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*J Immunol 2000; 164:1211-1215;*
doi: 10.4049/jimmunol.164.3.1211
http://www.jimmunol.org/content/164/3/1211

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Protein Tyrosine Phosphatase Activity Is Required for IL-4 Induction of IL-4 Receptor α-Chain

Hua Huang¹ and William E. Paul²

To investigate the role of protein tyrosine phosphatases in IL-4α-chain expression and signaling, we first established that SHP-1, but not SHP-2, coimmunoprecipitated with anti-IL-4α chain Abs in extracts prepared from resting lymphocytes. We further observed that the protein tyrosine phosphatase inhibitors Na₃VO₄ and pervanadate blocked the striking induction of IL-4α-chain expression that is mediated by IL-4. However, Na₃VO₄ did not diminish IL-4-induced Stat6 phosphorylation nor did it block the IL-4-mediated increase in IL-4α-chain mRNA. The striking inhibition in total cellular IL-4α-chain and in cell surface IL-4 receptors was associated with an inhibition of biosynthetic labeling of IL-4α-chain after a 30-min pulse with [³⁵S]methionine, indicating that reduction of IL-4α-chain protein resulted from either a diminished production of the receptor or a rapid degradation, possibly as a result of phosphorylation of the receptor in an early biosynthetic cellular compartment. Control of newly synthesized IL-4α-chain protein expression by phosphatase may provide a novel means to regulate IL-4 responsiveness.

The Journal of Immunology, 2000, 164: 1211–1215.

Interleukin-4 mediates its functions through binding to and stimulation of the IL-4 receptor. This receptor consists of the IL-4α-chain, which binds IL-4 with high affinity (1, 2), and the common γ-chain (γc) (3, 4), shared by the receptors for IL-2, IL-7, IL-9, and IL-15 (5–7). Upon interaction with IL-4, the IL-4α-chain and γc-chain-associated kinases JAK-1 and JAK-3 are activated (8–10). Activation of JAK1 and JAK3 results in the phosphorylation of IL-4α-chain and of the major substrates of the receptor, including Stat6 and insulin-receptor substrates 1 and 2 (11–13). Stat6 activation has been mainly implicated in the regulation of IL-4-induced gene activation (14, 15). It is the major regulator of the IL-4-induced up-regulation of the IL-4α-chain itself (16, 17).

Src homology 2 (SH2)³ domain-containing protein tyrosine phosphatases (PTPases; SHP-1 and SHP-2) have been shown to physically interact with growth factor and cytokine receptors and to participate in regulating their signaling. PTPases exert either positive or negative effects on growth and cytokine receptor signaling, depending on their cellular association context. SH2 domain-containing PTPases (SH2 PTPases) recognize the intracellular tyrosine inhibition motif (ITIM) consisting of a consensus sequence [I/VxYxxL], conserved in several families of inhibitory receptors (18, 19). When the tyrosine residue in the ITIM becomes phosphorylated, it recruits and activates an SH2 PTPase (20). The activated SH2 PTPase can then remove phosphate from tyrosine residues such as those found in the intracellular tyrosine activation motif of TCRs and B cell receptors. The dephosphorylation terminates TCR or B cell receptor signaling (21, 22).

SHP-1 has been reported to interact with the erythropoietin receptor, c-kit and the IL-3 receptor to inhibit signaling through these receptors (23–25). SHP-2, on the other hand, has been shown to interact with the IL-2R and the IL-5R, resulting in a positive signal (26, 27). However, when it interacts with CTLA-4, SHP-2 has been reported to deliver a negative signal (28).

SHP-1 has been reported to physically associate with the IL-4α-chain in the presence or absence of IL-4 stimulation (29, 30). However, the role of SHP-1 and other PTPases in IL-4 signaling has not been intensively studied. Here, we examined the effect of PTPases inhibitors on IL-4-induced Stat6 phosphorylation and IL-4-induced IL-4α-chain mRNA expression. Surprisingly, sodium vanadate prevented the increase in cell surface and total IL-4α-chain expression normally induced by IL-4. Furthermore, [³⁵S]methionine-labeling experiments indicate that sodium vanadate either inhibited IL-4α-chain biosynthesis or led to rapid degradation of the receptor.

