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New Programming of IL-4 Receptor Signal Transduction in Activated T Cells: Stat6 Induction and Th2 Differentiation Mediated by IL-4Rα Lacking Cytoplasmic Tyrosines

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Signaling by the IL-4 receptor α-chain (IL-4Rα) is a key determinant of the development of the Th2 lineage of effector T cells. Studies performed in tissue culture cell lines have indicated that tyrosines of the IL-4Rα cytoplasmic tail are necessary for the induction of Stat6, a transcription factor required for Th2 differentiation. Surprisingly, we have found that in activated T cells, IL-4Rα chains lacking all cytoplasmic tyrosines promote induction of this IL-4-specific transcription factor and efficient commitment to the Th2 lineage. Mutagenesis of a tyrosine-free cytoplasmic tail identifies a requirement for the serine-rich ID-1 region in this new program of IL-4R signal transduction observed in activated T cells. Additional findings suggest that an extracellular signal-regulated kinase pathway can be necessary and sufficient for the ability of such tyrosine-free IL-4Rα chains to mediate Stat6 induction. These results provide novel evidence that the molecular mechanisms by which a cytokine specifically induces a Stat transcription factor depend on the activation state of T lymphoid cells. Furthermore, the data suggest that one pathway by which such new programming may be achieved is mediated by extracellular signal-regulated mitogen-activated protein kinases. *The Journal of Immunology*, 2003, 171: 1891–1900.

Interleukin-4 is a pleiotropic cytokine that exerts crucial immunoregulatory effects on lymphoid and nonlymphoid cells (1, 2). A critical function of this cytokine is to promote the differentiation of activated T cells into Th2 effectors (3, 4). Naive CD4 T cells stimulated in the absence of IL-4 signaling are severely impaired in the development of progeny which secrete cytokines of the Th2 program (IL-4, -5, and -13) (5, 6). In contrast, the presence of IL-4 provides a powerful stimulus for activation of Th2 cytokine gene expression (7). The cytokines produced by a Th2 cell population are then able to promote allergic diseases (8), host resistance to extracellular parasites (9, 10), and protection against organ-specific autoimmunity mediated by the Th1 subset of T cells (11–14).

To exert these effects, IL-4 binds to cell surface receptor complexes of which the IL-4Rα chain is an obligate component (2, 15, 16). Studies with IL-4Rα-deficient mice establish a critical role of this protein in the development of a Th2 response as well as for the actions of IL-4 on nonlymphoid target cells (16, 17). IL-4 binding to IL-4Rα induces the Th2 program of cytokine genes in activated T cells by inducing the tyrosine phosphorylation of a transcription factor designated Stat6 (18–21). Thus, Stat6-deficient CD4 T cells are impaired in their ability to differentiate into Th2 effectors, while IL-4-independent Th2 development without IL-4R signaling was promoted by expression of constitutively activated Stat6 constructs in T lymphoblasts (22, 23).

Stat6 is a member of a family of transcription factors which serve highly specific developmental functions after their induction by cytokines (24, 25). The induction of Stat proteins in general, and Stat6 in particular, has been studied intensively in cell lines (25, 26), leading to a general model of how specificity is achieved for Stat induction (2, 25, 27, 28). In this model, cytokine engagement of the appropriate receptor leads to activation of noncovalently associated members of the Janus family of protein tyrosine kinases (28, 29), which then phosphorylate tyrosine residues in the cytoplasmic tail of the cytokine receptor. The standard mechanism by which specific coupling of a cytokine with Stat signaling arises through molecules containing appropriate Src homology 2 (SH2)6 and protein tyrosine-binding domains which form complexes after specific recognition of these phosphoryrosines in the cytoplasmic tail of a restricted subset of receptors (29–31). The presence of an SH2 domain represents a highly conserved feature of Stat transcription factors, but the SH2 domains of each Stat transcription factor encode different specificities for the amino acids flanking receptor phosphoryrosines (31, 32). Accordingly, tyrosine phosphorylation of the intracellular tail of the cytokine receptor provides a requisite scaffolding function, thereby creating biological specificity through the provision of ligand-induced docking sites (2, 33). In the case of IL-4, this means of achieving a highly specific functional linkage to Stat6 induction

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6Abbreviations used in this paper: SH2, Src homology 2; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; m, mouse; h, human; IRES, internal ribosomal entry site; GFP, green fluorescent protein; RLU, relative light unit; PKC, protein kinase C; EpoR, erythropoietin receptor.
predicts an absolute requirement for conserved tyrosines in the cytoplasmic tail of IL-4Rα.

