Multiple tyrosine residues in the intracellular domain of the common β subunit of the interleukin 5 receptor are involved in activation of STAT5

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Abstract In contrast to the general model of cytokine-induced JAK/STAT signaling, tyrosine phosphorylation of the IL-5R β chain seems to be dispensable for STAT activation in cells overexpressing exogenous STAT proteins. In this study we expressed IL-5 receptor mutants in 293 cells and studied IL-5-induced endogenous STAT-dependent transcription. Our results indicate that: (a) tyrosine phosphorylation of the IL-5R β chain is required for endogenous STAT5 activation, (b) multiple tyrosine residues are phosphorylated upon IL-5 stimulation, including Tyr^{577}, Tyr^{612}, Tyr^{695}, and Tyr^{750}, and (c) Tyr^{612}, Tyr^{695}, and Tyr^{750} are all capable of inducing activation of STAT5, demonstrating a high level of functional redundancy within the IL-5R β chain.

Key words: IL-5; IL-3; GM-CSF receptor; Signaling; Stat protein

1. Introduction

Cytokines such as interleukin-5 (IL-5), IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) play a crucial role in the proliferation, differentiation, and activation of blood cells and their precursors [1,2]. IL-5 is essential for eosinophil proliferation and differentiation [3] and is the key regulator of survival, migration, and activation of mature eosinophils [4–6]. The IL-5 receptor is composed of a specific IL-5R α chain which is only expressed on eosinophils and basophils [7] and a common βc subunit that is shared with the receptors for IL-3 and GM-CSF [8]. Both the α and βc chains are necessary for signal transduction by IL-5 [9–11].

IL-5 induces rapid and reversible tyrosine phosphorylation of cellular proteins in both hematopoietic cells and fibroblasts [6,9]. Tyrosine phosphorylated proteins include the βc subunit itself and proteins that belong to the ras-MAPK pathway and the JAK-STAT pathway [9,11–13]. Recent studies have shown that several tyrosine kinases of the Src-family, including $p59^{fyn}$ [14] and $p53/56^{lyn}$ [15], and others, such as Tec [16] and $p93^{fes}$ [17], are activated via the βc subunit in different cellular systems.

The Janus tyrosine kinase JAK2 is activated in response to IL-3, IL-5, and GM-CSF and specifically associates with the membrane-proximal region of the β chain [12,18]. JAK2 then activates the transcription factors STAT1, STAT3 and STAT5, depending on the cell type studied [11,13,19]. A number of studies have suggested that activated JAKs can phosphorylate tyrosine residues within the cytokine receptor, providing specific binding sites for the SH2 domain of the STAT transcription factors. Support for this model has been obtained from different receptor systems, including the IFN-γ [20], IL-2 [21], IL-4 [22], IL-6 [23], IL-9 [24], erythropoietin (EPO) [25,26], and growth hormone (GH) receptors [27]. By contrast, we and others have shown that deletion mutants of the common β that lack tyrosine residues, can still activate STAT1 and STAT3 (when overexpressed in COS cells) [11] and STAT5 (when overexpressed in BA/F3 cells) [13], suggesting that tyrosine phosphorylation is dispensable for STAT activation by IL-5.

In this paper we report that multiple tyrosine residues in the IL-5Rβ, including Tyr^{577}, Tyr^{612}, Tyr^{695}, and Tyr^{750}, can be phosphorylated upon IL-5 signaling. Tyrosine phosphorylation of either Tyr^{612}, Tyr^{695}, or Tyr^{750} is necessary and sufficient for maximal endogenous STAT activation, showing the existence of a high level of functional redundancy within the IL-5Rβ.