Materials and Methods

Animals and cell cultures

C57BL/6 mice were purchased from the National Cancer Institute, Bethesda, MD. Single-cell suspensions were prepared from spleen and lymph nodes. RBC were lysed by ACK (0.15 M NH₄Cl and 0.7 mM KH₂PO₄ lysis buffer (Biofluids, Gaithersburg, MD). For Na₃VO₄ inhibition experiments, lymph node cells (1 × 10⁶ for FACS analysis; 5 × 10⁴ for immunoprecipitation) were cultured in the presence of 0.5 ng/ml of IL-4 with or without addition of 100–250 μM Na₃VO₄ for 4–6 h. Pervanadate was prepared by mixing equimolar (20 mM) solutions of hydrogen peroxide and sodium orthovanadate for 20 min at room temperature immediately before use. Residual hydrogen peroxide was removed by incubation with catalase-conjugated agarose beads (Sigma, St. Louis, MO).

Immunoprecipitation and Western blot analysis

Single-cell suspensions of lymph node and spleen cells were prepared and stimulated with 5 ng/ml of IL-4 in complete RPMI 1640 at room temperature for the time indicated. The reaction was stopped with cold PBS containing 100 μM Na₃VO₄. For Stat6 immunoprecipitation, cells were lysed in hypotonic buffer (20 mM HEPES (pH 7.9)), 10 mM KCl, 0.1 mM EDTA, 10% glycerol, and 0.2% Nonidet P-40 freshly supplemented with 1 mM Na₃VO₄ 1 mM PMSF, 10 μg/ml aprotinin, leupeptin, and pepstatin.

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Received for publication July 29, 1999. Accepted for publication November 15, 1999.

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³ Abbreviations used in this paper: SH2, Src homology 2; PTPase, protein tyrosine phosphatase; ITIM, intracellular tyrosine inhibition motif.
After centrifugation at 4°C for 1 min, supernatants were collected as cytoplasmic extracts. For IL-4Rα-chain immunoprecipitation, cells were lysed with 0.5 ml of lysis buffer (50 mM HEPES, 0.5% Nonidet P-40, or 1% Brij 96, 5 mM EDTA, 50 mM NaCl, 10 mM sodium pyrophosphate, and 50 mM NaF) freshly supplemented with 1 mM Na3VO4, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin, and pepstatin or complete protease inhibitor loading buffer, separated in a 7.5% acrylamide gel, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) that were probed with FACS buffer and analyzed in a FACScan (Becton Dickinson, Mountain View, CA).

Results

**SHP-1, but not SHP-2, is associated with the IL-4Rα-chain**

It has been reported that SHP-1 is physically associated with IL-4R in a human B cell line (26) and that SHP-2 is physically associated with IL-4Rα-chain in murine B cells (31). To clarify the physical interaction between SH2-PTPases and IL-4Rα-chain in normal lymphocytes, we prepared cellular extracts from mixed spleen and lymph node cells, immunoprecipitated the IL-4Rα-chain with an anti-IL-4Rα-chain Ab (M2), and then immunoblotted with monoclonal anti-SHP-1 or with polyclonal anti-SHP-2 Abs.

We observed that SHP-1, but not SHP-2, is constitutively associated with the IL-4Rα-chain and that this association is not affected by the binding of IL-4 (Fig. 1A). The coimmunoprecipitation of SHP-1 by anti-IL-4Rα Ab was shown to be specific since such association was not detected in spleen and lymph node cell lysates prepared from IL-4Rα-chain knockout mice (Fig. 1B).

**Na3VO4 treatment enhances IL-4-induced Stat6 phosphorylation**

To understand the role of PTPases in early IL-4 signaling, we treated fresh spleen and lymph node cells with the PTPase inhibitor Na3VO4, either alone or in combination with IL-4, for 10, 20, or 60 min. Because Na3VO4 also inhibits nuclear PTPases, cytosolic Stat6 was separated from the nuclear Stat6 to examine the effect of Na3VO4 on IL-4Rα-chain-associated PTPases. The phosphorylation levels of Stat6 were measured. Na3VO4 alone did not cause detectable cytosolic Stat6 phosphorylation; however, when combined with IL-4, it increased the level of such phosphorylation. This effect diminished after 20 min of stimulation and was no longer detectable at 60 min (Fig. 2).

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**FIGURE 2.** Na3VO4 treatment increases IL-4-induced Stat6 phosphorylation. Pooled spleen and lymph node cells prepared from C57BL/6 mice were incubated with or without IL-4 (5 ng/ml) and/or Na3VO4 (100 μM) for 0–60 min. Lysates prepared from these cells were immunoprecipitated (IP) with anti-Stat6 Ab. Filters containing immunoprecipitated Stat6 were immunoblotted with anti-PY (4G10) and then stripped and reprobed with anti-Stat6 Ab.