Whereas these features of Stat6 induction are well supported by evidence from cell lines treated with cytokine alone, a central feature of IL-4/Stat6-induced lymphocyte differentiation is that it occurs only in activated cells. For instance, Th2 differentiation and the accompanying changes in cytokine gene transcription require TCR-mediated cellular activation. Because precedents from other receptor signaling systems suggest that prior signals can reprogram the mechanisms of receptor signaling (34), we hypothesized that similar reprogramming of IL-4Rα might occur after T cell activation. To investigate how the IL-4Rα promotes the development of Th2 cells, we used retroviral transduction of freshly activated IL-4Rα-deficient T lymphocytes with wild-type and mutated receptors. Transduction of a receptor and IL-4 treatment of the cells were required for the emergence of IL-4-producing cells. Surprisingly, however, mutated receptors lacking all cytoplasmic tyrosines efficiently promoted Th2 differentiation. Furthermore, Stat6 was induced by IL-4 even when activated T cells expressed a receptor lacking cytoplasmic tyrosines. For IL-4Rα chains lacking cytoplasmic tyrosines, the ability of IL-4 to induce Stat6 and Th2 development required an acidic serine-rich region. Using a T cell transfection model similar to those used to evolve the original paradigm of Stat activation, we provide evidence of one signaling pathway that is capable of triggering this new programming of IL-4Rα function. Specifically, an extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) may be necessary and sufficient for the functional coupling of Stat6 induction to these mutant receptors.

Materials and Methods

Mice

IL-4Rα-deficient mice on a BALB/c background (generous gift from N. Noben-Trauth, George Washington University, Washington, DC) were bred with DO11.10 TCR-transgenic BALB/c mice. IL-4Rα+/− and DO11.10-transgenic mice were genotyped by PCR (17). Mice were maintained in microisolator cages under specific pathogen-free conditions and used with institutional approval in accordance with applicable regulations.

Plasmids and mutation

Full-length huStat6 cDNA (35) was cloned into the MIT retrovector (36) and mouse (m) IL-4Rα cDNA (C57BL/6) into pcDNA3 and MSCV2.2-IREs-GFP (GFP-RV) (37). Stop codons, restriction sites, and amino acid substitutions were introduced into a mIL-4Rα cDNA by PCR using Pfu polymerase and DpnI (Stratagene, La Jolla, CA). For consistency with existing literature, numbering of the mIL-4Rα sequence rather than the amino acid residue number in the primary sequence was used in this study. The ID-1 region was mutated using site-directed mutagenesis.

Retroviral transduction and Th2 polarization

Single-cell suspensions were prepared from spleen and lymph nodes as described previously (41). Retrovirus-containing supernatants were collected 48 h after transfection of the PGN24 ecotropic packaging cell line with retrovectors plasmids and centrifuged (1 h, 10,000 × g) with peptide-activated splenocytes from IL-4Rα−/− DO11.10-TCR-transgenic mice as described elsewhere (37, 42). Cells were cultured in the presence of recombinant mIL-4 (10 ng/ml), recombinant human (h) IL-2 (10 ng/ml), anti-IFN-γ (2–10 ng/ml), and anti-IL-12 (2–10 ng/ml). Expression of IL-4Rα was determined by flow cytometric analysis 3 days after transduction. After 6–7 days of culture, cells were washed, sorted for GFP+ cells, and restimulated. Restimulations were performed using peptide-pulsed APCs (BALB/c, wild-type, or IL-4Rα deficient) or plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (2.5 μg/ml) for 48 h. Cytokine production was determined by ELISA of these culture supernatants while the frequency of cytokine-producing cells was measured by restimulation of Th2-polarized cells (APCs plus OVA233−339 peptide or PMA plus ionomycin) and intracellular staining (43, 44).

Gel mobility shift analysis

IL-4Rα−/− DO11.10 TCR splenocytes were activated with OVA peptide and infected with replication-defective internal ribosomal entry site (IRES)-green fluorescent protein (GFP) retroviruses encoding wild-type or mutated IL-4Rα cDNAs. Six days after transduction, GFP+ cells (106) were isolated by positive sorting using a FACSStarTM (BD Biosciences), divided equally, and recultured for 30 min in complete medium with or without IL-4 (30 ng/ml). After lysis with 0.5% Nonidet P-40, 50 mM Tris–Cl (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, 100 mM Na2VO4, 50 mM NaF, 1 mM DTT, 0.4 mM PMSF, 3 mg/ml aprotinin, 1 μg/ml leupeptin, and 10% glycerol, lysates were cleared of insoluble material by centrifugation (15,000 × g, 5 min). Mobility shift assays were performed using 5 μg of total protein, 1 μg of poly(dI-dC), and a Stat6-specific 32P-labeled double-stranded DNA probe from the mouse germline Ig H chain promoter as described previously (45).

