Activation of STAT5 by IL-4 relies on Janus kinase function but not on receptor tyrosine phosphorylation, and can contribute to both cell proliferation and gene regulation

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Abstract

We have investigated mechanisms and consequences of STAT5 activation through the human IL-4 receptor (IL-4R). By functionally expressing receptor mutants in the murine pro-B cell line Ba/F3, we could show that phosphorylated tyrosine residues within the IL-4R α chain are dispensable for IL-4-induced STAT5 activity. However, disruption of a membrane-proximal proline-rich sequence motif ('box1') in either subunit of the bipartite IL-4R abolished not only ligand-induced tyrosine phosphorylation of Janus kinases JAK1 and JAK3, but also IL-4-triggered activation of STAT5 and concomitant cell proliferation. A dominant-negative version of STAT5b, but not of STAT5a, interfered with IL-4-induced DNA synthesis in Ba/F3 cells, suggesting an involvement of STAT5b in the control of cell proliferation through IL-4R. Reporter gene experiments finally showed that transcription from promoters of STAT5 target genes can be specifically induced by challenging cells with IL-4, and that both STAT5a and STAT5b can contribute to IL-4-triggered transcriptional control.

Introduction

IL-4 is a pleiotropic modulator of the mammalian immune system, but also acts on various other tissues (1). The mediator of its function is the bipartite IL-4 receptor (IL-4R) which is present in the cytoplasmic membrane of IL-4 target cells. Lymphoid and myeloid blood cells express an IL-4R complex consisting of the IL-4R α chain (IL-4Rα) (2) and the common γ receptor chain (γc) (3), whereas in non-immune cells, IL-4Rα apparently forms a functional IL-4R employing an IL-13R subunit instead of γc (4). It is as yet unknown if IL-4Rα homodimers, which under experimental conditions also can trigger intracellular signal transduction (5,6), have a possible physiological role. All known receptor chains involved in IL-4-specific signaling belong to the cytokine receptor superfamily (7).

Ligand-induced dimerization of IL-4R subunits triggers various intracellular reactions which ultimately lead to complex cellular responses such as proliferation and differentiation. The best characterized cytoplasmic signaling route utilized by IL-4R is the JAK/STAT pathway (8). Upon stimulation of hematopoietic cells with IL-4, Janus kinases JAK1 and JAK3, which are associated with IL-4Rα and γc respectively (9,10), become tyrosine phosphorylated and, thus, activated. Phosphorylation substrates of the JAKs are both receptor chains, signaling mediators such as insulin receptor substrate 2 (IRS-2) and phosphoinositol-3 kinase, and, importantly, signal transducers and activators of transcription (STATs) (11). STAT proteins immediately transfer information from the stimulated receptor complex to the DNA: once tyrosine phosphorylated, they dimerize and translocate from the cytoplasm into the cell nucleus where they influence specific transcription at promoters containing STAT recognition motifs (12).
STAT6, which is specifically activated by both the IL-4R and the IL-13R complex (13-16) is crucial for IL-4- (and IL-13-) governed immunoregulation (17-19), and was for several years considered the only STAT protein involved in signaling through IL-4R. However, evidence has been provided for IL-4-induced activation of STAT5 in primary human B cells (20). We have recently demonstrated that IL-4R is connected to the STAT5 pathway in murine and human lymphoid tissue as well as in cell lines stably expressing the human IL-4R complex. IL-4 stimulation renders STAT5 fully functional with regard to nuclear translocation, DNA binding and specific gene regulation (21).

Two versions of STAT5 have been cloned: STAT5a was initially named ‘mammary gland factor’ and characterized as a regulator of milk protein genes in prolactin-stimulated mammary epithelial cells (22). STAT5b is present not only in mammary gland, but also in hematopoietic cells and liver tissue (23-25). Its C-terminal transactivation domain differs from that of STAT5a in a characteristic fashion (26), suggesting a distinct and compared with STAT5a possibly more general function in gene regulation. Recent work has revealed that STAT5 is activated by a wide spectrum of receptors for cytokines and growth factors in a variety cell types. These findings raise the question if and in what way STAT5 may contribute to basic processes influencing cell differentiation and cell growth. With regard to possible mechanisms underlying the control of STAT5 function it is interesting that all cytokine receptor systems sharing conserved signaling motifs underlying the control of STAT5 function it is interesting that all cytokine receptor systems sharing conserved signaling motifs.

