Protein-tyrosine Phosphatase Shp-1 Is a Negative Regulator of IL-4- and IL-13-dependent Signal Transduction*

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Binding of interleukin (IL)-4 to its transmembrane receptor results in the Jak-mediated tyrosine phosphorylation of a number of protein components of the IL-4 signaling cascade, including Jak1, Jak2, Jak3, Tyk2, IL-4Ra, IRS-1, IRS-2, and Stat6 in appropriate cell types. However, the protein-tyrosine phosphatases (PTPs) that dephosphorylate these proteins and terminate signaling remained unidentified. We have noted that IL-4-dependent activation of Stat6 is sustained longer in fibroblasts than in lymphoid cells. Because Shp-1, an SH2 domain-containing PTP, is expressed primarily in hematopoietic cells, we examined whether Shp-1 activity could regulate IL-4-dependent cell signaling. Expression of an Shp-1 transgene in NIH 3T3 cells markedly reduces both IL-4-dependent Stat6 activation and Stat6-mediated transcription of IL-4-responsive genes. In accord with this, IL-4 treatment of bone marrow-derived macrophages from viable motheaten mice that express subunit (Shp-1 null mutant) mice compared with normal pre-B cells derived from control animals. These data clearly implicate Shp-1 in the negative regulation of the IL-4/IL-13-activated Jak-Stat pathway.

Interleukin (IL)-4 activates the Janus kinase-signal transducer and activator of transcription (Jak-Stat) pathway leading to the transcriptional activation of specific genes including immunoglobulin-E, CD23, IL-1 receptor antagonist, class II major histocompatibility complex, suppressor of cytokine signaling (SOCS)-1, -2, and -3, cytokine-inducible SH2-containing protein CIS-1, IL-4 itself, and the α-subunit of IL-4 receptor (IL-4Ra) in appropriate cell types (1–6).

The cellular response to IL-4 is mediated through two types of receptor complexes (2, 3). The type I receptor comprises a heterodimer of IL-4Ra, a high-affinity IL-4-binding subunit and the gamma common (γc) chain (1–3, 6–9). The γc chain is shared by the cytokines IL-2, -4, -7, -9, and -15 (1–3, 6, 8, 9). IL-4Ra and γc physically associate with Jak1 and Jak3, respectively (2, 3, 6). Whereas IL-4Ra and Jak1 are expressed in all IL-4-responsive cell types, γc and Jak3 are expressed mostly in hematopoietic cells (2, 3, 6–9). In nonhematopoietic cells, IL-4 acts through the type II receptor complex comprised of IL-4Ra and IL-13Rα, a low-affinity binding protein for IL-13 (2, 3, 10, 11). IL-13 is believed to act through the type II IL-4 receptor complex (2, 3). Although Jak2 and Tyk2 are activated by IL-4 in certain cells, the IL-13Rα-specific Jak has not been identified (12, 13). Stat6 and IRS-1/IRS-2 are recruited to the activated IL-4Ra and become phosphorylated at critical tyrosine residues by the activated Jak1 molecules (2, 3, 14, 15). Dephosphorylation of these signaling proteins is a key mechanism for down-regulation of cytokine signaling; however, the protein tyrosine phosphatases (PTPs) that dephosphorylate the proteins in the IL-4/IL-13 signaling pathway remain to be identified.

We have proposed that more than one PTP is involved in the negative regulation of the Jak-Stat pathway activated by interferons (IFNs) and other cytokines (16–18). A global inhibition of PTP-activities by pervanadate (PV) results in the constitutive phosphorylation of Stat6, which depends on the activation of both Jak1 and IL-4Ra (18). This suggests that PTPs not only down-regulate cytokine-dependent activation of the Jak-Stat pathway but also inhibit the constitutive activation of the pathway (18). The SH2 domain-containing phosphatase Shp-1 has been implicated in the down-regulation of Jak-Stat signaling by a number of cytokines including IL-2, IL-3, erythropoietin (EPO), and IFN-α/IFN-β (19–24). However, the role of Shp-1 in IL-4/IL-13 signaling has not been defined. Here we present biochemical and genetic evidence that Shp-1 is involved in the negative regulation of IL-4/IL-13-dependent activation of Stat6 and subsequent induction of IL-4/IL-13-responsive gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Murine IL-4 and IL-13 were purchased from R & D Systems. Human IL-4 was a gift from the Schering-Plow Research Institute. Stat6 antibody was obtained from Santa Cruz Biotechnology Inc. Shp-1 antibody was purchased from Upstate Biotechnology, New York. Luciferase reporter constructs were obtained from Tularik Inc., California. NIH 3T3 and Daudi cell lines were grown as described elsewhere (18).