2. Materials and methods

2.1. Cell culture, reagents, and antibodies

293, Rat-1, and 3T3 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 8% heat-inactivated fetal calf serum (FCS). HL-60 cells were maintained in RPMI 1640 supplemented with 8% Hyclone serum (Gibco). Human IL-5 (hIL-5) was a kind gift of Dr. D. Fattah (Glaxo Wellcome Group Research, Stevenage, UK). Polyclonal rabbit antibodies against the IL-5R β chain were produced by immunization of rabbits with the histidine-tagged extracellular part of the IL-5R β chain; anti-phosphotyrosine monoclonal antibody (4G10) was obtained from UBI (Lake Placid, NY); monoclonal antibody against STAT1 (G16920) was obtained from Transduction Laboratories (Lexington, KY); antibodies against STAT3 (K-15), STAT5a (L-20) and STAT5b (C-17) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Synthetic oligonucleotides and plasmid construction

The following oligonucleotides were used in this study (only the upper strands are shown): the human ICAM-1 IRE (5′-AGCTTAGTTTCCGGGAAAGCAC-3′), the *c-fos* SIEm67 (5′-AGCTTCATTTCCCGTAAATCA-3′), the FcγRI (5′-AGCTTGAGATGTATTTCCCAGAAAAGA-3′) and the β-casein (5′-AGCTTAGATTTCTAGGAATTCAA ATCA-3′). These oligonucleotides were multimerized and cloned in the pBlCAT2 reporter. pSV-lacZ, expression vectors for the IL-5R α and β chains and deletion constructs of the βc were described previously [11,28]. Site-directed mutagenesis was performed as described by Kunkel [29].

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Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription; IL, interleukin; IL-5R, receptor for IL-5; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; SH domain, Src homology domain; ICAM-1, intercellular adhesion molecule-1

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2.3. Transient transfection, immunoprecipitation, Western blot analysis and gel retardation assay

Transient transfection experiments and CAT assays were performed as described previously [11,28]. Immunoprecipitation and Western blotting of STAT proteins from transfected 293 cells (20 cm^2 dishes, Nunc) or HL-60 cells were performed as described previously [11]. Nuclear extracts were prepared from unstimulated and IL-5-stimulated Rat-1 cells and 3T3 cells and assayed for STAT binding as described previously [11].

3. Results

To determine the domains in the IL-5R β chain involved in the activation of endogenous STAT proteins, several truncated forms (Fig. 1A) were transfected together with the IL-5 receptor α subunit and a reporter containing FcγRI STAT binding sites in 293 cells. Fig. 1B shows that truncation of the β chain down to amino acid 615 (β615) did not affect the level of IL-5 induced CAT activity. Deleting the next 4 amino acids encompassing the tyrosine phosphorylation site YLSL (β611) greatly reduced IL-5-induced CAT activity. A further reduction was observed using deletion mutant β456, which contains only the first seven amino acids of the intracellular domain. Similar results were obtained in Rat-1 and 3T3 cells (not shown). This indicates that amino acids 611–615 and amino acids 581–456 are important for IL-5-induced endogenous STAT activation.

In parallel, we tested IL-5-induced tyrosine phosphorylation of the βc deletion constructs. In Fig. 1C it is shown that β881 (the full-length receptor), β763, β627, and β615 are heavily phosphorylated after IL-5 stimulation. A clear drop in phosphorylation was observed in constructs lacking Tyr^{612}, like β581 and β611, suggesting that Tyr^{612} is phosphorylated. No tyrosine phosphorylation was observed in shorter constructs lacking Tyr^{577}, like β568 and β456, indicating that Tyr^{577} was also phosphorylated. Interestingly, this tyrosine residue is also phosphorylated in BA/F3 cells in response to GM-CSF [30]. The differences in the loading controls of Fig. 1C are partially caused by the fact that the longer mutants are less well expressed compared to the shorter constructs.

To determine which STAT was activated in 293 cells, we immunoprecipitated STAT1, STAT3, STAT5a and STAT5b from IL-5-treated 293 cells and assayed the phosphorylation on tyrosine residues. While STAT1, 3 and 5a were not activated by IL-5 (not shown), STAT5b was tyrosine phosphorylated in 293 cells after IL-5 stimulation (Fig. 1D). Similar results were obtained in Rat-1 and 3T3 cells (not shown).

