Constitutive association of JAK1 and STAT5 in pro-B cells is dissolved by interleukin-4-induced tyrosine phosphorylation of both proteins

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Abstract The bipartite human interleukin-4 (IL-4) receptor was functionally expressed in murine pro-B cells and activated by human IL-4 to evoke intracellular signaling. Mutual association of signal transducing proteins within the receptor complex was then studied in dependence of ligand stimulation. Besides ligand-induced receptor heterodimerization and contacts of the two IL-4 receptor subunits α and γ with Janus kinases JAK1 and JAK3 a prominent constitutive binding between JAK1 and signal transducer and activator of transcription STAT5 was detected. Since both these proteins become phosphorylated in response to IL-4 receptor stimulation, the influence of tyrosine phosphorylation on their mutual contact was analyzed. Association of JAK1 and STAT5 was found to occur exclusively between unphosphorylated proteins.

Key words: Signal transduction; Cytokine receptor; JAK/STAT pathway; Protein-protein interaction

1. Introduction

Interleukin-4 (IL-4), a multifunctional immune regulator, exerts its activity on target cells via the bipartite IL-4 receptor, which consists of the interleukin-4 receptor α-chain (IL-4Rα) [1], and the common γ-receptor chain (γc) [2,3]. Both receptor subunits are members of the cytokine receptor superfamily characterized by common structural [4] as well as functional properties [5].

Ligand-induced juxtaposition of cytoplasmic receptor domains triggers intracellular signal release and ultimately leads to cellular responses such as differentiation and proliferation. Heterodimers of IL-4Rα and γc [2] as well as experimentally induced homotypic dimers of IL-4Rα [6,7] can mediate the activation of various proteins involved in signal transfer from the cell membrane to the nucleus. Of key importance in this course of events is the coordination of specific tyrosine phosphorylation processes. Phosphorylation is operative in modulating the interactions between signaling proteins associated with the IL-4 receptor complex.

Upon IL-4 receptor chain dimerization, Janus kinases JAK1 and JAK3, which are already preassociated to the α- and γ-chain, respectively [8,9], are activated and become subject to autophosphorylation and/or transphosphorylation. Subsequently, various tyrosine residues within the cytoplasmic domains of the receptor chain as well as in associated signaling mediators become phosphorylated by the JAK kinases and probably to some extent also by the action of src type kinases. As a result of these reactions, a network of protein-protein interactions within the receptor complex and its vicinity is created that relies on contacts between protein recognition domains and phosphotyrosine-containing cognate sequence motifs within their binding partners.

The best characterized signaling transduction cascade triggered by the IL-4 receptor is the JAK/STAT pathway. Whereas STAT6 is known for several years to be a characteristic mediator of IL-4 signaling [10], we have recently shown that the IL-4 receptor is also able to activate STAT5 [11]. The mechanisms of STAT5 activation through the IL-4R have not yet been characterized.

In this study we have approached IL-4-induced signal transduction leading to STAT5 activation by an analysis of protein-protein interactions within the receptor complex. Coprecipitation experiments did not allow for the detection of an IL-4-evoked direct contact between STAT5 and either of the IL-4 receptor subunits. Instead, a prominent constitutive association between STAT5 and JAK1 was observed. This interaction showed a striking dependence on the tyrosine phosphorylation state of both proteins. These findings add novel mechanistical aspects to the understanding of IL-4-triggered activation of STAT5. In addition, they raise interesting speculations on the functional implications of tyrosine phosphorylation reactions in the temporal organization of ligand-induced signal transduction in cytokine receptor complexes.

2. Materials and methods

2.1. Cell culture and IL-4 stimulation of cells

The previously described cell line BAF-4α-pγ [6,12] is a derivative of the IL-3-dependent murine pro-B cell line Ba/F3 and stably expresses both subunits of the human IL-4 receptor. An aminoterminal epitope tag was fused to the human γc receptor chain that allows for specific immunoprecipitation of the heterologous receptor by using antibody P5D4 [12]. Cells were cultivated as described [6,12].

For cytokine stimulation experiments, samples of 3×10^7 cells were starved from murine IL-3 for 2 h and then incubated at 37°C for 10 min in 1 ml of medium containing no cytokine, or 7 nM of recombinant human IL-4 (a gift from W. Sebald, Würzburg).