Establishment of Jurkat cell lines

Jurkat cells were cultured in medium as described elsewhere (44). 293T and FNX cells (gift from Dr. G. Nolan, Stanford, CA) were cultured (37 C in 5% CO2) in complete DMEM. To create stably transduced polyclonal cell populations, VSV-G-pseudotyped retroviruses were produced by co-transfecting 293T cells with MiT-Stat6, pSV-gp, and pCMV-G vectors using the calcium phosphate method. Jurkat T cells were infected with virus-containing supernatants (collected after 48 h of culture at 35°C, 3% CO2) in the presence of polybrene (8 μg/ml for 6–16 h) and Thyl.1 progeny were isolated by FACS after 2–4 day. Jurkat cell populations stably transduced with Stat6 were divided and infected with VSV-G-pseudotyped retroviruses containing MSCV-GFP-mIL-4Rα constructs. After preparative FACS for Thy1.1 and GFP expression, cell surface expression of mIL-4Rα was determined using M1 anti-IL-4Rα as described above.

Flow cytometric analysis and intracellular staining

Biotinylated and fluorochrome-conjugated Abs against CD4 (R-PE), Thy1.1 (R-PE), IL-4 (allophycocyanin), IFN-γ (R-PE), anti-IgG (biotin), and streptavidin-PerCP were obtained from BD Pharmingen (San Diego, CA). M1 anti-IL-4Rα Ab (40) was a generous gift from Immunix (Seattle, WA). mIL-4Rα was detected by indirect immunofluorescent staining with unlabeled M1 Ab followed by biotinylated anti-IgG4,5,6 and then streptavidin-PerCP. For intracellular staining, Th2-polarized lymphocytes were restimulated for 6 h with peptide-pulsed BALB/c APCs (1 μg/ml with a 1:1 APC:T cell ratio) or PMA (50 ng/ml) plus ionomycin (1 μg/ml). Monesin (2 μM) was added to the cell culture during the last 4 h. Cells were stained with anti-IL-4Rα, fixed with 4% paraformaldehyde, and permeabilized in 0.5% saponin/1% BSA in PBS before staining with fluorochrome-conjugated anti-IL-4 and anti-IFN-γ. IL-18Rα expression was quantitated by three-step indirect immunofluorescent staining with anti-IL-18Rα (R&D Systems; Minneapolis, MN), biotinylated anti-IgG4,5,6 and streptavidin-PerCP. Fluorescent sorter analyses used a FACSCalibur and was analyzed using CellQuest (from both BD Biosciences, Mountain View, CA) or FloJo software.

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Western blot analysis

Stably transduced Jurkat T cells expressing wild-type and mutant mIL-4 receptors ($20 \times 10^3$) were transiently transfected with Stat6-dependent TK-luciferase, pcDNA3-Stat6 (35) and lacZ reporter plasmids using electroporation in a Bio-Rad Gene-Pulser (Bio-Rad, Hercules, CA). Similarly, Jurkat T cells stably transduced with mIL-4 receptors were cotransfected with Stat6 reporter, pcDNA3-Stat6 and lacZ reporter plasmids along with the dominant active ERK plasmid or pCMV5-Myc control. Cells were replated in RP10F, cultured for 16 h, divided into equal portions, and treated for 24 h with medium alone, mIL-4 (10 ng/ml), hIL-4 (10 ng/ml), each in the presence or absence of PMA (50 ng/ml) or PMA plus ionomycin (1 μg/ml). Extracts of these treated cells were assayed for luciferase activity in relative light units (RLU) (46). To quantitate induction of luciferase activities by IL-4, values induced by mIL-4 through mutated receptors were normalized for transfection efficiencies relative to the wild-type receptor (normalized fold induction = fold induction $\times$ (hIL-4 RLU(wt))/hIL-4 RLU(mutant)). Where indicated, cells were pretreated (100 μM, 30 min) with the MEK inhibitor U0126 (Promega, Madison, WI) or the p38 inhibitor SB202190 (10 μM; Alexis, San Diego, CA) before stimulation with cytokine and/or PMA.

Results

Efficient Th2 differentiation promoted by an IL-4Ra lacking the differentiation domain and cytoplasmic tyrosines

