R α-chain is, by itself, insufficient for full Stat6 activation.

The NPXY sequence at Y1 is important in the binding of potentially competitive PTB domain substrates to the IL-4Rα

The relatively low level of gene expression activity of Δ557.EAGYKAF appears to reflect the need to retain the structure of the gene regulation domain, or possibly the need for a more extended sequence to construct a fully competent Stat6 site. However, our studies did not directly assess the effect of replacing the NP at −3, −2 of the juxtaY1 sequence on the binding of possible competitive substrates. The related proteins IRS-1 and IRS-2 have been shown to interact with the insulin receptor I4R sequence NPXY (30, 31). To determine whether the NPXY of the I4R motif of the IL-4R was important in binding PTB domain proteins, we studied the requirements for these interactions using a yeast two-hybrid system and stable Δ557.EAGYKAF IL-4Rα transfectants.

Yeast strains expressing a “bait” construct composed of the IL-4R I4R motif linked to the insulin receptor β-chain kinase domain and a “prey” construct containing the full length IRS-1 molecule were established. Importantly, this construct allows tyrosine phosphorylation of the IL-4R I4R motif by the insulin receptor kinase (data not shown). Analysis of reporter gene expression using the yeast two-hybrid system described by Brent and colleagues (23) revealed a strong, direct interaction between IRS-1 and the tyrosine-phosphorylated I4R motif of the IL-4R (Table I). Mutation of the central tyrosine of the I4R motif (Y497) to F or E eliminated the interaction with IRS-1. Similarly, mutating the N at the −3 position (N494) to Q or E blocked the interaction of IRS-1 with the I4R motif. In contrast, mutation of the P at −2 (P495) to E or A or the S at +2 (S499) to T or R reduced IRS-1 binding only modestly as judged by reporter gene expression. Thus, the binding of the PTB domain protein IRS-1 is critically dependent on the N at −3 as well as on the central Y of the I4R motif (Y1).

To test more directly the importance of the NPXY in the interaction with PTB domain-containing signaling molecules, we assessed the ability of Δ557.EAGYKAF, which lacks the N at position −3, to activate IRS-2, the IRS-1 homologue expressed in...
hemopoietic cells. IRS-2 undergoes tyrosine phosphorylation upon IL-4 stimulation and subsequently associates with multiple downstream signaling proteins, including the regulatory subunit of PI-3K (p85) (32). Abs specific for the PI-3K p85 will co-precipitate IRS-2 from IL-4-stimulated, but not unstimulated, cells. As shown in Figure 7, human Δ557.WT receptors interacting with human IL-4 induced IRS-2 association with PI-3K to the same extent as did mouse IL-4 activation of the endogenous mouse IL-4R. However, human Δ557.EAGYKAF receptors were substantially less active in this assay. Therefore, substituting the “full” Y3 sequence for Y1 diminishes association of the competing protein IRS-2. Interestingly, IRS-2 association with PI-3K was not fully inhibited, which may be consistent with recent studies (31) indicating that IRS-2 (although not IRS-1) possesses an alternative means of interacting with the insulin receptor. Thus, the replacement of NP with EA in Δ557.EAGYKAF (and probably in Δ557.EA) results in a diminished but not absent activation of IRS-2 and thus may not fully ablate potential substrate competition at the Y1 site.

Discussion

We have previously defined two regions of the human IL-4Rα that are largely responsible for the control of IL-4-mediated growth and gene expression, respectively (14). The growth domain, located between aa 437–557 includes the 14R motif with its PTB domain-binding sequence, NPXY. This 14R motif is conserved in the receptors for IL-4, insulin, and IGF-1 (15), and all three of these receptors activate IRS-1 and IRS-2 (14, 33). Interestingly, the IL-4R truncation mutant Δ557, lacking the gene expression domain, was able to weakly activate IL-4-mediated gene expression in stable transfectants, while similar transfectants expressing receptors for insulin or IGF-1 did not (14). This led us to examine differences between these three receptors. We noted that the juxtaY1 sequence, while being conserved at the residues that define the 14R motif, was different at positions −1, +1, and +3 (where Y1 is residue 0) in the IL-4R compared with homologous residues in the insulin and IGF-1 receptors (Fig. 1). Given that STAT activation has been shown to be dependent upon Y residues and key surrounding amino acids (34), we anticipated that the juxtaY1 sequence would determine the ability of a truncated IL-4Rα to activate Stat6 and the genes that it regulates.