2.2. Immunoprecipitation and immunoblotting

Samples of starved or cytokine-treated cells were pelleted and sub-

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Abbreviations: γc, common receptor γ-chain; DMEM, Dulbecco's modified essential medium; EPO, erythropoietin; FCS, fetal calf serum; G418, geniticin; GM-CSF, granulocyte-macrophage colony-stimulating factor; hIL-4, human interleukin-4; hIL-4R, human interleukin-4 receptor; mIL-4, murine interleukin-4; mIL-3, murine interleukin-3; hIL4Rα, human interleukin-4 receptor α-subunit; IgG, immunoglobulin G; IL, interleukin; JAK, Janus kinase; RPMI, Rosswell Park Memorial Institute; PCR, polymerase chain reaction; pTyr, phosphotyrosine; PTB domain, phosphotyrosine binding domain; SH2 domain, src homology domain type 2; STAT, signal transducer and activator of transcription

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jected to cell lysis and immunoprecipitation as described [6,12]. Cleared lysates were incubated with 1–5 μg of specific antibody for 3 h at 4°C. Mouse monoclonal antibodies used were X14/38 to hIL-4Rα [13], P5D4 recognizing epitope-tagged human γc [12] and E34-1 directed to murine JAK3 [14]. Rabbit antisera used were anti-JAK1 (Transduction Laboratories) and anti-STAT5a [15]. Immunocomplexes were collected from lysates with 20 μl of anti-mouse-IgG-agarose or protein A-Sepharose (Sigma) and assayed by Western blot as described [12] using peroxidase-coupled anti-pTyr antibody RC20 (Transduction Laboratories) or the above-mentioned primary antibodies and peroxidase-coupled anti-mouse and anti-rabbit IgG (Sigma) for detection. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham). Stripping of blots for reprobing was done 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 all other antibodies) followed by extended washing.

3. Results and discussion

3.1. Analysis of protein-protein interactions within the IL-4 receptor complex reveals a constitutive association between STAT5 and JAK1

We have recently demonstrated the activation of STAT5a and STAT5b through the human IL-4 receptor (hIL-4R) [11]. To gain insight into molecular mechanisms underlying this signaling pathway, we wished to determine which protein-protein interactions are involved in the contact between STAT5 and the IL-4 receptor. To this end, we employed a Ba/F3-derived cell line stably expressing a functional bipartite human IL-4R that consists of hIL-4Rα and human γc with an amino-terminally fused epitope tag (BAF-4α-pγ) [6,12]. We performed immunoprecipitations from lysates of both starved and hIL-4-stimulated BAF-4α-pγ cells using antibodies to various constituents of the hIL-4R complex. Precipitates obtained with antibodies to human IL-4Rα, epitope-tagged human γc, JAK1, JAK3, and STAT5a were subjected to Western blot analysis (Fig. 1). Controls employing only protein A-Sepharose or anti-mouse IgG agarose were included to rule out any possible unspecific precipitations (lanes 1–4). We first probed blots resulting from the indicated Ipps with anti-STAT5a in order to identify interaction partners among the components of the receptor assembly (panel I). Apart from the anti-STAT5 precipitates serving as a positive control in this test (lanes 13 and 14), a strong STAT5a signal was observed in anti-JAK1 precipitates from both untreated and stimulated cells (lanes 9 and 10), indicating a specific interaction of the two proteins. No coprecipitation of STAT5a with hIL-4α, γc or JAK3 could be detected. The blot was subsequently stripped from anti-STAT5a and reprobed with anti-JAK1 and, in order to control for successful precipitations and equal loading, with anti-hIL-4Rα, anti-JAK3 and anti-γc (panels II–V). As expected, an intense JAK1 signal was seen in the anti-JAK1 precipitates (panel II, lanes 9 and 10). Again indicating a specific binding between JAK1 and STAT5a, JAK1 was coprecipitated by anti-STAT5a (lanes 13 and 14). Coprecipitation of JAK1 with anti-STAT5a yielded clear, but much weaker signals than the reverse experiment. We attribute this finding to the fact that in Ba/F3 cells STAT5a is by far more abundant than JAK1 (data not shown). The weak expression level of Janus kinases and both hIL-4 receptor subunits could also be the reason why a coprecipitation of JAK1 with hIL-4Rα and JAK3 with epitope-tagged γc, respectively, could not be detected on the original blots that had previously undergone incubation with anti-STAT5a and removal of this antibody (data not shown). Similar difficulties were encountered with attempts to show coprecipitation of IL-4Rα with γc. To still demonstrate these various protein-protein contacts, the delicate Ipps and subsequent Western blots (marked with a triangle) were performed again separately and subjected immediately to detection with the respective antibodies without prior stripping. Negative controls without antibodies were always included

