Interleukin-4 (IL-4) Induces Phosphatidylinositol 3-Kinase (p85) Dephosphorylation

IMPLICATIONS FOR THE ROLE OF SHP-1 IN THE IL-4-INDUCED SIGNALS IN HUMAN B CELLS

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Farhad Imani, Kelly J. Rager, Branimir Catipovic, and David G. Marsh

From the Division of Clinical Immunology, Department of Medicine, The Johns Hopkins University School of Medicine, Asthma and Allergy Center, Baltimore, Maryland 21224

IL-4 is a potent cytokine with pleiotropic effects on many cell types. The biological effects of IL-4 include induction of IgE class switching, induction of proliferation in T and B cells, and up-regulation of CD23 and major histocompatibility complex class II molecules on human B cells (1–4).

The IL-4 receptor complex is present on many hematopoietic and non-hematopoietic cell lines (5). Although many members of the cytokine receptor family, including the IL-4 receptor (IL-4R), lack protein kinase consensus domains, ligand binding to these receptors results in tyrosine phosphorylation as well as dephosphorylation and subsequent biological responses (6, 7).

Early studies of Morla et al. (8) reveal IL-4-induced tyrosine phosphorylation of proteins of 110 and 170 kDa in a murine mast cell line, IC 2.9. Subsequently, Wang et al. (9) reported IL-4-induced tyrosine phosphorylation of a 170-kDa polypeptide, termed 4PS in murine myeloid cell lines. Keegan et al. (10) reported that 4PS may be antigenically and functionally similar to the insulin receptor substrate-1. Additional evidence of IL-4-induced signal transduction events was demonstrated by the IL-4-induced association of the 85-kDa subunit of phosphatidylinositol 3-kinase (p85) with the phosphorylated form of 4PS; however, p85 itself was not phosphorylated (11).

Further studies have shown that IL-4 treatment induces the association of IL-4R with the γ chain of the IL-2 receptor complex (12) that participates in IL-4-mediated signaling by γ chain-associated JAK kinases (13, 14). However, experiments by He and Malek (15) provide evidence for the presence of two distinct IL-4R-mediated signaling events, γ chain-dependent and -independent, providing an explanation for the pleiotropic effects exerted by IL-4. Also, Hou et al. (16) reported that IL-4 could activate a tyrosine-phosphorylated DNA-binding protein termed IL-4-Stat (Stat-6) that is involved in IL-4-mediated transcription. Recently, Stat-6 knockout mice have been developed, and these mice are deficient in biological responses to IL-4 treatment (17).

In this report we provide evidence for IL-4-induced protein tyrosine dephosphorylation of the 85-kDa subunit of the PI 3-kinase (p85). Also, we report the association of SHP-1, previously known as PTP-1C, with the IL-4R and PI 3-kinase (p85). It therefore appears that the IL-4-induced protein tyrosine dephosphorylation may play a role in the IL-4-induced signaling pathways.

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centrifugation at 16,000 × g. The pelleted membrane fractions were washed once with isotonic buffer and solubilized in 1 × SDS-sample buffer, or the aliquots were frozen at −80 °C.

For the kinetics of IL-4-induced dephosphorylation, Ramos cells (5 × 10^6/ml) were serum-starved for 1.5 h before the addition of 10 ng/ml recombinant human IL-4. At indicated time points, cells were harvested and lysed in buffer containing 1% Nonidet P-40, 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 10 mM benzamidine, 100 µg/ml TPCK, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO₄, and 2 mM β-glycerol phosphate. The nuclei and cell debris were removed by centrifugation at 10,000 × g for 5 min. Equal amounts of cell extracts, as determined by BCA protein determination assay (Pierce), were prepared by the addition of an equal volume of 2 × SDS-PAGE buffer containing 5% β-mercaptoethanol and boiling for 2 min. The proteins were subjected to electrophoresis through 10% SDS-PAGE and electrotransferred to nitrocellulose membrane for Western blot analysis.