**Isolation of Bone Marrow-derived Macrophages**—Macrophages were prepared from bone marrow of C57BL/6J normal (+/+) and viable motheaten (me/me) mice as described (25). (L-929 cell-conditioned medium (LCM) as a source of macrophage colony-stimulating factor (M-CSF).) The bone marrow-derived macrophages (BMDM) were grown for 5–6 days in the presence of 30%.

**Isolation of Pre-B Cells**—The pre-B cell lines from motheaten (me/me) and normal littermate mice (+/+) were generated as described (26).

**Transfection of 3T3 Cells**—NIH 3T3 cells were transfected with pBluescript plasmid containing the murine Shp-1 cDNA and membrane fusion protein CIS-1, IL-4 itself, and the α-subunit of IL-4 receptor (IL-4Ra) in appropriate cell types (1–6).

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puromycin-resistant gene, and colonies were isolated by puromycin selection (27). Transient transfection of NIH 3T3 cells and luciferase assay were performed as described (18).

Electrophoretic Mobility Shift Assay and Western Blot Analysis—Preparations of pervanadate solution and whole cell extract (WCE) were described previously (17). Electrophoretic mobility shift assays (EMSA) were performed using a duplex N6-GAS (upper strand: 5’-GATCGCTCTTCTTCCCAGGAACTCAATG-3’) probe as described previously (17, 18, 28, 29). Western blot analysis was performed using WCE as described earlier (18).

RESULTS

Differential Activation of Stat6 by IL-4 in Hematopoietic Versus Nonhematopoietic Cells—To understand the negative regulation of IL-4 signaling, we determined the time course of IL-4-dependent Stat6 activation in hematopoietic and nonhematopoietic cell lines. Binding of IL-4 to its receptor on the cell surface results in Jak-dependent phosphorylation of tyrosine 641 residue of latent Stat6 and subsequent homodimerization of phospho-Stat6 molecules through the SH2 domain-phosphotyrosine interaction (28–30). Stat6-dimer translocates to the nucleus and binds to recognition site GAS and activates transcription (28–30). The phosphorylation and activation of Stat6 can be monitored by measuring the in vitro binding of Stat6 homodimer to N6-GAS, which is not recognized by any other activated Stat proteins (18, 28, 29).

Stat6 activation by IL-4 at varying times (0.5–8.0 h) was measured by EMSA in NIH 3T3 and Daudi cells (Fig. 1A). In both cell lines, Stat6 activation attained a similar maximum level at ~0.5 h of cytokine treatment. At 2 h, while more than 50% activation of Stat6 persisted in NIH 3T3 cells, Daudi cells retained less than 20% activated Stat6. Even after 8 h of IL-4 treatment, 30% activated Stat6 remained in NIH 3T3 cells compared with less than 10% activated Stat6 sustained in Daudi cells (Fig. 1B), suggesting that regulation of Stat6 activity in hematopoietic cells may be different from other cell types.

