Regulation of the Dephosphorylation of Stat6

PARTICIPATION OF TYR-713 IN THE INTERLEUKIN-4 RECEPTOR α, THE TYROSINE PHOSPHATASE SHP-1, AND THE PROTEASOME*

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Signal transducer and activator of transcription 6 (Stat6) plays an important role in interleukin (IL)-4-induced responses. To analyze the regulation of Stat6 phosphorylation, cells were cultured in the continuous presence of IL-4 or after a pulse and washout. In the continual presence of IL-4, Stat6 remained phosphorylated for an extended period. After IL-4 removal and inhibition of the Janus family kinase, tyrosine-phosphorylated Stat6 decayed at a rate dependent upon the length of IL-4 stimulation. The decay of tyrosine-phosphorylated Stat6 was similar in the presence or absence of either cycloheximide or actinomycin D. In the absence of functional Src homology-containing phosphatase-1 (SHP-1), the early loss of tyrosine-phosphorylated Stat6 was substantially reduced. Furthermore, the rate of loss of tyrosine-phosphorylated Stat6 in cells expressing a mutation of the human IL-4 receptor α in the immunoreceptor tyrosine-based inhibitory motif sequence (Y5F) was dramatically decreased compared with wild-type cells. The early rate of decay was similar in the presence or absence of MG132, an inhibitor of the proteasome, but the later rate of decay was decreased 5-fold. These results suggest that the loss of tyrosine phosphorylation of Stat6 is regulated by the action of SHP-1 and the proteasome but is not dependent on new protein synthesis.

Interleukin (IL)-4 is a multifunctional cytokine that plays several critical roles in the regulation of immune responses. IL-4 initiates these cellular responses by binding to and signal-
family members modulate signaling by several mechanisms that include blocking access of the Stats to receptor-binding sites and ubiquitination of the Janus kinases and their subsequent targeting to the proteasome (19). Recently, it has been shown that the de-ubiquitinating enzyme DUB-2 can prolong Stat5 phosphorylation induced by IL-2 (20). The SOCS family is also believed to regulate the Jak/Stat pathway by interfering with the function of the Jak kinases (17, 18, 21). Furthermore, IL-2-induced SOCS-2 has been shown to inhibit IL-2 responses in a classical negative feedback loop (22). Overexpression of SOCS-1 in a murine B-cell line resulted in decreased Jak1 phosphorylation, decreased Stat6 phosphorylation, and decreased CD23 expression (15). SOCS proteins may be acting in a classic negative feedback loop to suppress the Jak-mediated tyrosine phosphorylation of Stat6.

Although there is considerable understanding of the IL-4-mediated tyrosine phosphorylation of Stat6, little is known about the regulation of its inactivation. Therefore, to characterize the regulation of Stat6 tyrosine phosphorylation, we analyzed the rate of dephosphorylation after IL-4 removal and Jak kinase inhibition in a variety of cell types. The results presented herein indicate that the loss of tyrosine-phosphorylated Stat6 is regulated by the rapid action of a tyrosine phosphatase and the action of proteasome-mediated protein degradation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human epithelial lung carcinomas Calu-3 and A549 were maintained in Dulbecco’s modified Eagle’s medium (BioWhitaker, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Human monocytic THP-1 cells were maintained in RPMI (BioWhitaker, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 0.05 mM 2-mercaptoethanol. IL-3-dependent myeloid cell line 32D expressing the huIL-4Rα wild-type (WT) or a Y173F mutation (23, 24) were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% WEHI-3B supernatant. Murine SHP-1 WT and mutant cells are IL-3-dependent primitive hematopoietic precursor cells isolated from the yolk sac (9.0–9.5 days post-coitum) of WT or mcre/mcre embryos and immortalized by a homeobox-containing gene Hox11 (25, 26). They were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Murine B-cell lymphoma M12.4.1 and murine T-hybridoma A1.1 were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 0.05 mM 2-mercaptoethanol. IL-3-dependent myeloid cell line 32D expressing the huIL-4Rα wild-type (WT) or a Y173F mutation (23, 24) were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% WEHI-3B supernatant. Murine SHP-1 WT and mutant cells are IL-3-dependent primitive hematopoietic precursor cells isolated from the yolk sac (9.0–9.5 days post-coitum) of WT or mcre/mcre embryos and immortalized by a homeobox-containing gene Hox11 (25, 26). They were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, and 10% WEHI-3B supernatant.