**Immunoprecipitations, Western Blot Analysis, and Densitometric Scanning—**Ramos cells at 5 × 10^6/ml were washed twice and lysed for 5 min in buffer containing 1% digitonin or 1% Nonidet P-40, 25 mM Tris-HCl (pH 7.8), 150 mM NaCl, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 10 mM benzamidine, 100 µg/ml TPCK, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO₄, and 2 mM β-glycerol phosphate. The nuclei and cell debris were removed by centrifugation at 10,000 × g for 5 min. The cell extracts were precleared once with protein A-Sepharose. After the addition of appropriate antibodies, the reactions were then allowed to continue for 4 h at 4 °C. To remove the antibody-antigen complexes, prewashed protein A-Sepharose (Pharmacia Biotech Inc.) or goat anti-mouse IgG-agarose (Sigma) was added to the reaction. After incubation for 1 h, the complexes were removed by centrifugation at 2,000 × g and washed extensively with buffer containing 0.5% digitonin or 0.5% Nonidet P-40, 25 mM Tris-HCl (pH 7.8), and 150 mM NaCl. Samples were prepared by the addition of an equal volume of 1 × SDS-PAGE buffer containing 2.5% β-mercaptoethanol to the washed resin and boiling for 2 min before electrophoresis through 10% SDS-PAGE.

For Western blot analysis, equal amounts of protein from detergent extracts, as determined by BCA protein assay (Pierce), or immunoprecipitated proteins were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked using buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 3% bovine serum albumin for 24 h at 4 °C. Monoclonal antibody to phosphotyrosine (anti-Tyr(P)) mAb 4G10. The results showed that the PI 3-kinase (p85) may participate in the IL-4-induced signaling, we speculated that the IL-4-induced dephosphorylated p85 polypeptide may in fact be the 85-kDa subunit of the PI 3-kinase (p85). To determine this, we performed Western blot analysis using mAb to PI 3-kinase (p85). Ramos cells were left untreated or were treated with 10 ng/ml IL-4. After a 1-min incubation, detergent extracts were prepared, and an equal amount of protein was subjected to SDS-PAGE and Western blot analysis using anti-phosphotyrosine mAb 4G10 (Fig. 2A, left panel). The blot was then stripped and subjected to immunodetection using anti-PI 3-kinase (p85) mAb. The results revealed that p85 migrated with identical mobility to PI 3-kinase (p85) (Fig. 2A, right panel), suggesting that the two polypeptides may be the same.

Subsequently, to confirm that IL-4 treatment could induce the dephosphorylation of PI 3-kinase (p85), we performed immunoprecipitation studies. Ramos cells were left untreated or were treated with 10 ng/ml IL-4 for 1 min. After preparation of detergent extracts and immunoprecipitation using anti-PI 3-kinase (p85), the proteins were resolved by SDS-PAGE. After electrotransfer, the proteins were visualized by Western blot analysis using anti-phosphotyrosine mAb 4G10 (Fig. 2B, left and middle panels). An identical blot was also probed with mAb to PI 3-kinase (p85) (Fig. 2B, right panel). The results revealed that the PI 3-kinase (p85) was dephosphorylated upon IL-4 treatment of Ramos cells. Interestingly, an IL-4-induced dephosphorylated polypeptide at approximately 170 kDa was also coimmunoprecipitated with PI 3-kinase (p85) (Fig. 2B, middle panel).

**IL-4 Treatment Can Induce Specific Translocation of SHP-1 to the Cellular Membrane Fraction—**Our data showed that PI 3-kinase (p85) was rapidly dephosphorylated by IL-4 treatment; therefore, we attempted to determine the phosphatase that may be involved in this event.

In concert with the signaling molecules, phosphatases are thought to play a key role in cellular proliferation and differentiation (19, 20). For example, both SHP-1 and SHP-2 have been shown to the cellular membrane fraction (21, 22).

Interestingly, IL-4R shares common signal transduction molecules such as 4PS and PI 3-kinase (p85) with the insulin receptor (10, 11, 23). Based on the reported similarities between IL-4R and the insulin receptor, we performed Western blot experiments using monoclonal antibodies to two candidate protein-tyrosine phosphatases, SHP-1 and SHP-2. Ramos cells were serum-starved for 1 h and treated with 5 ng/ml recombinant human IL-4 for 15 min before cell disruption and fractionation. The membrane and cytoplasmatic fractions were subjected to Western blot analysis using anti-SHP-1 and anti-SHP-2. The results revealed that the level of SHP-1 increased in the mem-
SHP-1 Associates with the IL-4R as Well as with PI 3-Kinase