Both molecular mechanisms and cellular effects of IL-4-induced STAT5 activation have not yet been characterized. Apart from the strict requirement for γc to be present within the receptor complex (21), structural determinants for productive interaction between STAT5 and IL-4R are not known. Possible roles of STAT5 in IL-4-triggered cell proliferation and differentiation remain to be elucidated. In this study we have addressed these questions using a series of mutated human IL-4R subunits stably expressed in the murine pro-B cell line Ba/F3. We analyzed the interdependence between STAT5 activation and phosphoryrosine-SH2 contacts to the receptor protein and investigated the possible involvement of STAT5 in IL-4-influenced proliferation and differentiation in this cellular model system.

**Method**

**DNA constructs**

Recombinant DNA work was performed following standard procedures (35). Expression plasmids pKCR-4α and pKCR- γ, encoding human IL-4Rα and N-terminally epitope-tagged human γc, respectively, have been described (5,36). Expression constructs encoding hIL-4Rα truncation mutants hIL-4RαL612stop, S405stop and K234ane as well as ‘box1’ mutant hIL-4RαL241A/P242A/P244A were described elsewhere (37). For reasons of convenience, the triple alanine mutant is designated hIL-4Rα (box1 mut) throughout this study.

The generation of hybrid receptor genes 4α/γ and pγα and the respective pKCR-derived expression plasmids was detailed before (5). A mutant hybrid gene encoding 4α/γP266A/I268A/P269A (named 4α/γ (box1 mut) in this study) was constructed as follows. From plasmid pKCR-4α/γ (5) an XhoI–HindIII fragment representing the intracellular domain of wild-type human γc was removed and replaced by a mutant fragment containing the three altered codons P266A, I268A and P269A. The latter DNA fragment was generated by recombinant PCR using mutagenic primers SB1 (5’CCGACATGGCCCGAGCTGCCACCTGAAG-3’) and SB2 (5’CTTCAGGGTGCGCGCTCGGGCATTGCCAGTCGCG-3’).

Reporter gene constructs (STAT5-RE)6–luciferase and oncostatin M (OSM)–luciferase have been described (26,38). The mIL-2Rα–luciferase construct pGL3-IL-2Rα contains a described 2.5 kb mIL-2Rα promoter fragment (39) cloned into pGL3 basic (Promega, Madison, WI) and was obtained from P. Reichenbach (ISREC, Epalinges). pXM-derived expression plasmids encoding wild-type versions and dominant-negative mutants of STAT5a, STAT5b and STAT6 have been described (26,40).

**Cell culture and generation of stably transfected cell lines**

Ba/F3 cells and Ba/F3-derived cell lines were cultured as described previously (36). Stable transfection of Ba/F3 cells with hIL-4R constructs has been described in detail (5,36). Surface expression of all receptor constructs was verified and quantified by reacting cells with mAb directed to the respective extracellular receptor domains. Cell-bound antibodies were detected by chemiluminescence elicited by peroxidase-coupled secondary antibodies (41).