The development of activated CD4 T cells into Th2 cells is one of the most critical differentiation events promoted by IL-4 binding to the IL-4Ra chain. To dissect the requirements of the receptor cytoplasmic tail in this process and in particular to investigate the requirement for that portion of the receptor which has been essential for Stat6 induction in cell lines (26, 47, 48), we established a reconstitution system using lymphoid cells from IL-4Ra-deficient mice. DO11.10 TCR-transgenic cells homozygous for a disrupted IL-4Ra locus were activated in vitro using OVA323–335 peptide, divided shortly after activation, and infected with replication-defective retroviruses encoding wild-type or mutated mIL-4Ra cDNAs. Of note, cell surface staining showed that the transduced wild-type receptor was expressed only on GFP+ cells and was not overexpressed compared with the endogenous IL-4Ra gene in wild-type cells (Fig. 1, A and B). After infection, cells were plated in Th2 conditions during further culture and then assayed for the capacity to produce IL-4 upon restimulation. Although a delay in IL-4 addition to cultures after T cell activation may reduce the efficiency of Th2 development (49), the expression of transduced receptors was well able to mediate Th2 differentiation (Fig. 1C), consistent with preliminary findings that IL-4 could induce significant Th2 differentiation of wild-type T cells when its addition was delayed 2 days after T cell activation (data not shown). These data indicated that for DO11.10 T cells activated with antigenic peptide, induction of significant IL-4 gene activation was dependent on IL-4Ra expression. To determine whether the IL-4-binding ectodomain alone was sufficient to promote Th2 differentiation, we tested a mutated IL-4Ra chain deleted at a membrane-proximal site. Consistent with an essential role for the IL-4Ra cytoplasmic tail, transduction of DO11.10 IL-4Ra-∆cyt cells with a wild-type mIL-4Ra cDNA permitted the development of IL-4 production, whereas cells expressing a mutant receptor lacking almost the entire cytoplasmic tail (∆cyt) produced no IL-4 upon restimulation (Fig. 2, A and B). Despite its failure to restore activation of IL-4 production, IL-4Ra(∆cyt) was expressed at the cell surface at levels greater than or equal to the wild-type polypeptide (Fig. 2C). To determine whether the IL-4 production data were reflective of Th2 differentiation, immunofluorescent staining of intracellular cytokines was performed. Among the transduced (GFP+) cells, only cells that expressed an IL-4Ra developed the capacity to accumulate detectable levels of IL-4 protein, and IL-4-positive cells lacked detectable IFN-γ (Fig. 2B).

In resting tissue culture cells, the highly specific induction of Stat6 by IL-4 appears to require a scaffolding function of the tyrosines in the IL-4Ra cytoplasmic tail. Thus, IL-4 binding leads to receptor tyrosine phosphorylation and induction of Stat6-requiring tyrosines in the second, third, and fourth positions, conserved in...
FIGURE 2. IL-4Rα lacking the differentiation domain and cytoplasmic tyrosines efficiently mediates development of IL-4-producing cells. A. Mutant IL-4 receptors promote development of the indicated IL-4 production capacity in T cells. DO11.10−/−IL-4R−/− splenocytes were activated with OVA123-339 peptide and transduced with retrovectors encoding the indicated wild-type (WT) and IL-4Rα mutants (diagrammed to left). After 6 days of culture under conditions polarizing toward Th2 development, GFP+ T cells were restimulated with OVA123-339-pulsed APCs followed by measurement of IL-4 production in culture supernatants. Mean ± SEM results of independent experiments, in each of which values were normalized to the IL-4 production of cells transduced with wild-type IL-4Rα (100%). Th2-polarized splenocytes from IL-4R−/− and IL-4Rα+/- mice were used as negative and positive controls, respectively. To the right, unnormalized ELISA quantitation of IL-4 production in an experiment representative of four independent transductions is shown (** sample, n = 2 experiments, construct not used in the experiment shown). Similar results were obtained using sorted and unsorted (GFP+/−) cells and with restimulation using OVA123-339 peptide presented by IL-4Rα−/− APC or plate-bound anti-CD3/anti-CD28. B. Efficient Th2 differentiation promoted by a tyrosine-free IL-4Rα. Cells generated as in A were grown in Th2-polarized conditions, restimulated 6 h with APCs plus peptide, and processed for staining of intracellular IFN-γ and IL-4. Representative profiles of GFP+ cells are presented. No IL-4+ cells developed among the GFP− population. The percentage of Th2 (IL-4+ IFN-γ−) cells in each sample is indicated in the circled regions. C. Mutant receptors are expressed at levels comparable to those of wild type. Three days after transduction with the indicated constructs (performed as in A), expression of IL-4Rα on primary IL-4Rα−/− T cells was determined using the M1 mAb against mIL-4Rα ectodomain. Immunofluorescent analyses were used to generate histograms of IL-4Rα on GFP− and GFP+ cells in the CD4+ T cell gate. Note that ~50% of cells transduced with wild-type IL-4Rα exhibited expression levels within the range of activated T cells from wild-type mice.
transduced T cells, both as assayed using a bulk population of transduced cells and with restimulation of the GFP+ population after preparative cell sorting (Fig. 2A). This finding was not due to overexpression of the mutated receptors, because each of these proteins was expressed at a level comparable to that of the transduced wild-type IL-4Rα, which in turn was present at levels no higher than endogenous receptors on wild-type T cells (Fig. 2C). Furthermore, we have noted no difference between IL-4Rα heterozygous (+/-) and T cells (+/+) in measurements of IL-4 production or the frequency of Th2 cells (data not shown). Finally, the receptor mutant truncated at a membrane-proximal site in the box 1 sequence (Δcyt) failed to restore any IL-4 production or generate any IL-4+ cells. This lack of activity was not due to deficient expression: deletion of the cytoplasmic tail led to cell surface expression at least equal to that of wild-type receptors (Fig. 2C). Together, these data indicate that ligand binding can promote Th2 differentiation in the absence of all cytoplasmic tyrosines of IL-4Rα, but residues between box 1 and the Δ437 end point are essential for this process.