Reagents—Recombinant murine IL-4 and recombinant huIL-4 were obtained from R & D Systems (Minneapolis, MN). AG490 was obtained from Calbiochem and was stored at −20 °C in MeSO. MG132 was obtained from Biomol (Plymouth Meeting, PA) and was stored at −80 °C in MeSO. Cycloheximide was obtained from Sigma and was dissolved in DMSO to use. Actinomycin D was obtained from Biowhi and was stored at −20 °C in ethanol. Lactacystin, clasto-lactacystin β-lactone, Z-LLF-CHO, Z-Val-Phe-CHO, and ALLM were all obtained from Calbiochem and were dissolved in MeSO prior to use. Sodium orthovanadate was obtained from ICN (Aurora, OH) and was dissolved in PBS prior to use. anti-IL-4 monoclonal antibody, 1B11, was a gift from W. Watson and Dr. W. E. Paul (Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda).

Lymphocyte Isolation—Human blood was diluted in PBS, and peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque. Cells were activated with plate-bound OKT3 (gift from Dr. Yufang Shi) for 72 h at 37 °C to obtain primary activated T-lymphocytes. Human B-cells were purified from peripheral blood mononuclear cells with the B-cell isolation kit according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA).

Subcellular Fractionation—Briefly, cells were stimulated with IL-4 (10 ng/ml) at 37 °C and subjected to a pulse-washout experiment. The cells were washed three times in complete 37 °C medium, resuspended at 2 × 10^6 cells/ml, and then cultured in the absence of cytokine. Subcellular fractionation was performed using NE-FER nuclear and Cytoplasmic extraction reagents according to the manufacturer’s protocol (Pierce).

Immunoprecipitation and Immunoblotting—Briefly, cells were stimulated with IL-4 (10 ng/ml) at 37 °C and subjected to a pulse-washout experiment. The cells were washed three times in complete 37 °C medium, resuspended at 2 × 10^6 cells/ml, and then cultured with sodium orthovanadate, MG132, lactacystin, actinomycin D or cycloheximide at 37 °C. The reaction was terminated by dilution in ice-cold PBS, and cells were lysed in HEPES buffer (50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 10 mM pyrophosphate, 1 mM Na2VO4, and protease inhibitors). The soluble fraction was immunoprecipitated with anti-Stat6 (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were washed in lysis buffer and solubilized in SDS sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride membrane. The membranes were then probed with a monoclonal anti-phospho-tyrosine antibody, RC20-HRPO (Transduction Laboratories, Lexington, KY). The bound antibody was detected using enhanced chemiluminescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Where indicated, the blots were stripped and probed with control antibodies. Band intensities were analyzed using the public domain software NIH Image. Data were graphed, and slopes were analyzed using CA-Cricket Graph III.

RESULTS

Kinetics of Dephosphorylation of Stat6—Even though Stat6 is one of the major signaling molecules activated by IL-4, there has been little research into the kinetics and regulation of Stat6 tyrosine phosphorylation. It has been shown that B-cells must be in contact with IL-4 for an extended period to become activated, and the minimum time for half-maximal activation of biological responses is 12 h (27). It has also been shown that transient Stat6 activation after exposure to IL-4 is not sufficient to induce CD23 expression (28). In addition, B-cells produce little IgE unless IL-4 is present for at least 2 days of culture (29). These responses require the activity of Stat6. It has been reported that Stat6 DNA binding activity is rapidly activated in B-cells by IL-4 and remains that way for up to 24 h (6). To analyze the kinetics and longevity of Stat6 tyrosine phosphorylation, we analyzed several different B-lymphocyte lines. M12.4.1 cells (a murine B-lymphoma), Ramos cells (a human B-cell line), and normal human B-cells purified from peripheral blood were treated with IL-4 for various times, and tyrosine phosphorylation of Stat6 was analyzed by immunoprecipitation and immunoblotting. In the continuous presence of IL-4, Stat6 was rapidly phosphorylated and remained phosphorylated for extended periods (up to 16 h in this experiment) (Fig. 1A). These results are consistent with previous studies showing Stat6 activation in murine B-cells for 24–48 h after IL-4 treatment (6) and suggest that if IL-4 is continuously present, the overall pool of Stat6 remains tyrosine-phosphorylated.