Two potential possibilities may account for this cell-type-specific differential kinetics of IL-4-dependent Stat6 activation. First, the cell lines that support sustained Stat6 activation in response to IL-4 or IL-13 may not express either the γc chain or Jak3 or both and, hence, are responsive to these cytokines through the type II IL-4 receptor complex, whereas in Daudi cells, IL-4 acts through the type I receptor complex (2, 6, 8, 9). Daudi cells did not respond to IL-13 (data not shown), indicating that the type II IL-4 receptor complex is not functional in these cells. Besides differential usage of the receptor complex, the number of functional receptors may contribute to the duration of IL-4 signaling in these two cell types (31). The second possibility is that differential expression of Shp-1, an SH2 domain-containing protein-tyrosine phosphatase that is predominantly expressed in hematopoietic cells and has been shown to down-regulate other cytokine signals, may account for the difference in Stat6 activation kinetics (20–24). In support of the latter possibility, Western blot analysis shows that Shp-1 protein is barely detectable in NIH 3T3 cell extract while Daudi cells express high levels of Shp-1, and IL-4 treatment did not alter the levels of Shp-1 protein in these cell lines (Fig. 2A).

Shp-1 Down-regulates IL-4/IL-13-dependent Jak-Stat Signaling—To examine further the involvement of Shp-1 in the IL-4-dependent signaling of Stat6, we overexpressed murine Shp-1 in NIH 3T3 cells. Stable cell lines were isolated by drug (puromycin) selection, and a representative clone that expressed Shp-1 at high levels (Fig. 2A) was selected for further analyses. In response to IL-4 or IL-13, the DNA-binding activity of Stat6 was markedly reduced in Shp-1-overexpressing cells compared with the corresponding control cell line (Fig. 2B). It is interesting to note that PV-mediated activation of Stat6 is also significantly (>3-fold) reduced in cells expressing the Shp-1 transgene compared with the control cells (Fig. 2B).
Negative Regulation of IL-4/IL-13 Signaling by Shp-1

These results suggest that Shp-1 is involved in the down-regulation of IL-4/IL-13-responsive gene expression through the down-regulation of the IL-4/IL-13-dependent Stat6 activation.

To confirm the involvement of Shp-1 in IL-4/IL-13 signaling, we determined cytokine-dependent Stat6 activation in cells derived from Shp-1-deficient mice. The viable strain of motheaten (me/m-e) mice have a homozygous mutation in the Shp-1 gene that results in the expression of Shp-1 protein with substantially reduced PTP activity (32). Upon IL-4 treatment, BMDM from me/m-e mice show remarkably enhanced DNA-binding activity of Stat6 compared with BMDM from control littermates (Fig. 3, A and B). These data clearly indicate that Shp-1 is involved in the down-regulation of the IL-4/IL-13-dependent Stat6 activation.

Shp-1 associates with IL-2Rα, IL-3Rα-chain, and EPO receptor upon treatment of cells with the respective cytokine (19–24). Therefore, Shp-1 fulfills the function of an upstream PTP that we have designated PTP-x that dephosphorylates the activated Jaks and the receptor subunits (17, 18). We have shown previously that PTP-x not only down-regulates cytokine-induced signals but also inhibits the constitutive signals that result from the basal activity of the cytokine receptor-associated Jak(s) (18). However, in the IL-2, IL-3, and EPO-system, Shp-1 does not constitutively associate with the receptors (19–23). If Shp-1 constitutively associates with IL-4 receptor subunits and inhibits the basal activity of IL-4 receptor-associated Jak(s), one would expect to see constitutive Stat6 activation in cells that are derived from the mev mice. These mice have a frameshift mutation in the Shp-1 gene and do not express detectable Shp-1 activity (32). To test for constitutive Stat6 activation, pre-B cell lines were established from mev and control mice (26). We found that pre-B cells from mev mice do not support constitutive activation of Stat6, and even a brief exposure to IL-4 does not enhance Stat6 activation in mev pre-B cells compared with control pre-B cells (Fig. 3C). However, after 4 h of IL-4 treatment, the activated Stat6 level is significantly increased in mev pre-B cells over that of the control cells (Fig. 3C). These results further confirm that Shp-1 is involved in the down-regulation of IL-4 signaling but suggest that an additional PTP activity is also involved in the negative regulation of IL-4- and IL-13-dependent Jak-Stat signaling.