By transferring sequences from the human insulin receptor or introducing a Y→F mutation at Y1 in the Δ557 mutant of IL-4Rα, we show that Y1 and its juxta tyrosine sequence are required for the CD23 expression elicited by this receptor. While this result indicated that juxta tyrosine sequences control Stat6 activation, Y1 is a weak Stat6 activator. To locate strong Stat6 activation sequences, we looked to the gene expression domain.

Located between aa 557 and 657, the gene regulation domain possesses three conserved Ys (Y2, Y3, Y4) whose juxta tyrosine residues are evolutionarily conserved and homologous to one another. These residues are equidistantly spaced 27 aa from one another; this spacing is conserved among the human, mouse, and rat IL-4R, while other Y spacing is not (35). All three Ys are embedded in the sequence GYK/QXF. Interestingly, the weak Stat6 activator Y1 is surrounded by the similar sequence AYRSF. This preservation of the Y+3 residue in all STAT-activating sequences may be particularly important, as this location has been shown to play an important role in SH2 domain binding (36), the mechanism for STAT association with receptors.

Wang et al. (16) demonstrated that the entire gene regulation domain could be moved to a truncated IL-2Rβ. In response to IL-2 stimulation, transfectants expressing this receptor activated CD23, but did not proliferate. This important finding both confirmed the function of the domain and showed it was a mobile structure. We wanted to further these results by determining the minimal Stat6 activation cassette in the gene regulation domain.

Hou et al. (27) showed that peptides expressing extended sequences surrounding Y3 and Y4 inhibited the induced DNA-binding activity of Stat6 and we demonstrated that all three Ys function in Stat6 activation and gene expression. Thus, we suspected that the difference in juxta tyrosine sequences between the IL-4R growth and gene regulation domains would explain the failure of the growth domain to activate Stat6 and the genes it regulates. Surprisingly, transferring the conserved juxtaY3 sequence GYKAF to Y1 in Δ557 had no effect on the ability of this mutant to activate Stat6.

A possible explanation of thus unexpected result could be that the NPXY sequence within the 14R motif would allow the binding of PTB domain-containing proteins, including IRS-1 and IRS-2. If these large proteins were also attempting to “dock” to phosphorylated Y1, this might inhibit Stat6 binding. Thus, we substituted EA (found at −3 and −2 in the juxtaY3 sequence) for NP in the juxtaY1 sequence, effectively destroying the PTB domain recognition site. Indeed, data from a yeast two-hybrid analysis indicated that mutations at the N (−3) and P (−2) residues greatly decrease IRS-1 binding to the IL-4R 14R motif, giving hope that this mutation would block IRS-2 binding to IL-4R in M12 cells. However, Δ557.EA mutants also showed no increase in gene expression abilities.

Since neither mutation alone enhanced gene expression, we transferred the entire sequence to IL-4R, making a Δ557.EAGYKAF mutant that possessed the conserved juxta tyrosine sequence, and should lack IRS-2 activation capacity. In fact, we found that this mutant could elicit some association of IRS-2 with PI-3K p85, although at reduced levels compared with the wild-type receptor. This result is in keeping with recent findings of a second receptor association domain present in IRS-2 but not IRS-1 (31). How this domain binds the IL-4R remains unknown, and the more complex question of IRS-2 association and its effects on Stat6 activation remain to be answered. Nonetheless, Δ557.EAGYKAF did show enhanced Stat6 DNA binding activity and could activate all of the genes tested. Thus, the sequence EAGYKAF can serve as a Stat6 activation sequence and is mobile, at least within the IL-4R.