**FIGURE 3.** Pervanadate treatment increases IL-4-induced IL-4Rα-chain mRNA expression. Pooled spleen and lymph node cells from C57BL/6 mice or from Stat6 knockout mice were treated with or without IL-4 and nothing, Na3VO4 (NV 100 μM), or freshly prepared pervanadate (PV 200 μM) for 4 h. Total RNA was prepared from these cells using RNAzol (Tel-Test, Friendswood, TX). Ten micrograms of total RNA was electrophoresed in individual lanes of a 1% agarose gel, transferred to a nitrocellulose filter, and probed with a 32P-labeled IL-4Rα-chain cDNA probe.
Effect of PTPases on Stat6-dependent IL-4Rα-chain mRNA induction

IL-4Rα-chain mRNA is rapidly up-regulated by IL-4. This induction fails to occur in splenocytes from mice that lack Stat6 (Fig. 3). To examine whether the Na₃VO₄-induced increase in Stat6 phosphorylation in response to IL-4 resulted in enhanced Stat6-dependent gene induction, we treated fresh spleen and lymph node cells with Na₃VO₄ or with pervanadate, either alone or in combination with IL-4, for 4 h and measured IL-4Rα-chain mRNA. Pervanadate induced a marked increase in IL-4Rα mRNA whereas Na₃VO₄ had only a modest effect.

Effect of PTPases on IL-4Rα-chain protein expression

We cultured fresh spleen and lymph node cells in the presence or absence of IL-4 overnight with or without Na₃VO₄. In contrast to its effect on IL-4-induced IL-4Rα-chain mRNA, Na₃VO₄ inhibited IL-4-mediated up-regulation of cell surface IL-4Rα-chain, as detected by immunofluorescence staining with the M1 monoclonal anti-IL-4Rα-chain Ab. Na₃VO₄ had no such effect on IL-2Rβ-chain expression (Fig. 4).

Na₃VO₄ had a similar effect on total cellular IL-4Rα-chain as detected by immunoprecipitation using the M2 monoclonal anti-IL-4Rα-chain Ab and immunoblotting with a rabbit anti-IL-4Rα-chain; pervanadate had an even more striking inhibitory effect. As a control, Na₃VO₄ had no effect on the amount of Stat6 protein in these cells (Fig. 5).

Despite the striking reduction in the amount of IL-4Rα-chain protein in cells treated for 4 h with IL-4 and Na₃VO₄, these cells showed as much or more phosphorylation as did cells treated with IL-4 alone. Interestingly, a low concentration of Na₃VO₄ was sufficient to diminish IL-4-induced IL-4Rα-chain expression but failed to enhance IL-4-induced IL-4Rα-chain tyrosine phosphorylation (Fig. 6).

**Na₃VO₄ inhibits IL-4Rα-chain biosynthesis**

To examine biosynthesis of the IL-4Rα-chain, we stimulated spleen and lymph node cells for 4 h with medium alone, with IL-4

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**FIGURE 4.** Na₃VO₄ treatment inhibits IL-4-induced enhancement of surface IL-4Rα-chain expression. Pooled spleen and lymph node cells prepared from C57BL/6 mice were incubated with or without IL-4 and/or Na₃VO₄ overnight and then analyzed by flow cytometry for IL-4Rα expression using the monoclonal anti-IL-4Rα-chain Ab M1. As a control, cells were also stained with FITC-conjugated anti-mouse IL-2Rβ-chain Ab.

**FIGURE 5.** Na₃VO₄ treatment inhibits IL-4-induced enhancement in total cellular IL-4Rα expression. Pooled spleen and lymph node cells from C57BL/6 mice were treated with nothing, IL-4, IL-4 plus Na₃VO₄, or IL-4 plus pervanadate (PV) for 6 h. Lysates prepared from these cells were immunoprecipitated (IP) with M2 or with polyclonal anti-Stat6 Ab and immunblotted with rabbit anti-IL-4Rα-chain Ab or with anti-Stat6 Ab.

**FIGURE 6.** Na₃VO₄ treatment dramatically increases IL-4-induced IL-4Rα tyrosine phosphorylation. Pooled spleen and lymph node cells from C57BL/6 mice were treated with medium alone, IL-4, or IL-4 plus Na₃VO₄ (100–500 μM) for 4 h. Lysates prepared from these cells were immunoprecipitated (IP) with M2 and immunoblotted with rabbit anti-IL-4Rα Ab. The filters were stripped and blotted with monoclonal anti-PY Ab (4G10).
or with IL-4 plus Na$_3$VO$_4$. After a 1-h culture period in methionine-cysteine-deficient medium, the cells were cultured with $^{[35]$S]methionine for 30 min. Lysates were prepared and immunoprecipitated with M2. Immune complexes were separated on a 7.5% polyacrylamide gel. Radiolabeled IL-4R-α-chain was detected by autoradiography.