Th2 development in these in vitro assays is highly dependent on the nuclear induction of Stat6 but a Stat6-independent pathway has also been described (19–23, 37). To determine whether the specific induction of Stat6 could be affected by an IL-4Rα chain lacking cytoplasmic tyrosine residues, activated T cells transduced with IL-4Rα cDNAs were expanded in IL-2 and used for mobility shift assays after sorting for GFP+ cells. Under these conditions, Stat6-binding activity was detected in cells expressing either the wild-type receptor or the mutant Δ437(Y0F), but only if stimulated with IL-4 (Fig. 3). In contrast, the expression of a ligand-binding domain (Δcyt) was not able to promote Stat6-binding activity. These data show that the specific induction of Stat6 by IL-4 required IL-4Rα expression but was mediated by a chain lacking the cytoplasmic tyrosines which confer specificity in nonactivated tissue culture cells.

IL-4 induction of Stat6 mediated by an IL-4Rα chain lacking cytoplasmic tyrosine residues depends on activating signals in the T cell

Previous analyses of the mechanism of Stat6 induction by IL-4Rα have used tissue culture cell lines without other concurrent stimuli, and in this setting induction requires the conserved phosphotyrosines Y2, Y3, and Y4 (2, 47, 50). Two potential reasons why the present results with Th2 differentiation might differ from previous studies are a 1) a disparity between tissue culture cell lines and primary mouse T cells or 2) an alteration in the functional characteristics of the receptor triggered by other signaling pathways, e.g., those induced after T cell activation. To distinguish between these possibilities, we tested the function of key IL-4Rα mutants using the human tissue culture cell line Jurkat. After transfections with a Stat6-dependent reporter gene and mIL-4Rα cDNAs, the ability of receptors to activate the Stat6 pathway was assayed. Wild-type mIL-4Rα mediated IL-4 induction of the Stat6 reporter construct, whereas anti-CD3 pretreatment enhanced this IL-4-induced transcriptional activity but did not in itself cause promoter activity (Fig. 4A). Importantly, a mutated IL-4Rα chain lacking all tyrosine residues, Δ437(Y0F), was able to induce Stat6-dependent reporter gene activity in Jurkat cells subjected to TCR cross-linking but not in resting controls (Fig. 4A). In similar experiments, the Δ437(Y0F) mutant was unable to transduce significant induction of Stat6 reporter activity in resting Jurkat T cells treated with mIL-4, whereas significant induction of the Stat6 reporter construct was triggered by mIL-4 treatment of T cells activated by a protein kinase C (PKC) mimetic and calcium ionophore (Fig. 4B). Comparable results were obtained whether the receptors and Stat6 were introduced by transient transfection or by stable retrovector-mediated transduction of receptors and Stat6 into the cells, and in each instance receptor expression among the mutants was similar to that of wild-type and to that on primary mouse T cells (data not shown). Furthermore, this effect could be provided by PMA alone (Fig. 4B, right panel). These findings indicated that transcriptional activity dependent on functional Stat6 could be induced via an IL-4Rα lacking cytoplasmic tyrosines. Consistent with earlier studies, the mutated receptor did not respond to IL-4 treatment of resting cells, but only after cellular activation.

For further biochemical evidence that Stat6 can be induced by IL-4 binding to IL-4Rα chains lacking tyrosines, the ability of Δ437(Y0F) to mediate Stat6 induction was tested using stably transduced Jurkat T cells. Unlike the transduced primary cells, sufficient protein could be obtained to perform immunoblots for detection of the induced, tyrosine-phosphorylated Stat6. Consistent with the reporter gene data, induction of anti-phospho-Stat6 by mIL-4 was detected in nuclear extracts of PMA-activated but not resting T cells (Fig. 4C). Collectively, the data indicate that an IL-4Rα devoid of cytoplasmic tyrosines could induce transcriptionally active Stat6 in a process mobilized by signaling pathways downstream from T cell activation or a PKC agonist. This induction of Stat6 DNA-binding activity in activated T cells (primary or Jurkat) required IL-4 treatment, the IL-4Rα ectodomain, and residues of the cytoplasmic tail distal to the box 1 sequence. Furthermore, the presence of another cytokine whose receptor uses the common γ-chain, IL-2, did not lead to any Stat6 induction. We conclude that in activated primary T cells, specific induction of Stat6 by IL-4 must be transduced via an IL-4Rα chain but this specificity can be achieved without the tyrosine residues of the IL-4Rα chain.