Although introducing the extended Y3 sequence allows enhanced gene expression, Δ557 receptors expressing this sequence do not maximally induce such activation. Indeed, the Δ557.EAGYKAF mutant only weakly activates Erk and fails to induce maximal CD23 expression even at ~19 nM IL-4, the highest concentration tested. Transfectants expressing this receptor require greater than 150-fold more IL-4 to induce CD23 expression on 50% of test cells than is required by transfectants expressing wild-type receptor. By contrast, transfectants expressing full length receptors in which 2 of the 3 Ys within Stat6 sites have been mutated to F achieve close to full activation at <1 nM IL-4 and require only 3- to 10-fold more IL-4 for induction of 50% activity than transfectants expressing the wild-type receptor. The superiority of these point mutants might represent the need to maintain more extended sequences, either because the Stat6 docking site is actually larger than that inferred from sequence homology or because such sequences are necessary to retain three dimensional structure.

Alternatively, the point mutants might be superior to Δ557.EAGYKAF because they actually express one strong Stat6-binding site (Y2, Y3, or Y4) and one weak Stat6-binding site (the Y1 site), whereas Δ557.EAGYKAF expresses only one site. This does not appear to be the case. A truncated receptor...
(Δ579) in which the truncation occurs just after the end of the conserved sequence in the Y2 Stat6-binding site functions, similar to the Δ557.EAGYKAF rather than to the double point mutants, is consistent with the need for more extended conserved sequences rather than with the need for a strong and a weak Stat6 site.

In this regard, it is important to point out that other studies of the mobility of receptor elements have generally not examined the relative efficiency of receptors expressing the transplanted elements or the ability of these receptors to activate STAT-specific gene expression. Thus, Gerhart et al. (37), Stahl et al. (34), and May et al. (38) have each shown that short receptor-derived consen sus elements can direct activation of Stat1, Stat3, and Stat5, respectively, but only to the extent of assessing STAT tyrosine phosphorylation or DNA binding. While these are two crucial events in the use of the JAK-STAT pathway, the end effect of gene expression has remained unknown.

We show that an IL-4Rα-derived consensus motif can confer Stat6 activation to a receptor that normally only weakly activates Stat6, and more importantly, that this activation results in the expression of three Stat6-specific genes. Further, our study offers evidence that Stat6 activation and the resulting gene expression can occur as a graded event, resulting in partial gene activation that requires a higher concentration of IL-4 than does the wild-type IL-4Rα. The genes affected showed a range of expression, with the more sensitive CD23 and I-A expression elicited to a higher degree than Ie. Thus, our study demonstrates that a short consensus element cannot only direct specific activation of Stat6 but that the degree of Stat6 activation determines the extent of Stat6-dependent gene expression.

These studies point to the capacity of individual units of the receptor to behave as modules, but also indicate that their optimal function depends upon retention of certain elements of their native structure. Indeed, it is striking that the three evolutionary variants of the IL-4Rα that have been studied show a high degree of conservation in both the number and the placement of the Stat6-binding sites within the gene regulation domain. This strongly suggests that the structural organization of the receptor is key to its efficient function. Indeed, understanding the significance of the equal spacing of the Stat6-binding sites in the absence of significant sequence conservation of the Y2-Y3 and Y3-Y4 interval sequences to one another or among species may be critical to understanding the normal functioning and the evolution of the receptor.

It has recently been reported that a polymorphism exists in the juxta-Y2 sequence in humans and that individuals with an R at the +1 position (GYREF) are at much higher risk of atopic disease than individuals with a Q at that position (21). The authors suggest that this reflects a diminished capacity of the R polymorphic form to bind the phosphatase SHP-1 (SRC homology phosphatase-1). An alternative possibility is that the Q polymorphic form is a weaker Stat6 site. An examination of the detailed dose response properties of the Y2F mutant (14) could be very revealing. If the principal function of the Q polymorphic form is as a SHP-1 docking site, then the Y2F mutant might be expected to be more effective as an IL-4R than the wild-type form, since the SHP site would have been eliminated.

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