**Immunoprecipitation and Immunoblotting**

For immunoprecipitation, Ba/F3-derived cell lines were starved for 2 h in medium without cytokine, then samples of 3×10^6 cells were kept at 37°C for 10 min in 1 ml of RPMI containing no cytokine or 7 nM of hIL-4 respectively. Cleared lysates were prepared as described (36) and incubated with 0.5-3 μg of specific antibody for 3 h at 4°C. The following antibodies were used: mouse monoclonals X14/38 to hIL-4R (42), P5D4 to epitope-tagged human γc (36) and E34-1 to murine JAK3 (43); rabbit antiserum to JAK1 (44), and STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were precipitated from lysates with 15 μl of Protein A-Sepharose (Sigma, St. Louis, MO) and analyzed by Western blot as described (5) using peroxidase-conjugated antibody RC20 to phosphoryrosine (Transduction Laboratories, Lexington, KY) or the above-mentioned specific antibodies for detection. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK). Blots were regenerated for reprobing by incubating the membranes with 0.1 M glycine (pH 2.9) at room temperature for 20 min (for RC20) or with 62.5 M Tris–HCl (pH 8.5), 100 mM β-mercaptoethanol, 2% SDS at 50°C for 30 min (for other antibodies) followed by extended washing. Immunodetection of STAT5 in whole-cell lysates from transiently transfected cells was performed as described (26,37). The equivalent of 3×10^5 BAF-4α-γ cells was loaded per gel lane; a rabbit antisemur to amino acids 350–480 of human STAT5a was used for probing of the blots (26). Isotype-specific immunoprecipitation and detection of
STAT5a and STAT5b was carried out as described previously (21).

**Cell proliferation assay**

Cytokine-induced proliferation of stably transfected cell lines was measured by [3H]thymidine incorporation into de novo synthesized DNA as described (36). Proliferation assays with BAF-4α-py cells in combination with transient expression of STAT proteins were performed as follows. Cells (3.5×10⁶) were transfected with 50 µg of expression plasmid by electroporation using a Eurogentec Easyjet instrument. Two consecutive electric pulses of 600 V/25 µF and 150 V/2.100 µF were applied. Cells were then kept in medium with mIL-3 for 24 h to allow for recovery. After this period, cells were washed, counted and subjected to standard proliferation tests. As a modification to the described procedure (36), only 5×10⁵ live cells instead of 4×10⁶ in the standard protocol were used per measurement. The fraction of efficiently transfected live cells was controlled to be >70% by parallel transfection with an expression plasmid encoding green fluorescent protein and microscopic inspection of cells under UV light after 24 h. Equivalent expression of employed STAT constructs was tested by Western blot analysis of Ba/F cells transfected with identical amounts of each plasmid under milder electroporation conditions (single pulse 250 V/ 960 µF).

**Reporter gene assay**

BAF-4α-py cells were transiently transfected with 10 µg of luciferase reporter gene plasmid (in some experiments together with 10 µg of STAT5 or STAT5 mutant expression plasmid) by electroporating 6×10⁶ cells at 250 V and 960 µF using a BioRad gene pulser. After transfection, cells were cultured in medium containing 10% FCS for 2 h. Cells were then washed and incubated for 20 h (or 36 h in experiments employing co-transfection with STAT5) with medium/10% FCS without cytokine or with mIL-3 or hIL-4 respectively as described (36). Luciferase activity was then measured as described (22) and normalized by determining the protein concentration of the extract.

**Results**

**Both IL-4-induced cell proliferation and activation of JAK kinases is not dependent on tyrosine phosphorylation of IL-4Rα**

Ligand-induced tyrosine phosphorylation of cellular proteins including IL-4R chains coincides with the activity of JAK kinases and the stimulation of cell proliferation (5,37). We first correlated tyrosine-bearing structural modules of hIL-4Rα with the capability to support hIL-4-dependent cell proliferation and activation of JAK1 and JAK3. To this end, four derivatives of the murine pro-B cell line Ba/F3 that stably express different mutant hIL-4R complexes were subjected to stimulation with hIL-4 and subsequently analyzed with regard to cell proliferation and tyrosine phosphorylation of JAK1 and JAK3. The cell lines employed co-express full-length human γ, along with individual hIL-4Rα variants with successive C-terminal truncations. BAF-4α-py cells express wild-type hIL-4Rα, in BAF-4αL612stop-py and BAF-4αS405stop-py cells, the receptor molecules lack regions containing the two C-terminal or all cytoplasmic tyrosine residues respectively, whereas cell line BAF-4αK234anc-py expresses a hIL-4Rα variant devoid of the entire intracellular domain (Fig. 1A). Surface expression levels of the two longer hIL-4Rα mutants investigated in this experiment had previously been shown to be in a similar range as the full-length receptor chain, only the ‘tailless’ mutant hIL-4RαK234anc appears on cells in a considerably higher copy number (37). As Fig. 1(B) shows, all cell lines except for BAF-4αK234anc-py respond to hIL-4 by cell proliferation. These data are consistent with results obtained by others (45–47) and clearly demonstrate that intracellular tyrosines on the side of hIL-4Rα are not essential for the transmission of a proliferative signal. However, the response amplitude evoked by the hIL-4Rα mutant bearing only 173 cytoplasmic amino acids (BAF-4αS405stop cells) was significantly lower compared to wild-type.