An ERK MAPK pathway can render tyrosine-free IL-4Rα competent to induce Stat6

The preceding data suggested that one or several signaling pathways must be sufficient to reprogram mutated IL-4Rα chains which lack tyrosines, inasmuch as IL-4 led to nuclear mobilization of phospho-Stat6 mediated by these mutant receptors. To identify one such signaling pathway, we focused on the observation that activation of PKCs by PMA and consequent signals converted the nonfunctional Δ437(Y0F) mutant on resting Jurkat cells into a receptor able to activate Stat6 (Fig. 4, B and C). Signaling pathways downstream from PKC activation in T cells include the ERK and...
MAP kinases. Inhibition of the ERK pathway using U0126 (Fig. 5A) and PD98059 (data not shown) blocked the ability of PMA to render the tyrosine-free IL-4Rα mutant competent to in-duce Stat6, a nonspecific, Stat-independent decrease in overall transcriptional activity notwithstanding. In contrast, the p38 inhibitor SB202190 had no effect on IL-4R signaling to Stat6 even though p38 MAPK was activated by PMA (Fig. 5C). Control experiments using activation site-specific anti-phospho-peptide Abs confirmed the inhibition of phosphotransfer at the doses used (Fig. 5C). Of note, as in previous studies, ERK activation was not triggered by IL-4 alone (52), although IL-4 did induce the p38 MAPK pathway. This finding indicates that altered receptor function required an active ERK pathway in this model, an activation which the IL-4 receptor itself could not provide. To complement these results, transfection experiments were performed using a constitutively active form of ERK (39). Dominant active ERK bypassed the need for PMA, so that IL-4 action on the Δ437(Y0F) receptor mutant was able to activate Stat6-dependent transcription without upstream activation of signaling by PMA (Fig. 6). Since this transcriptional activation depended on IL-4, an IL-4R, and Stat6, we conclude that activity of the ERK pathway can be both necessary and sufficient to promote Stat6 induction when recruitment of the latent transcription factor is independent from association with phosphotyrosines of IL-4Rα.
FACS staining revealed that forms of IL-4R found to be inhibited by IL-4 in a Stat6-dependent manner (57). Tyrosines could mediate IL-4 repression of IL-18R/H9251/H11002 whereas mutation of the ID-1 motif (E/H9251/S437) crippled the ability of the tyrosine-free IL-4R A normal ID-1 region is required for Stat6 induction and Th2 development in the absence of tyrosines in IL-4Rα cytoplasmic tail

The identification of this novel form of Stat6 induction, independent of a requirement for IL-4Rα phosphotyrosine residues but requiring activation events (T cell or ERK activation) and the proximal cytoplasmic tail, raised the question which portion of the receptor was necessary for the pathway. Deletion of a region termed the ID-1 crippled the ability of IL-4Rα to promote proliferation in nonlymphoid cells (53–55), while loss of an apparent c-Fes docking site in the adjacent ID-2 region had no effect on proliferation (56). Deletion and substitution mutations (ΔID1 and (ID1) E→S−, respectively) were introduced into the Δ437(Y0F) IL-4Rα receptor mutant (Fig. 7A). These constructs were tested for their ability to mediate IL-4 effects on Stat6-dependent transcription and Th2 development. Each means of mutating this region crippled the ability of the Δ437(Y0F) IL-4Rα to promote nuclear induction of phospho-Stat6 (Fig. 7B) as well Stat6-dependent gene transcription (Fig. 7C) in activated Jurkat T cells. Expression of the IL-18Rα, which can promote Th1 differentiation, has been found to be inhibited by IL-4 in a Stat6-dependent manner (57). FACS staining revealed that forms of IL-4Rα lacking cytoplasmic tyrosines could mediate IL-4 repression of IL-18Rα expression, whereas mutation of the ID-1 motif (E→S−) in the Δ437(Y0F) receptor abrogated this effect (Fig. 8A). Furthermore, mutation of the ID-1 region eliminated the ability of the tyrosine-free IL-4Rα to promote Th2 development (Fig. 8B). Together, the findings identify the ID-1 region as a key component of this activation-induced molecular program of IL-4Rα signaling.

Discussion

The present study demonstrates that in activated T lymphocytes, IL-4Rα can specifically mediate Stat6 phosphorylation and Th2 development by a novel pathway which is independent of phosphotyrosines and the previously identified Stat6 recruitment mechanism of this cytokine receptor chain. This competence of an IL-4Rα chain lacking all cytoplasmic tyrosines required two elements. First, T cell activation altered IL-4R function, in that Stat6 and Th2 differentiation were induced and IL-18Rα expression inhibited in the primary T cells expressing only IL-4Rα. Of note, this MAPK signaling pathway also potentiated IL-4 induction of Stat6 and promoter activity mediated through a wild-type IL-4Rα. Second, a region of conserved serine/acidic residues, previously designated “ID-1" and implicated in IL-4-induced proliferation, needed to be intact. Together, the data provide evidence of a new means by which the competence to mediate specific Stat induction and undergo Th2 differentiation is regulated. This new mechanism depends on cross-talk between the IL-4R and other signaling pathways. In addition to