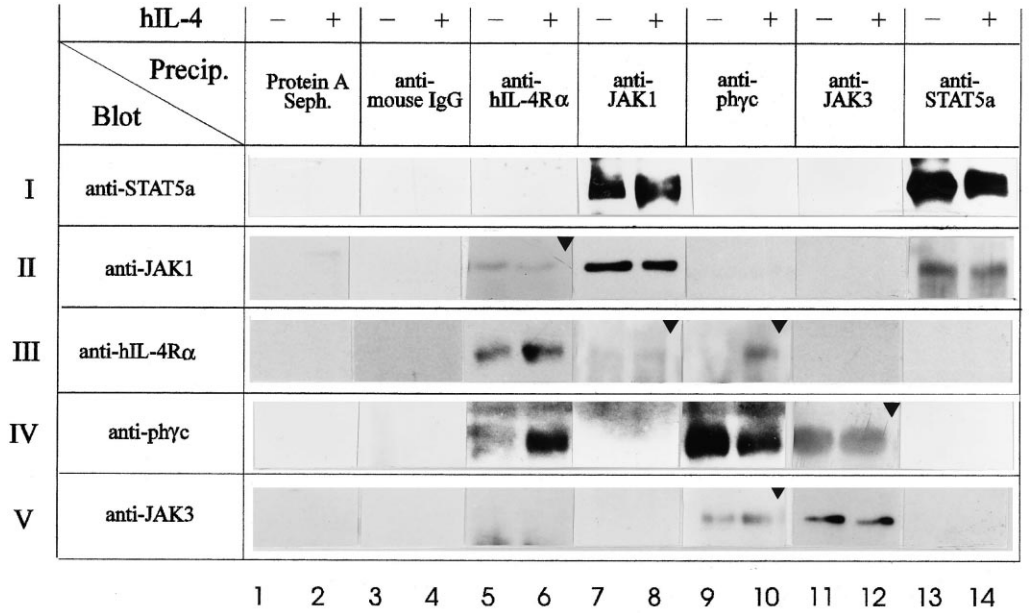

Fig. 1. Analysis of protein-protein interactions in the hIL-4 receptor complex in BAF-4α-pγ cells by coimmunoprecipitations. Starved cells were either left untreated or stimulated with 7 nM hIL-4 as indicated. From lysates immunoprecipitations were performed applying the antibodies indicated in the top row. As specificity controls, precipitations with protein A-Sepharose and anti-mouse IgG were included. Precipitates were subjected to SDS-PAGE and Western blot. The membranes were probed with anti-STAT5a and then successively with antibodies to JAK1, hIL-4Rα, epitope-tagged hγc, and JAK3 as indicated to the left. Sections marked with a filled triangle were derived from separate blots that had not undergone previous detections with other antibodies (for details see text).

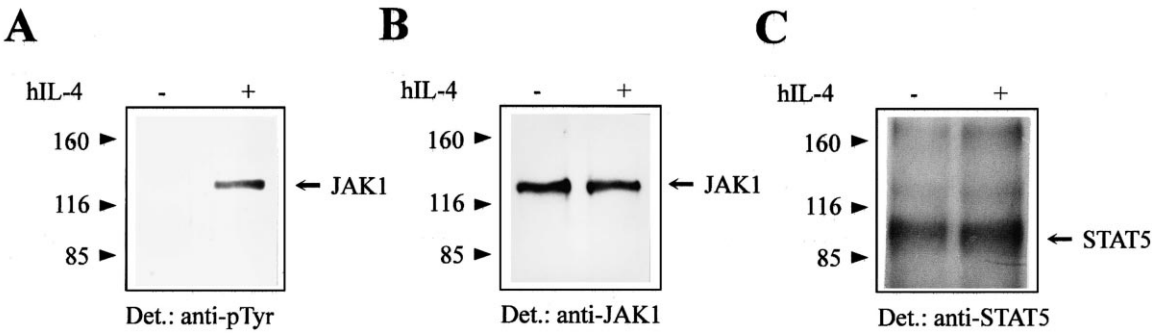

Fig. 2. Effect of STAT5 tyrosine phosphorylation on the protein-protein interaction with JAK1. Starved BAF-4α-pγ cells were left untreated or stimulated with hIL-4 as indicated and subjected to immunoprecipitation with anti-JAK1 followed by Western blot. The blot was successively probed with antibodies to (A) pTyr, (B) JAK1 and (C) STAT5.