Despite containing no cytoplasmic tyrosine residue within the mutant hIL-4Rα chain, BAF-4αS405stop cells still showed specific hIL-4-dependent tyrosine phosphorylation of both JAK1 and JAK3 (Fig. 1C), although to a somewhat lesser extent than the cell lines expressing the L612stop- or wild-type α receptor subunit analyzed in parallel. These results indicate that hIL-4R-mediated cell proliferation and JAK activation both occur independently of receptor phosphorylation and coincide semiquantitatively with respect to signal intensity.

**STAT5 activation through IL-4R occurs independently from tyrosine phosphorylation of the receptor α chain**

Various signaling processes in cytokine receptor complexes are controlled by protein–protein interactions specified through contacts between phosphotyrosine-containing sequence motifs and SH2 domains of respective binding partners (8). Since hIL-4Rα mutant S405stop is able to transmit intracellular signals although it is devoid of any cytoplasmic tyrosine residue, it was important to determine which signaling factors it still can activate. Recently we had identified STAT5 as a signal mediator triggered by IL-4R (21). Therefore we employed the different truncation mutants of hIL-4Rα shown in Fig. 1(A) to address the structural determinants for hIL-4-induced STAT5 activation.

Using the respective cell lines, we tested which receptor variants were able to trigger tyrosine phosphorylation of STAT5 upon hIL-4 stimulation. Figure 2 shows that in cell lines BAF-4α-py, BAF-4αL612stop-py and BAF-4αS405stop-py, hIL-4 treatment resulted in activation of STAT5α as judged by tyrosine phosphorylation.

These results demonstrate that neither Y688, which is embedded within a putative STAT5 docking site (see Discussion), nor any other cytoplasmic tyrosine residue of hIL-4Rα is required to mediate hIL-4 induced activation of STAT5. hIL-4R-dependent STAT5 phosphorylation thus parallels hIL-4-induced activation of JAK1 and JAK3 (compare Fig. 1C).

Disruption of the ‘box1’ motif in either subunit of IL-4R simultaneously abolishes activation of JAK1, JAK3 and STAT5 as well as support of cell proliferation

We next set out to study the relationship between JAK1/JAK3 phosphorylation, on the one hand, and IL-4-induced STAT5 activation...
IL-4-induced STAT5 activation

Fig. 1. hIL-4-induced cell proliferation and tyrosine phosphorylation of JAK1 and JAK3 in Ba/F3 derived cell lines expressing truncated versions of hIL-4Rα. (A) Schematic representation of mutant hIL-4R complexes expressed by the cell lines used in this experiment. hIL-4Rα is shown in dark grey, human γc in white. Black segments represent the transmembrane domains of both receptor chains, the PS5D4 epitope-tag N-terminally fused to γc appears as a light grey box. Tyrosine residues present in the intracellular domain of hIL-4Rα chains are represented by asterisks. (B) hIL-4-induced proliferation of cell lines depicted under (A). Cells were left untreated or incubated with 7 nM hIL-4 for 24 h. Cytokine-induced cell proliferation was determined by measuring [3H]thymidine incorporation. The proliferative responses are expressed relative to the extent of proliferation induced by a saturating concentration of mIL-3, which was set as 100%. Results are representative for at least five independent experiments. (C) hIL-4-induced tyrosine phosphorylation of JAK kinases in the cell lines depicted in (A). Starved cells were either left untreated or stimulated with 7 nM hIL-4 for 10 min as indicated. JAK1 (top) or JAK3 (bottom) were immunoprecipitated from cell lysates and analyzed for tyrosine phosphorylation by Western blot. Equal loading of gels was verified by reprobing the blots with specific antibodies to JAK1 and JAK3 respectively (not shown).