Discussion

In this study, we confirm that IL-4 strikingly induces expression of the IL-4R-α-chain. This had been initially observed by flow cytometric analysis of the IL-4-binding capacity of T cells and B cells stimulated with IL-4 (16); this effect was subsequently shown not to occur in cells derived from Stat6-deficient mice (17), indicating that the gene encoding the IL-4R-α-chain was IL-4-dependent and Stat6 inducible. Indeed, Kotanides et al. (32) showed that IL-4 induces IL-4R mRNA in an HT-2 cell line and induces expression of reporter genes in transfected HeLa cells through a Stat6-binding element found in the IL-4R-α-chain promoter.

We demonstrated that lymph node and spleen cells harvested from normal mice showed a substantial increase in IL-4R-α-chain mRNA, total cellular IL-4R-α-chain protein, and cell surface IL-4R-α-chain as early as 4 h after stimulation with IL-4. Indeed, the degree of induction of IL-4R-α-chain mRNA or total protein is ~10-fold, as shown by comparing cells incubated in IL-4 plus IL-2 with cells incubated in IL-2 alone.

The biological significance of this induction has not been established, but it may be important in many IL-4-induced biological responses, particularly those in which prolonged IL-4 exposure is needed. Among these, one of the most striking is the requirement of IL-4 to be present for 48–72 h to efficiently induce CD4$^+$ T cells stimulated through their Ag receptors to develop into IL-4-producing (Th2) cells (33). During that time, the increased Stat6 signal that would result from increased expression of membrane IL-4R-α-chain may be critical, and a portion of the time needed for induction of the Th2 phenotype may reflect that needed to increase the degree of IL-4R-α-chain expression as a result of the action of IL-4. The actual time required for cells to commit to the Th2 phenotype might be substantially shorter if the experiments began with T cells in which the IL-4R-α-chain had already been up-regulated.

Based on our observation of the striking association of the PT-Pase SHP-1 with the IL-4R-α-chain in freshly isolated mouse spleen and lymph node cells and a previous report of such an association in the human B cell line, Ramos (29), we tested the effects of Na$_3$VO$_4$ and pervanadate on IL-4-mediated functions. We were surprised to observe a striking inhibition in the up-regulation of total cellular IL-4R-α-chain protein and cell surface IL-4Rs. However, such treatment did not diminish the degree of IL-4-induced Stat6 phosphorylation; indeed, Stat6 phosphorylation was increased in the presence of Na$_3$VO$_4$ 10 and 20 min after addition of IL-4. In keeping with this increase in Stat6 phosphorylation, pervanadate enhanced the IL-4-induced increase in IL-4Ra mRNA levels, as determined by Northern blotting. The blockade of IL-4Rα up-regulation by phosphatase inhibitors does not necessarily implicate SHP-1 as important in this process; the action of another phosphatase could be critical for IL-4-induced IL-4Rα up-regulation.

Although we anticipated that vanadate might diminish IL-4R-α-chain expression by causing rapid loss of the receptor from the cell surface, we observed that, in the presence of vanadate, there was diminished biosynthetic labeling of IL-4R-α-chain after a 30-min pulse with $^{[35]$S]methionine. This strongly suggests that vanadate impairs translation of IL-4R-α-chain mRNA or causes rapid degradation of newly synthesized IL-4R-α-chains. This effect appears to be specific since vanadate does not diminish the degree of expression of either Stat6 or IL-2β-chain.

Interestingly, it has recently been reported that in the presence of vanadate, the erythropoietin receptor is phosphorylated in the endoplasmic reticulum (34). Although, we have not been able to demonstrate phosphorylation in a comparable pool of IL-4R-α-chains, possibly because of the relatively low level of their expression, total cellular IL-4R-α-chain phosphorylation is increased in the presence of high concentrations of Na$_3$VO$_4$ despite the striking inhibition in total cellular IL-4R-α-chain protein. Mutational analysis should allow a determination of whether tyrosine phosphorylation of IL-4R-α-chain might target it for rapid degradation.

Prior analysis of expression of human IL-4R-α-chains in the mouse B lymphoma cell line M12 did not reveal any striking effects on the degree of IL-4R-α-chain expression when either the first or the second, third, and fourth conserved tyrosines were mutated to phenylalanine. In that experiment, the last (fifth) tyrosine was not examined; interestingly, this tyrosine is within a sequence homologous to ITIM (35–38).

Acknowledgments

We thank Nancy Noben-Trauth for providing IL-4R knockout mice and Cynthia Watson and Jane Hu-Li for their excellent technical assistance.

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