to assure specificity (data not shown). In this way, constitutive associations between receptor chains and JAK kinases (panel II, lanes 5 and 6; panel IV, lanes 11 and 12; panel V, lanes 9 and 10) as well as ligand-dependent intersubunit contacts (panel III, lanes 9 and 10; panel IV, lanes 5 and 6) could readily be detected.

These results comprehensively mirror protein-protein interactions that underlie the IL-4-driven JAK/STAT5 pathway(s). Interestingly, no ligand-induced association of STAT5 with the IL-4 receptor chains that would be stable enough to result in coimmunoprecipitation could be detected. Instead, we observed an intense and constitutive binding of STAT5 to JAK1. Examples have emerged for STAT activation without the apparent necessity for phosphotyrosines on the side of the activated receptor [16,17]. Tyrosine phosphorylation of the receptors for GM-CSF and EPO are dispensable for at least some degree of ligand-induced STAT5 phosphorylation and activation [16,18]. We could recently show that activation of STAT6 is not dependent on tyrosine residues contained in the sequence of IL-4Rα chain [19] and have also observed that STAT5 does not require IL-4Rα tyrosine phosphorylation (Friedrich et al., submitted for publication). Together with results presented in this work, these earlier findings point to a possible function of the STAT5-JAK1 complex in IL-4R signal transduction. Our data and interpretations are in full agreement with a publication by Fujitani et al., who identified STAT5 as a major binding partner for JAK1 in two-hybrid screen and demonstrated that STAT5, unlike STAT3, cannot be coprecipitated with the cytokine receptor subunit gp130 [20]. Moreover, these authors could show that phosphotyrosines on the side of the receptor were not essential for STAT5 activation and concluded that signaling can occur through the immediate interaction of STAT5 with gp130-bound JAK1.

3.2. *Association of JAK1 and STAT5 occurs exclusively between unphosphorylated proteins*

Since STAT5 had been found to contact JAK1 in BAF-4α-pγ cells irrespective of hIL-4 stimulation and this association is of possible importance for signaling, we were interested to assess to what extent IL-4-induced tyrosine phosphorylation of the two binding partners influences their mutual interaction. Anti-JAK1 immunoprecipitates from lysates of starved and hIL-4-stimulated BAF-4α-pγ cells were analyzed for tyrosine phosphorylated proteins by Western blot (Fig. 2A). A single phosphorylated protein in the size range of 120 kDa appeared upon hIL-4 stimulation which by reprobing of the blot (Fig. 2B) with anti-JAK1 was proven to be JAK1. No tyrosine phosphorylation of proteins in the size range of 90 kDa was detectable (Fig. 2A), but reprobing of the blot with an antibody to STAT5 demonstrated that considerable amounts of STAT5 had been coprecipitated with JAK1 (Fig. 2C). To more thoroughly analyze the effect of tyrosine phosphorylation on the interaction between STAT5 and JAK1, we also performed the reverse experiment, i.e. immunoprecipitates obtained with anti-STAT5 were consecutively probed with anti-pTyr (Fig. 3A), anti-STAT5 (Fig. 3B) and anti-JAK1 (Fig. 3C). In accordance with the data documented in Fig. 2, JAK1 was present in the anti-STAT5 precipitates only in its unphosphorylated form (Fig. 3C); it could not be detected by anti-pTyr (Fig. 3A).