To assess whether Tyr^{612} and Tyr^{577} are important for STAT5 induction, we mutated tyrosine 612 to phenylalanine (612F) and tyrosine 577 to glycine (577G) in the β627 deletion mutant (β627YY). The single mutations of Tyr^{577} (β627GY) or Tyr^{612} (β627YF) result in a dramatic decrease of phosphorylation, while mutation of both residues completely inhibited IL-5-induced phosphorylation (Fig. 2A). We next mutated tyrosines 577 and 612 in the context of the full-length receptor (β881GFYYYY). Interestingly, when stimulated with IL-5, this mutant β chain was still phosphorylated (Fig. 2B). This indicated that other tyrosines were phosphorylated. To identify these tyrosines, the same mutations were made in β826

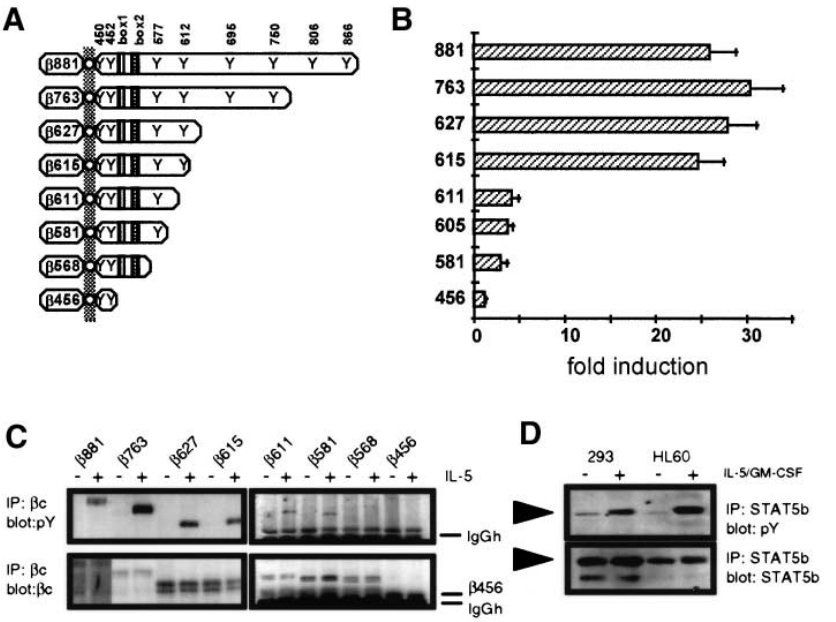

Fig. 1. IL-5-induced activation of STAT5 and tyrosine phosphorylation of IL-5R β-chain deletion mutants in 293 cells. (a) Structure of the full-length IL-5R β-chain and deletion mutants used in this study. Intracellular tyrosine residues (Y) are indicated by numbers, box 1 and box 2 are indicated by shaded rectangles, the extracellular domains are partially indicated. (b) 293 cells were transfected with the IL-5Rα chain (1 μg) and truncated βc receptors (1 μg) together with a 4xGAStkCAT reporter construct (2 μg). 24 h post-transfection, IL-5 (10^{-10} M) was added for another 16 h. Fold induction represents CAT activity in IL-5-treated cells relative to untreated cells, and is the mean of three independent experiments. (c) 293 cells expressing wild-type or mutant β chains were incubated without (−) or with 5×10^{-10} M hIL-5 (+) for 15 min at 37°C. Whole-cell lysates were immunoprecipitated with a polyclonal antibody against the β chain, and immunoprecipitates were analysed using anti-pY (top panel) or anti-βc (bottom panel) antibodies. βc proteins appear as two bands, of which only the upper band is phosphorylated after IL-5 stimulation. The lower bands might represent incorrectly processed forms of βc which are not expressed at the cell surface, and can therefore not respond to IL-5. (d) 293 cells were transfected with the IL-5Rα and β chain and incubated without (−) or with 5×10^{-10} M hIL-5 (+) for 15 min at 37°C. HL-60 cells stimulated with GM-CSF served as a positive control. Whole-cell lysates were immunoprecipitated with a STAT5b antibody. The immunoprecipitates were analyzed with anti-pY (top panel) or anti-STAT5b (bottom panel).