The IL-4-induced dephosphorylated p85 represents the 85-kDa subunit of PI 3-kinase. A, Ramos cells were serum-starved and treated with 10 ng/ml IL-4. After 1 min posttreatment, cells were lysed, and equal amounts of the detergent lysates were subjected to 10% SDS-PAGE and Western blot analysis. First, the tyrosine-phosphorylated polypeptides were probed using anti-Tyr(P) (α p-Tyr) mAb [4G10] (left panel). After visualization by ECL, the blot was stripped and reprobed with mAb specific to the 85-kDa subunit of the PI 3-kinase (right panel). B, Ramos cells (5 × 10⁶) were serum-starved and either left untreated or treated with 10 ng/ml IL-4 for 1 min. Cells were then lysed in buffer containing Nonidet P-40. The lysates were then subjected to immunoprecipitation using anti-p85 (α p85) mAb. Left panel, cellular extract prepared from IL-4-treated and untreated Ramos cells probed with anti-Tyr(P) (α p-Tyr) mAb [4G10]. Middle panel, immunoprecipitation (IP) using anti-p85 (α p85) mAb probed with anti-Tyr(P) (α p-Tyr) mAb [4G10]. Right panel, a blot identical to the middle panel was probed with anti-p85 (α p85) mAb.

SHP-1 but not SHP-2 association with the cellular membrane fraction is increased after IL-4 treatment. Ramos cells were serum-starved for 1 h before treatment with 5 ng/ml IL-4. After 10 min of incubation, cellular membrane (Mb) and cytoplasmic fractions (Cyto) from equal numbers of cells (1 × 10⁶) were subjected to 12% SDS-PAGE and Western blot analysis using mAbs to SHP-1 (left upper panel) and to SHP-2 (right upper panel). The immunoblotted proteins were visualized by horseradish peroxidase-conjugated goat anti-mouse IgG and the enhanced chemiluminesence technique. The positions for doublet SHP-1 and SHP-2 bands are indicated. Jurkat cell extract (Transduction Laboratories) was used as a control. Data are representative of three different experiments.

DISCUSSION

We have used the highly IL-4-sensitive subtype (2G6.4C10) of the human Burkitt’s lymphoma B cell line Ramos. These cells are IgM⁺ and respond to IL-4 treatment by up-regulation of CD23 (24). In this paper we report the IL-4-induced tyrosine dephosphorylation of an 85-kDa polypeptide in Ramos cells.

This polypeptide appears to be identical to the 85-kDa regulatory subunit of PI 3-kinase (p85). Immunoprecipitation studies revealed that IL-4 treatment induced tyrosine dephosphorylation of PI 3-kinase (p85). Interestingly, an IL-4-induced tyrosine-dephosphorylated polypeptide at approximately 170 kDa was also coimmunoprecipitated with anti-PI 3-kinase (p85) (Fig. 2B, middle panel). At present the identity of the 170-kDa polypeptide (p170) is unknown, but it is possible that this molecule may represent insulin receptor substrate-2.

Also, IL-4 treatment of Ramos cells resulted in specific translocation of SHP-1 to a cellular membrane fraction. Zhao et al. (25) reported SHP-1 phosphorylation and translocation to the cellular membrane by phorbol ester treatment of a human promyelocytic leukemia cell line. Furthermore, by performing during the immunoprecipitation resulted in a significant increase in the association of SHP-1 with the IL-4R, suggesting an IL-4-induced association of SHP-1 with the IL-4R. Normal rabbit serum failed to immunoprecipitate SHP-1 (Fig. 4A, upper panel). To determine whether SHP-2 could also be coimmunoprecipitated with the IL-4R, the blot was then stripped and subjected to probing with mAb to SHP-2. The result revealed that SHP-2 was not associated with the IL-4R in Ramos cells (Fig. 4A, lower panel).

It has been reported that SHP-1 and SHP-2 play a role in the insulin receptor signaling (21, 22). Since we observed the IL-4-induced PI 3-kinase (p85) dephosphorylation and SHP-1 association with the IL-4R, we tested the possibility that SHP-1 or SHP-2 may also interact with PI 3-kinase (p85) in Ramos cells. IL-4-treated Ramos cell extracts were prepared as above, and immunoprecipitation was performed with anti-PT 3-kinase (p85) mAb. Western blot analysis of the immunoprecipitated proteins was performed using, first, anti-SHP-1 (Fig. 4B, upper panel) and then, after stripping with SHP-2 (Fig. 4B, lower panel), mAb. The results revealed that SHP-1 could specifically associate with PI 3-kinase (p85) in Ramos cells. Control mAb (IgG1) failed to immunoprecipitate SHP-1 (Fig. 4B).
IL-4 induces p85 dephosphorylation