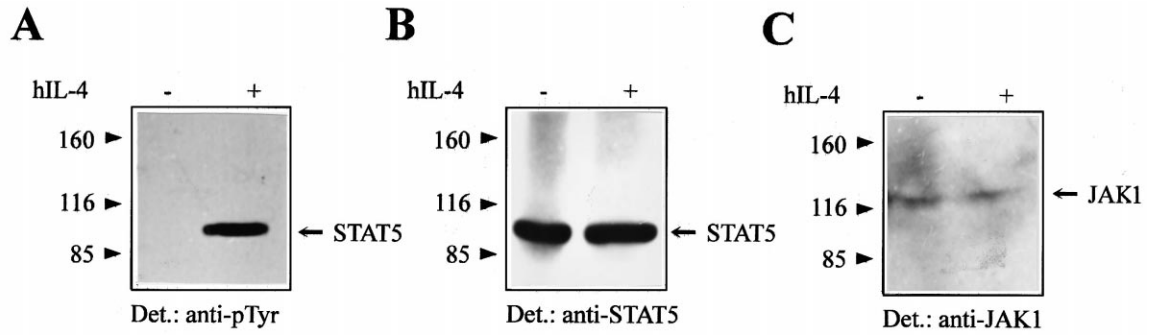

Fig. 3. Effect of JAK1 tyrosine phosphorylation on the protein-protein interaction with STAT5. Starved BAF-4α-pγ cells were left untreated or stimulated with hIL-4 as indicated and subjected to immunoprecipitation with anti-STAT5 followed by Western blot. The blot was successively probed with antibodies to (A) pTyr, (B) STAT5 and (C) JAK1.

We conclude from the two complementary precipitation experiments that tyrosine phosphorylation of JAK1 and/or STAT5 precludes their binding to each other. In functional terms of cytokine signaling, we interpret these results such that the mechanism of STAT5 activation by JAK1 involves a rapid release of the STAT protein from the contact with the kinase once both proteins have become tyrosine phosphorylated upon activation of the receptor system. Fujitani et al., who have first demonstrated a direct interaction between STAT5 and JAK1 [20], did not address the influence of tyrosine phosphorylation of the two proteins on their mutual contact. By deletion analysis these authors have shown that a determinant located in the amino acid 680–688 region of STAT5 is critical for binding to JAK1. Interestingly, Y694, the major phosphoacceptor site in the STAT5 molecule, is not essential for this association. This finding implies that tyrosine phosphorylation of STAT5 does not regulate its affinity for JAK1 in a positive fashion. It is, however, conceivable that the regulatory tyrosine at position 694 may have a negative influence on JAK1 binding upon phosphorylation. In its phosphorylated form it promotes dimerization of STAT5 through pTyr-SH2 domain crossbridging between two individual molecules [21]. As a consequence of this event, a nearby JAK contact interface could be distorted, leading to the dissolution of the JAK/STAT association.

On the side of the JAKs, the JH2 or pseudokinase domain proved the crucial substructure for the interaction with STAT5 [20]. Although tyrosine phosphorylation of Janus kinases is correlated with activation, the underlying mechanisms are not well understood. Catalytic activity appears to be controlled by phosphorylation of two tyrosine residues within the putative activation loop of the kinase domain, but autophosphorylation of the protein occurs at a multitude of additional sites [22]. We do not know yet whether dissociation of STAT5 and JAK1 results from phosphorylation of only one or both of the proteins. Our results, however, leave open the possibility that tyrosine phosphorylation, perhaps within the JH2 domain, may modulate the properties of JAK1 with respect to its participation in protein-protein interactions.

Recently some other cases have emerged in which tyrosine phosphorylation mediates against intermolecular protein-protein association. The affinity between erythrocyte membrane protein band 3 and aldolase is decreased by tyrosine phosphorylation, probably by blocking through electrostatic repulsion [23,24]. Bradshaw et al. showed that interaction of the cytoplasmic tail of CD152, a mediator of T cell activation, with a clathrin-associated protein is negatively regulated by tyrosine phosphorylation [25]. Various other studies employing epitope-specific antibodies, protein binding assays and structural methods have provided evidence for a determinant influence of tyrosine phosphorylation on protein conformation [26–31].

For serine/threonine phosphorylation, versatile regulative functions in intracellular information transfer are well established. Likewise, the role of protein tyrosine phosphorylation clearly appears to exceed positive control involving SH2 and PTB domains where phosphorylation is required for binding. Future studies will unravel to what extent negative regulation of protein-protein interactions by tyrosine phosphorylation is a mechanism operative in signal transduction in general and in cytokine receptor signaling in particular.

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