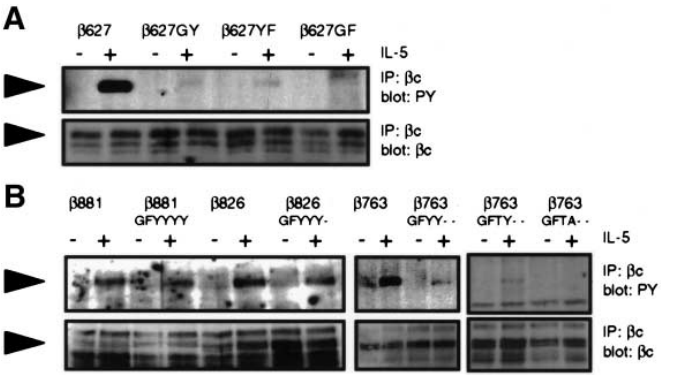

Fig. 2. IL-5 induces phosphorylation of Tyr^{577}, Tyr^{612}, Tyr^{695} and Tyr^{750} of the βc chain. 293 cells expressing wild-type or mutant β chains were incubated without (−) or with 5×10^{-10} M hIL-5 (+) for 15 min at 37°C. β chain phosphorylation was assayed as described for Fig. 1C. (a) Mutation of Tyr^{577} (627GY), Tyr^{612} (627YF) or both (627GF) in the β627 (627YY) context. As described for Fig. 1C, β627 appears as two bands, of which only the upper band (indicated by an arrowhead) is phosphorylated after IL-5 stimulation. (b) Mutation of Tyr^{577} and Tyr^{612} (GF) in the β881, β826 and β763 deletion constructs, and mutation of Tyr^{577}, Tyr^{612} and Tyr^{695} (GFTY) or Tyr^{577}, Tyr^{612}, Tyr^{695} and Tyr^{750} (GFTA) in the β763 context. β881, β826, and β763 run as a double band (indicated by an arrowhead) in between two unspecific bands. These unspecific bands are more obvious in the left panel, since this panel was exposed longer than the β763 panel.

(β826GFYYY-) and β763 (β763GFYY- -), which eliminated Tyr^{866} and Tyr^{806}, respectively. Both these receptor mutants are phosphorylated (Fig. 2B). β763GFYY- - contains only two tyrosine residues: Tyr^{695} and Tyr^{750}. To discriminate between these, three mutants were constructed: β763GFTY- - only containing Tyr^{750}, β751GFYF- - only containing Tyr^{695}, and β763GFTA- - containing no tyrosine residue. Phosphorylation studies with these constructs showed that both Tyr^{695} and Tyr^{750} could be phosphorylated, albeit at low levels (Fig. 2B and not shown). Taken together, tyrosine residues 577, 612, 695, and 750 in the IL-5Rβ are phosphorylated upon IL-5 stimulation.

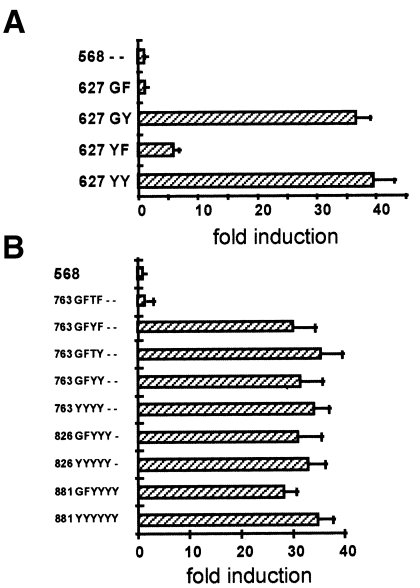

Fig. 3. Tyrosine redundancy in the β chain for STAT activation. 293 cells were transfected with 1 μg IL-5R α chain and 1 μg of the mutant β receptors together with 2 μg 4xGAStkCAT reporter construct, after which IL-5-induced STAT activation was measured as described for Fig. 1B. (a) Mutation of Tyr^{577} (627GY), Tyr^{612} (627YF) or both (627GF) in the β627 (627YY) context clearly shows that Tyr^{612} is important for STAT5 activation. (b) Mutation of Tyr^{577} and Tyr^{612} (GF) in the β881, β826 and β763 deletion constructs and mutation of Tyr^{577}, Tyr^{612} and Tyr^{695} (GFTY) or Tyr^{577}, Tyr^{612}, Tyr^{695} and Tyr^{750} (GFTA) in the β763 (YYYY) context shows that Tyr^{612}, Tyr^{695} and Tyr^{750} are able to mediate STAT5 activation.