activation and cell proliferation, on the other hand, more thoroughly. Both hIL-4R subunits contain a membrane-proximal proline-rich cytoplasmic consensus motif (‘box1’) which is known to be involved in productive contact between cytokine receptors and JAK (48,49). We employed two Ba/F3-derived cell lines that express hIL-4R complexes with mutated ‘box1’ motifs in the α or γ subunit respectively (Fig. 3A): cell line BAF-4α (box1 mut)-py expresses a bipartite hIL-4R with three amino acid positions in the box1 motif of hIL-4Rα changed to alanines (37). The newly generated line BAF-4α/γ (box1 mut)-py/γ/4α (see Methods) carries an equivalent triple amino acid alteration in the box1 of human γc, but in the context of a modified receptor configuration with mutually exchanged intracellular domains. This setup allows for an analysis of human γc function without interference by the endogenous murine γc (5). Both cell lines as well as two cell lines expressing the respective wild-type receptor controls were left untreated or stimulated with hIL-4 and subsequently
analyzed for ligand-dependent cell proliferation (Fig. 3B) as well as for tyrosine phosphorylation of JAK1, JAK3 and STAT5 (Fig. 3C). Clearly, disruption of the box1 motif in either hIL-4Rα or hIL-4Rγ subunit had simultaneous drastic effects on all cellular responses to hIL-4: the capability of the two receptor variants to phosphorylate JAK1 and JAK3 was completely abolished, both box1 mutants were also found to be incapable to support any significant degree of cell proliferation. With regard to IL-4-induced STAT5 activation, the box1 mutation in the α chain resulted in a complete loss of this response, whereas the receptor complex with the mutated box1 in the intracellular part of γc triggered only a very small extent of STAT5 phosphorylation.

**STAT5b but not STAT5a interferes with IL-4-induced cell proliferation**

STAT5 can still be activated through the ‘tyrosine-less’ S405stop mutant of hIL-4Rα, which is also able to promote a basic level of hIL-4-induced cell proliferation (cf. Figs 1B and 2). Some recent studies on cytokine receptor signal transduction have attributed to STAT5 a function correlated with the support of mitogenesis (50–52). To assess a possible involvement of STAT5 in hIL-4-induced cell proliferation, dominant-negative versions of STAT5a and STAT5b respectively were expressed along with the bipartite human IL-4R complex in Ba/F3 cells. Expression plasmids encoding C-terminally truncated versions of STAT5a, STAT5b and, as a control, STAT6 were introduced in BAF-4α-py cells (stably expressing the bipartite human IL-4R) by transient transfection before both IL-4- and IL-3-induced cell proliferation was determined. STAT mutants STAT5aΔ749, STAT5bΔ754 and STAT6Δ777 lack C-terminal sequences essential for transcriptional transactivation, and were shown to block the specific functions of their endogenous wild-type counterparts upon overexpression (40). As further controls, genes encoding full-length versions of the three STAT proteins were also transfected and resulting cells were analyzed with regard to cytokine-induced proliferation. Transfection efficiency was monitored by parallel transfection of cells with an expression plasmid encoding green fluorescent protein (data not shown).

As Fig. 4 shows, for STAT5a, no major difference was evident between cells transfected with genes for wild-type proteins or transactivation-negative STAT proteins, neither with respect to IL-3- nor hIL-4-induced cell proliferation. Overexpression of wild-type STAT6 had no particular effect on cytokine-dependent proliferation, whereas truncated STAT6 somewhat enhanced thymidine incorporation, primarily in response to IL-4. Importantly, proliferative responses to both cytokines appeared clearly impaired when cells transfected with STAT5bΔ754 were compared with those expressing wild-type STAT5b. Although the repressive effect of STAT5bΔ754 towards hIL-4 was weaker than that seen with IL-3 as a growth factor, it clearly suggests an involvement of STAT5b in hIL-4-mediated cell proliferation.