**FIGURE 5.** Activation of an ERK MAPK pathway can be necessary for Stat6 inducibility by a tyrosine-free-IL-4Rα. A–C, Abrogation of receptor potentiation by inhibition of ERK but not p38 MAPK. Jurkat T cells expressing wild-type (WT) or mutant IL-4R were transiently transfected with a Stat6-dependent luciferase reporter. Twenty-four hours later, solvent/vehicle, the MEK1 inhibitor U0126 at a final concentration of 100 μM (A) or an inhibitor of p38/MAPK, SB202190 at 10 μM (B), were added to the cultures as indicated. After 30 min, cells were stimulated either with mIL-4, hIL-4, PMA plus ionomycin, or each cytokine with PMA/ionomycin. After a 24-h further incubation, luciferase activity in cell extracts was quantitated. One experiment, representative of four with similar results, is presented. C, Inhibition of MAPK pathways (U0126 and SB202190 blockade of ERK, p38/MAPK, respectively). PMA or IL-4 induction of MAPK signaling pathways was analyzed by Western blot analysis. Aliquots of each sample of Jurkat cells used in A and B were pelleted, resuspended in 2× SDS sample buffer, and subjected to immunoblot analysis using specific anti-phosphopeptide Abs directed against activating sites in these MAPK (phospho-ERK, phospho-p38), followed in each instance by measurement of loading by stripping and reprobing with anti-ERK and -p38, respectively.

**FIGURE 6.** Activation of the ERK MAPK pathway can be sufficient for IL-4-dependent Stat6 inducibility by a tyrosine free-IL-4Rα. Dominant active ERK enhances the competence of wild-type (WT) and tyrosine-free IL-4Rα. Jurkat T cells expressing wild-type or mutant IL-4Rα were transiently transfected with a dominant, constitutively active ERK construct (39). Transfected cells were treated either with medium alone, mIL-4, PMA, or mIL-4 plus PMA and processed as in the preceding panels. Luciferase activities from a representative experiment are presented.
showing that normal activation of T cells can reprogram the molecular mechanisms by which latent Stat transcription factors are selected for specific induction by a cytokine receptor, the findings suggest that physiologic activation of MAPK may participate in this process.

Previous studies using cell lines have characterized a Stat6 activation motif in the IL-4R which could function as a mobile Stat6 activation cassette (2, 26, 47, 48, 50). Insofar as the elimination of cytoplasmic tyrosines from IL-4R rendered IL-4 induction of Stat6 less efficient even in activated T cells (Fig. 3), it is likely that phosphoryl-Y2, 3, or 4 still plays a significant role in activating latent Stat6 in the activated T cells. However, we showed that signals generated by a tyrosine-free IL-4R can still trigger Stat6 activation in activated primary T cells and in the T cell line Jurkat. It has been reported that Stat6 phosphorylation may be mediated by IFN-α in a B cell-specific manner (58). However, no cytokine other than IL-4 has been shown to induce Stat6 in T cells. Furthermore, any mechanism whereby Stat6 is induced by a cytokine other than IL-4 in our experiments is difficult to reconcile with the lack of nuclear Stat6 in the activated T cells untreated with IL-4, which were grown under conditions identical to the Th2 differentiation cultures. Finally, a ligand-binding domain and cytoplasmic tail containing box 1 (Janus kinase 1 binding site) and the ID-1 domain were required for Stat6 induction. IL-13 is an alternative ligand that can trigger IL-4R, but T cells do not express IL-13Rα (59); therefore, this cytokine is unlikely to have substituted for IL-4. Notwithstanding these points, it is conceivable that the mechanisms whereby T cell activation leads to the new molecular program of Stat6 induction via tyrosine-free IL-4R chains in some way involve multimeric complexes which include receptor chains from other cytokine receptors.

The Th2 differentiation data presented here show that mutants of the IL-4R chain that cannot mediate Stat6 induction in unstimulated T cells (Fig. 4) or other cell types (2, 26, 47, 48) become competent to do so after T cell activation or events that occur subsequent to such activation (Figs. 2 and 3). Whether by direct or indirect pathways, therefore, T cell activation reprograms IL-4R signal transduction to Stat6. Some precedent for such cross-talk has been observed for Stat5 induction by IL-4. IL-4 treatment of resting T cells does not induce Stat5 whereas this member of the Stat transcription factor family can be induced by IL-4 after T cell activation, a process we have found to be dependent on the inducible c-jun proto-oncogene and the latent NF-κB/Rel proteins (60–62). Signals generated by the TCR and the IL-4R synergize to promote Th2 development. Cross-talk between the process of T cell activation and IL-4R signaling also is suggested by studies finding diminished Th2 differentiation when cells were unable to achieve full activation states. A transgene encoding a catalytically inactive Lck protein, or the absence of CD28 costimulation, decreased the efficiency of IL-4R-mediated Th2 differentiation (63–65). Thus, some but not all aspects of cross-talk may be directly downstream from TCR signal transduction.