To determine whether tyrosine phosphorylation of the βc chain is involved in STAT5 activation, we tested the different mutations in 293 cells. In the context of β627, only mutation of Tyr^{612} (β627YF) resulted in a clear drop in CAT activity after IL-5 stimulation, while mutation of only Tyr^{577} (β627GY) had no effect (Fig. 3A). The double mutant β627GF did not induce any increase CAT activity. Therefore, although both tyrosines are phosphorylated after IL-5 stimulation (Fig. 2A), only Tyr^{612} plays a major role in STAT activation in the context of β627. Surprisingly, mutation of Tyr^{612} and Tyr^{577} in the context of β881, β826 and β763 did not result in a decrease in IL-5-induced CAT activity (Fig. 3B). Moreover, mutants containing only Tyr^{695} (763 GFYF) or Tyr^{750} (763 GFTY) were capable of inducing STAT activity (Fig. 3B). Importantly, β763GFTA- - was the only receptor mutant in this panel that is not phosphorylated after IL-5 stimulation (Fig. 2B) and does not support IL-5-induced CAT activity. Therefore, phosphorylation of either Tyr^{612}, Tyr^{695} or Tyr^{750} is both necessary and sufficient for STAT activation by IL-5.

4. Discussion

The major conclusion from our study is that tyrosine phosphorylation of the IL-5R β chain is essential for endogenous STAT5 activation. This is in contrast with previous data where tyrosine phosphorylation of the β chain was not important for STAT activation in COS and BA/F3 cells [11,13]. However, in these studies the STAT proteins were overexpressed, while we have studied activation of endogenous STAT proteins. Interestingly, when STAT proteins are overexpressed in 293 cells, receptor phosphorylation is dispensable for IL-5-induced STAT activation (data not shown). An alternative mechanism for activation of overexpressed STAT proteins can be the recruitment of STAT5 to activated JAK2 or other cytoplasmic tyrosine kinases without binding to the receptor, resulting in tyrosine phosphorylation and activation of STAT proteins.

Previous work has shown that Tyr^{577} and Tyr^{750} of the β chain are phosphorylated in BA/F3 cells after GM-CSF stim-

ulation [30,31]. Both residues are implicated in the activation of the SH2 containing adapter protein Shc and the phosphatase SHP-2 (also known as PTPD1 or Syp). Tyrosine phosphorylation of either Shc and SHP-2 leads to the activation of RAS and subsequently to transcriptional activation of the *c-fos* gene [30,31]. The tyrosine motifs 612 (YLXL) and 750 (YXXL) exhibit a clear homology with sequences found in other cytokine receptors which are involved in STAT5 activation [32], while the sequence of Tyr^{695} (YVSS) lacks this homology. The finding that each of these tyrosines was sufficient to mediate maximal STAT activity indicates that different sequences within a receptor can serve as a binding site for STAT5. These data show, together with the involvement of Tyr^{577} and Tyr^{750} for Shc and SHP-2 activation, that distinct sets of tyrosines are involved in different signaling routes. We can also conclude that the activation of a specific signaling route can be controlled by multiple tyrosines, showing the partial functional redundancy of these residues, as was also described for STAT activation by the EPOR and GHR [25–27]. Finally, the redundancy and specificity of the tyrosine residues in the activation of different signal transduction routes suggest that these pathways are involved in distinct cellular functions. Although STAT5 is implicated to activate specific genes involved in proliferation and differentiation of hematopoietic cells, the precise function(s) of STAT proteins in IL-5 signaling still remains to be elucidated.

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