**IL-4R can mediate gene transcription driven by STAT5-regulated promoters**

Recently, cytokine-induced transcription of several genes was shown to be mediated through the activation of STAT5 (38,52,53). These STAT5 target genes contain STAT5 binding sites in their promoter regions that specify STAT5-determined transcriptional regulation and include the OSM gene (osm) (38) and the IL-2Rα gene (54). An engineered promoter construct containing six consecutive STAT5 response elements [(STAT5-RE)6] also responds to activated STAT5a and STAT5b (26). To show that a signaling pathway via STAT5 can link IL-4R to transcriptional activation of STAT5-specific target genes, we introduced luciferase reporter genes fused to the regulatory region of the OSM promoter, the IL-2Rα promoter and (STAT5-RE)6 in BAF-4α-py cells, and subsequently stimulated them with hIL-4. Since IL-3 receptor of Ba/F3 cells triggers STAT5 activation, transcription through STAT5 induced by mIL-3 stimulation can serve as a positive control (52). Figure 5(A) shows that both mIL-3 and hIL-4 can evoke specific transcription from all three STAT5 target promoters. The clear response towards hIL-4, although weaker than that obtained with mIL-3 as a stimulus, indicates the potential of the hIL-4R to mediate specific gene regulation by means of STAT5 as a signal transmitter. Since there has been a report on the activation of STAT3 by IL-4 in human B cells (20), we ruled out the possibility that STAT3 might contribute to the transcriptional regulation of the transfected promoters. We could not detect any IL-4-dependent STAT3 phosphorylation in Ba/F cells (data not shown).

Since only STAT5b but not STAT5a appeared to be involved in the control of IL-4-induced cell proliferation (Fig. 4), we analyzed if the two isoforms had also distinct properties with regard to IL-4-dependent gene regulation. The OSM promoter, which has been characterized as a very specific STAT5 target promoter (38), was used to assess the individual contributions of STAT5a and STAT5b to cytokine-induced transcriptional activity. Expression plasmids encoding wild-type or truncated, dominant-negative versions of STAT5a or STAT5b were transfected together with the OSM reporter construct into BAF-4α-py cells. After 36 h, mIL-3- and mIL-4-inducible luciferase activity was determined (Fig. 5B). Full-length versions of both STAT5 isoforms increased the absolute luciferase level induced by both cytokines (Fig. 5B, left). Relating cytokine-dependent luciferase activity to the values determined for non-stimulated cells (Fig. 5B, right) revealed that expression
of both wild-type STATs enhanced IL-4-induced transcriptional activation of the OSM promoter. Dominant negative versions of both STAT5a and STAT5b reduced relative IL-4-induced transcription to a moderate, though significant degree. From these results we conclude that both STAT5 isoforms can contribute to IL-4-dependent gene regulation.

To ensure that STAT5 proteins were sufficiently well expressed upon transient transfection, BAF-4α-pγ cells co-transfected with OSM reporter and STAT5 expression plasmids (see above) were subjected to Western blot analysis employing an antibody to STAT5 after 36 h. Transfection with full-length STATs enhanced the expression levels of the respective endogenous proteins (Fig. 5C, lanes 2 and 4). Expression of truncated STAT5a (Fig. 5C, lane 3) and STAT5b (Fig. 5C, lane 5) resulted in the appearance of antibody-reactive bands with the expected migration behavior. The
lower abundance of truncated STATs in comparison to their full-length counterparts can be attributed to impaired mIL-3-dependent growth of cells expressing these mutants within the total cell pool (cf. Fig. 4). Reporter gene expression in the above described experiment is restricted to transfected cells. The major fraction of successfully transfected cells has most probably taken up both reporter gene construct and STAT expression plasmid simultaneously. The results are therefore consistent with the notion that expression of the STAT5 proteins is causal for the absolute and relative alterations in hIL-4-induced reporter gene transcription.

Discussion

Signal transduction through STAT proteins is a characteristic feature of cytokine receptors and most probably determines cellular responses to their respective ligands to a major part. The basic principles of STAT function have been unraveled in recent last years. However, mechanisms of STAT activation within cytokine receptor complexes appear to be more variable and complex than originally believed, and the role of different STATs in the control of general cellular processes such as proliferation and differentiation is still only partly understood.