To determine whether Shp-1 expression regulates the transcription of IL-4/IL-13-responsive genes, an IL-4/IL-13-responsive luciferase reporter construct TPU474 (29) and a Shp-1 expression plasmid (27) were cotransfected into NIH 3T3 cells. IL-4 treatment (20 ng/ml for 15 h) resulted in more than an 8-fold increase in luciferase activity in the absence of exogenous Shp-1 expression (Fig. 4). On cotransfection with the Shp-1-expression plasmid, the TPU474-derived IL-4-dependent as well as IL-4-independent (basal) luciferase activity was ablated in these cells (Fig. 4). These results clearly indicate that Shp-1 overexpression can down-regulate the transcription of IL-4/IL-13 responsive genes. Taken together, our results show for the first time that Shp-1 is a negative regulator of IL-4/IL-13-responsive gene expression through the down-regulation of cytokine-activated levels of phospho-Stat6 molecules.

DISCUSSION

In general, cytokine signals are limited in both magnitude and duration. Tyrosine dephosphorylation of signaling proteins
has been recognized as a key mechanism underlying the negative regulation of cytokine signaling. Based on our previous findings that the IFN-γ-dependent Jak-Stat signaling is differentially inhibited by two different groups of PTP inhibitors, we have postulated that at least two PTP activities are involved in the negative regulation of the Jak-Stat signaling (17, 18). The activated Jak5 and cytokine receptors are proposed to be inactivated by an upstream phosphatase PTP-x, whereas PTP-y dephosphorylates activated Stats (possibly in the nucleus). Using the IL-4/IL-13 system as a paradigm, we have recently shown that PTP-x not only down-regulates cytokine-induced signals, but inhibits spontaneous (constitutive) activation of the Jak-Stat pathway that results from the basal kinase activity of the cytokine receptor-associated Jak molecules (18).

The PTPs that regulate either IFN-γ- or IL-4-dependent Jak-Stat signaling have remained unknown. The SH2 domain-containing PTPs are plausible candidates for PTP-x, and two mammalian SH2 domain-containing PTPs, Shp-1 and Shp-2, have been identified (24). Shp-2 binds to IRS-1 and IRS-2 through an SH2 domain-phosphotyrosine interaction (2, 3, 14, and 15). Activated IL-4Rα recruits IRS proteins and activates the phosphatidylinositol 3-kinase pathway, leading to proliferation of responsive cells (2, 14, 15). Shp-2 is expressed in all cell types and is implicated in the positive regulation of cell signaling by receptor tyrosine kinases, including the receptors for insulin, insulin-like growth factor, platelet-derived growth factor, and epidermal growth factor, where it primarily functions as an adapter protein (2, 15, 24, 33, 34). In contrast, Shp-1 is involved in the negative regulation of IL-2, -3, -4, and -6, and IFN-α/IFN-β signaling (19–24). Using cells overexpressing, under-expressing, or deficient in Shp-1 activity, we have demonstrated herein that IL-4-dependent Stat6 activation and subsequent activation of IL-4-responsive gene expression are down-regulated by Shp-1 in different cell types.

A question that remains to be addressed is whether Shp-1 functions as PTP-x or PTP-y in the previous model (17, 18). It has recently been reported that a gain-of-function point mutation in IL-4Rα (glutamine 576 to arginine) leads to an enhanced IL-4 signaling in human cells (35). A phosphopeptide derived from the mutant IL-4Rα (encompassing the point mutation) has reduced binding affinity for Shp-1 compared with the wild-type peptide as determined in an in vitro binding assay (35). These findings suggest that Shp-1 may associate with the IL-4Rα and function as PTP-x in IL-4/IL-13 signaling cascade. However, Shp-1 deficiency in cells does not result in the constitutive activation of IL-4 receptor complex (Fig. 3). Therefore, Shp-1 is unlikely to associate constitutively with IL-4 receptor complex and inhibit the basal kinase activity of associated Jak. This suggests that another PTP also functions as PTP-x in the IL-4/IL-13 system (17, 18). Although we have provided compelling evidence that Shp-1 is a negative regulator of IL-4/IL-13-dependent Jak-Stat signaling, an identification of Shp-1 sub-strate(s) in this signaling cascade is necessary to understand mechanisms underlying Shp-1-mediated down-regulation of IL-4/IL-13-dependent gene expression in both hematopoietic and nonhematopoietic cells.

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