In the tissue culture model, where Stat6 induction mediated by the IL-4R chain gains independence from cytosolic phosphotyrosines of this receptor chain, the ERK MAPK signaling pathway can be necessary (Fig. 4, B and C) and sufficient (Fig. 5). This kinase relay has not previously been implicated in positive regulation of IL-4R signaling.
signal transduction. Activating phosphorylation of ERK is detectable 1 h after TCR stimulation and peaks around 4 h, but it is still readily detectable 20 h after TCR ligation (64). However, T cell activation is followed by complex signaling events that may be triggered by cytokine release (e.g., IL-2) or changes in cell surface receptors and ligands in the population. Thus, it is unclear in this and other studies whether events downstream from T cell activation are direct or indirect. In an apparent contrast to data on potentiation of IL-4R signaling, TCR engagement was followed by transient inhibition of IL-4 signaling (66). Using PMA treatment of T cells and the U0126 inhibitor provided evidence that this evanescent inhibition required ERK activation but lasted only ~12 h and required the coordinate contribution of a Ca²⁺/calcinemur phosphatase pathway (66). In our studies, however, the influence of T cell activation on IL-4 signaling was analyzed over 20 h after TCR engagement (in retroviral transductions of primary T lymphocytes) or activation of Jurkat T cells. Thus, whatever inhibitory effects may have influenced IL-4Rα would have subsided. Indeed, control experiments with the Jurkat model show that PMA leads to a transient inhibition of Stat6 induction by the wild-type IL-4R and this effect is followed by potentiation of Stat6 induction after 12~20 h (L.M.S. and M.B., unpublished observations). Whereas the present evidence is that an ERK MAPK pathway was necessary and sufficient for receptor potentiation, it is not clear whether the identical pathway is used for transient inhibitory effects inas much ionomycin alone could provide potent IL-4R inhibition while PMA alone could not (66). Of note, our studies demonstrated that ERK-mediated enhancement of Stat6 transcriptional activity was mediated through a transduced wild-type mIL-4R and the endogenous hIL-4R, rather than acting only on IL-4Rα lacking tyrosine motifs in its cytoplasmic tail. Together, these findings suggest a complex and time-dependent network of MAPK cross-regulation of the IL-4R, which does not induce ERK in T cells (Fig. 5 and Ref. 52).

Although there may be other mechanisms which could prove involved in other settings, our results provide evidence of a functional capacity of the ERK MAPK pathway to reprogram receptor function and show that this effect required an intact ID-1 region. In a tyrosine-free IL-4Rα, mutation of several conserved serine and glutamic acid residues of the ID-1 sequence completely eliminated the competence of the IL-4R to induce transactivation of a Stat6-dependent reporter or to mediate Th2 differentiation. There is little precedent for this ability of cytokine receptor mutants lacking tyrosine motifs to activate latent Stat transcription factors in primary cells. An analysis of the erythropoietin receptor (EpoR) demonstrated that its distal region and tyrosines were not essential for Stat5 activation or erythropoiesis in vivo (67). However, the molecular mechanism(s) by which a tyrosine-free mutant EpoR could induce Stat5 have not been elucidated. The present findings raise the possibility that the competence of this mutant EpoR was mediated by the activation of ERK through other growth factor/cytokine receptors on primary erythroid progenitors, thereby potentiating the tyrosine-free EpoR in a manner analogous to the mutated IL-4R on T cells.

Whereas the regulatory role of phosphotyrosines in cytokine receptors is extensively documented, emerging data suggest that receptor serine phosphorylation may also be important (68). Consistent with this, we show that isosteric substitutions in the ID-1 region of the IL-4R abrogated induction of Th2 development and the ERK-primed Stat6 activation in T cells expressing tyrosine-free mutants of IL-4Rα. This substitution analysis of the ID-1 region showed that serine and glutamate residues were important for the receptor function, suggesting that serines within this region might be direct targets of ERK or that signal-transducing molecule cutes associate with the ID-1 region as a consequence of TCR signals. Although the present study has focused on IL-4Rα induction of Stat6 in mediating a critical differentiative event in T cells, IL-4Rα is present in a large variety of cell types with pleiotropic functions (69). IL-4 and IL-13 act through IL-4Rα to mediate other important responses in vivo, such as the induction of chemokines or mucus hypersecretion by airway epithelial cells in the asthmatic response (70, 71). Importantly, ERK may be activated in airway epithelial cells of the inflamed lung by IL-4 or the concurrent action of other cytokines (72). As such, our findings raise the possibility that ERK can modulate the competence or activity of IL-4 and -13 receptors in such nonlymphoid cells.

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References


44. Wang, H., W. E. Paul, and A. D. Keegan. 1996. IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor alpha chain. Immunity 3:143.


47. Aronica, M. A., S. Goenka, and M. Boothby. 2000. IL-4-dependent induction of Bcl-2 and Bcl-xL in activated Th2 cells through a STAT6- and PI3-kinase independent pathway. Cytokine 12:578.


50. Wang, H., W. E. Paul, and A. D. Keegan. 1996. IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor alpha chain. Immunity 3:143.