Interestingly, many cytokine receptors are connected to signaling pathways mediated by more than one STAT species. Initially, STAT6 was assumed to be the sole representative of the STAT family to be triggered by IL-4R. This view must be modified, since we and others have observed that IL-4 evokes STAT5 activity in various cell types (20,21). While we previously showed a strict requirement of the IL-4-induced STAT5 response for the presence of the γc subunit in the IL-4R complex (21), determinants for STAT5 activation within IL-4Rα have not yet been addressed. The interface of cytokine receptor chains for the interaction with STAT factors has been assigned to pTyr-based sequence motifs serving as docking sites for SH2 domains (55). In the cytoplasmic domains of the erythropoietin (EPO) receptor, IL-2Rβγ, and the prolactin receptor, specific phosphorylated tyrosine residues have been shown to be crucial for the docking of STAT5 to the respective receptor chains and for its subsequent activation (29,56,57). From these studies, a preference of STAT5 for the sequence motif YXXL emerged. hIL-4Rα contains six intracellular tyrosines, one of which (Y688) is located within the sequence context YSAL. However, this study revealed an apparent independence of IL-4-induced STAT5 activation from phosphotyrosines in the primary structure of the receptor α chain which could provide docking sites for an SH2 domain.

Other examples have emerged for STAT activation without the apparent necessity for phosphotyrosines on the side of the activated receptor, although the underlying mechanisms have not been elucidated (24,58). Notably, we have recently found activation of STAT6 through IL-4R to also not rely on intracellular tyrosines of hIL-4Rα (37). Particularly interesting in the context of the results on STAT5 presented in this report was the finding that tyrosine phosphorylation of the receptors for granulocyte macrophage colony stimulating factor and EPO is dispensable for at least a basal degree of ligand-induced STAT5 phosphorylation and activation (24,56).

An alternative mechanism for the triggering of STAT5 in cytokine receptor complexes directly involving JAK kinases was recently proposed (59). These authors showed that mutants of signal transducing cytokine receptor chain gp130 devoid of all cytoplasmic tyrosines can still activate STAT5, and characterized immediate protein–protein interactions between overexpressed JAK kinases and STAT5. Our results on IL-4R are in line with a mechanism characterized by a direct phosphorylation of STAT5 through receptor-associated JAK1 and/or JAK3. Whereas tyrosine phosphorylation of IL-4R was found dispensable for STAT5 activation, the integrity of contact sites for kinases JAK1 and JAK3 in hIL-4Rα and γc respectively proved an absolute requirement for this reaction. In support of our interpretation, we have observed a strong direct contact between JAK1 and STAT5 in Ba/F3 cells (65). It will be a challenge for future investigations to characterize the underlying reactions and, in addition, to understand how signal specificity with regard to STAT involvement in the absence of specific tyrosine-containing sequence motifs in the receptor α chain can arise.