**Fig. 4.** SHP-1 can be coimmunoprecipitated with IL-4R and p85. A, 5 x 10⁷ Ramos cells were harvested and lysed in lysis buffer containing digitonin. Immunoprecipitations in the presence or absence of IL-4 were performed using anti-IL-4R (aIL-4R) serum or normal rabbit serum (NRS). The protein-antibody complexes were removed by the addition of protein A-Sepharose. The immunoprecipitated proteins were resolved by 10% SDS-PAGE and subjected to Western blot analysis using anti-p85 (a p85) mAb (upper panel), then the blot was stripped and probed with mAb to SHP-2 (lower panel). The immunoblotted proteins were visualized by horseradish peroxidase-conjugated goat anti-mouse Ig and the enhanced chemiluminescence technique. Cell extract (Cell Ext.) was used as a control. B, Ramos cells were treated with IL-4 at 10 ng/ml. After 1 min, cells were lysed as above, and immunoprecipitations were performed using anti-p85 (a p85) mAb or isotype-matched IgG1 control. Immunoprecipitated proteins were resolved and visualized as above.

In immunoprecipitation studies we showed that SHP-1 could specifically associate with IL-4R as well as with PI 3-kinase (p85), suggesting a role for this phosphatase in IL-4-induced signaling pathways.

The biological responses induced by IL-4 are transmitted by high affinity IL-4R present in low abundance (10²–10³ copies/cell) on many hematopoietic and non-hematopoietic cells (26). Cross-linking and immunoprecipitation studies of cell surface radiiodinated cells showed that IL-4 treatment induces the association of IL-4R and the γ chain shared by the IL-2, IL-5, IL-7, and IL-9 receptors (12). Since the IL-4R chain does not possess any protein kinase activity, IL-4-induced signals are thought to be mediated through IL-4R-associated protein kinase(s).

Further evidence of IL-4-induced signal transduction was demonstrated by the observation that the 85-kDa subunit (p85) of PI 3-kinase was associated with the phosphorylated form of insulin receptor substrate-2 (4PS), suggesting that this interaction may be critical to IL-4-induced activation (11). Hou et al. (16) revealed the presence of an IL-4-activated transcription factor termed Stat-6. Stat-6 is phosphorylated upon IL-4 treatment, and the phosphorylated form of Stat-6 is translocated to the nucleus where it can bind and activate the transcription of IL-4-responsive genes.

In contrast to the reported IL-4-induced protein phosphorylation events, in vivo phosphorylation studies by Mire-Sluis and Thorpe (7) in human TF-1 cells showed the IL-4-induced dephosphorylation of an 80-kDa phosphotyrosine polypeptide. Pretreatment of cells with TGF-β, a known down-regulator of IL-4-induced IgE class switching (28, 29), blocked this dephosphorylation (30). Although it is not yet determined, it is possible that p80 (7) is the same polypeptide as p85, and the difference in the molecular weight is due to different electrophoresis conditions and molecular weight markers.

The discrepancy in the reports of the IL-4-induced signals may represent the differences between human and mouse systems. Alternatively, these differences in the IL-4-induced signals may be due to the different cell types used in the experiments.

In our experiments, we have detected rapid protein tyrosine dephosphorylation upon IL-4 treatment. At this point we do not know the exact physiologic relevance of the IL-4-induced PI 3-kinase (p85) dephosphorylation. But based on previous reports showing tyrosine phosphorylation of PI 3-kinase (p85) by growth factors such as platelet-derived growth factor and insulin (31, 32), it is possible that the IL-4-induced inhibition of leukemic and normal B cell proliferation (27) may be due to the IL-4-induced PI 3-kinase (p85) dephosphorylation. Alternatively, based on our preliminary results showing that Na⁺VO₄₃, a potent protein-tyrosine phosphatase inhibitor, could block the IL-4-induced IgE germline transcript expression, it is tempting to speculate that protein tyrosine dephosphorylation may be necessary for the IL-4-induced signaling that leads to IgE class switching in human B cells. Further experiments are necessary to determine the exact role of PI 3-kinase (p85) dephosphorylation in the IL-4-induced signaling events.

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