A further intriguing question concerns the cellular and physiological role of STAT5 in the context of IL-4-induced signaling. Clearly, STAT6 determines the specificity of IL-4-mediated reactions to a large extent: STAT6 knockout mice are impaired in the development of Th2 cells, in the regulation of CD23, IL-4Rα and MHC class II, and in Ig class switching to IgE (17–19). The functions of STAT5 apart from its prominent role in the control of lactation are not yet well defined. The two forms of this factor are involved in signaling through a considerable number of cytokine receptors and therefore are likely to contribute to cellular functions of general relevance. A number of genes have been identified whose expression is specifically controlled by interaction of STAT5 with their...
Fig. 5. Cytokine-induced transcription from STAT5-responsive promoters in BAF-4α-pγ cells. (A) BAF-4α-pγ cells were transiently transfected as described in Methods with (i) a (STAT5-RE)₆–luciferase reporter construct containing six consecutive STAT5 response elements, (ii) with an OSM promoter–luciferase construct or (iii) with a mIL-2Rα promoter–luciferase construct as indicated. After incubation with medium/FCS containing no cytokine, mIL-3 or hIL-4 as indicated, luciferase activity was determined and normalized for total protein content of cell extracts. Data represents the mean of three experiments. (B) The OSM reporter construct was transfected together with expression plasmids encoding STAT5a or STAT5b or the respective dominant-negative mutants STAT5aD749 or STAT5bD754 into BAF-4α-pγ cells as indicated. Mock transfection with the expression plasmid alone served as control for the effects of heterologous STAT proteins. After 36 h, cells were incubated for 1 h in medium without cytokine (grey bars), with mIL-3 (white bars) or with hIL-4 (black bars) before luciferase activity was determined and normalized for total protein. Results are expressed both as absolute units of luciferase activity (left) and as relative stimulation indices (factor of cytokine-induced luciferase induction in comparison to non-stimulated cells, right). Data represents the mean of three experiments. (C) Analysis of expression of STAT5a/b and STAT5a/b truncation mutants in transiently transfected BAF-4α-pγ cells. Cells were transfected with the OSM reporter construct in conjunction with the indicated STAT5 expression constructs as described under (A). Empty expression vector served as negative control (mock). Cell lysates obtained 36 h after transfection were subjected to Western blot analysis using a polyclonal serum to STAT5 for detection as described under Methods. Immunoprecipitates obtained with specific antibodies to STAT5a and STAT5b served as positive controls (lanes 6 and 7). For optimal visualization of relevant bands, longer exposures for lanes 3, 4 and 5 are shown. Positions of wild-type and mutant STAT5 proteins are marked by arrows.

respective promoter regions (38,52,53). While we have demonstrated elsewhere that IL-4 regulates the expression level of these STAT5 target genes (21), we showed in this report definitely that IL-4R mediates transcriptional activity of STAT5-driven promoters. Both STAT5a and STAT5b can contribute to IL-4-dependent gene regulation. It will be important to correlate the functions of known and as yet unknown STAT5 target genes with responses of cells and tissues to IL-4.

The role of STAT5 in the mediation of cytokine-induced mitogenesis and cell proliferation as well as in differentiation processes in hematopoietic cells is a matter of debate. While some groups found a correlation between STAT5 activation and cytokine induced cell proliferation (29,50,60–62), others concluded from their results that a contribution by STAT5 is not essential for a mitogenic response (31,63). Evidence has been provided for the involvement of STAT5 in the
differentiation of certain cell types along the erythroid pathway (61,63).

In this report we present evidence for a supportive role of STAT5b, but not of STAT5a in IL-4R-mediated proliferation of Ba/F3 cells. This observation at first sight is in disagreement with results by Mui et al., who ascribe a role in the control of Ba/F cell proliferation to STAT5b only in conjunction with signaling evoked by IL-3, but not by IL-4 (52). However, it is possible that these authors did not detect the effect of their dominant-negative STAT5b construct on IL-4-triggered cell proliferation due to relatively weak inducible expression. We have introduced STAT5 mutants into Ba/F3 cells by transient transfection and were able to establish a higher intracellular concentration of heterologous STAT than by means of inducible expression in stably transfected cell clones employed in parallel (data not shown). This allowed us to readily measure the less pronounced effect of the STAT5b mutant on IL-4-dependent cell proliferation. However, data obtained using inducible expression for STAT5 variants also revealed an influence of STAT5b on cell IL-4-triggered cell proliferation in our hands (S. Wietek and K. Friedrich, unpublished results).

Our results are consistent with the view that STAT5b function, perhaps in an additive or synergistic mode with IRS-2, contributes to IL-4-induced DNA synthesis in pro-B cells. A more extended mutational analysis of receptor complex components and careful investigations on STAT-involving downstream signaling events are needed to address this issue.

Very recently, STAT5a/b knockout mice were described (64). While the most obvious effects of STAT5 deficiency appeared to be associated with the physiological functions of growth hormone and prolactin, the immune system was also affected. In particular, the white cell count was decreased and the proliferation of peripheral T cells was largely reduced. With regard to our results it will be very interesting to characterize T cell responses to IL-4 in these animals.

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Abbreviations

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<td>γc</td>
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References

IL-4-induced STAT5 activation