As long as IL-4 is present, a constant level of tyrosine-phosphorylated Stat6 is maintained. This implies the presence of an equilibrium between the tyrosine phosphorylation of Stat6 and the dephosphorylation of phospho-Stat6. The tyro-
Tyrosine phosphorylation of Stat6 by AG490. The level of tyrosine-phosphorylated Stat6 was assessed at various time points following cytokine removal and addition of AG490 (Fig. 2A). After removal of IL-4, the levels of detectable Stat6 tyrosine phosphorylation declined over time in a similar fashion in the presence or absence of AG490 indicating that the removal of IL-4 was sufficient to stop significant forward phosphorylation. Similar results were obtained when an anti-IL-4 antibody, 11B11, was used to terminate IL-4 signaling. Although it is known that the Jak kinases drive the tyrosine phosphorylation of Stat6, it is not known what mechanisms are involved in the dephosphorylation of Stat6. To determine whether Stat6 could remain phosphorylated in the absence of IL-4 signaling the forward reaction, we first determined the concentration of AG490, an inhibitor of the Jak kinases, capable of blocking the tyrosine phosphorylation of Stat6 (Fig. 1B). We found that 25 μM AG490 blocked the tyrosine phosphorylation of Stat6 by 83%, and 100 μM AG490 blocked tyrosine phosphorylation of Stat6 by 100%.

To determine whether Stat6 could remain phosphorylated in the absence of IL-4 signaling, M12.4.1 cells were treated with IL-4 for either 15 min or 2 h; the cytokine was washed away, and the cells were then cultured in the absence of cytokine but in the presence or absence of 50 μM AG490 for the indicated times. Stat6 was immunoprecipitated from whole cell lysates using anti-Stat6 antibody. Immunoprecipitates were analyzed by Western blot using the anti-phosphotyrosine antibody RC20-HRPO. The blot was stripped and reprobed using an anti-Stat6 antibody.

Results suggest that as long as IL-4 is present, Stat6 remains tyrosine-phosphorylated, and after IL-4 removal, the tyrosine phosphorylation of Stat6 declines over time. The results also suggest that the half-life of tyrosine-phosphorylated Stat6 can be dependent upon the length of IL-4 treatment in several cell types.

It is possible that the regulation of tyrosine-phosphorylated Stat6 differs between cytoplasmic and nuclear compartments. Therefore, M12.4.1 cells were stimulated with IL-4, and cytoplasmic and nuclear fractions were prepared by subcellular fractionation. In the absence of IL-4 signaling, Stat6 does not become tyrosine-phosphorylated, and there is noticeably more total Stat6 protein in the cytoplasmic compartment compared with the nuclear compartment (Fig. 3A, 1st and 2nd lanes). Upon IL-4 signaling, Stat6 becomes tyrosine-phosphorylated which is evident in both compartments (Fig. 3A, 3rd and 4th lanes), but there was much more tyrosine-phosphorylated Stat6 in the cytoplasmic compartment than the nuclear compartment. There was also an increase of total nuclear Stat6 protein after IL-4 treatment over the amount observed in the unstimulated nuclear fraction, which indicates some movement of Stat6 from the cytoplasm into the nucleus upon IL-4 signaling.

### Footnote

3 E. M. Hanson, unpublished observations.
Cells were treated with 10 ng/ml murine or human IL-4 for either 15 min or 2 h; the cytokine was washed away, and the cells were cultured in the absence of cytokine. Densitometry was performed using NIH Image 1.62 in order to calculate the ratio of phosphorylated Stat6 protein to total Stat6 protein. CA-Cricket Graph III was used to generate the best-fit lines. Results are representative of three experiments.

The half-lives of Stat6 phosphorylation in various cell types

| Cell line name | Cell type or tissue source | Half-life IL-4 for 15 min | Half-life IL-4 for 2 h |
|----------------|---------------------------|--------------------------|-----------------------|
| M12.4.1        | Murine B-cells            | 95                       | 65                    |
| U937           | Human monocytic           | 100                      | 65                    |
| THP-1          | Human monocytic           | 70                       | 60                    |
| Primary T cells| Human blood               | 150                      | 100                   |
| A1.1           | Murine T hybridoma        | 60                       | 60                    |
| A549           | Human epithelial, lung carcinoma | 135           | 135                   |
| Calu-3         | Human epithelial, lung carcinoma | 100           | 100                   |

To analyze the decay of tyrosine-phosphorylated Stat6 in the nuclear compartment, M12.4.1 cells were treated with IL-4 for either 15 min or 2 h; the cytokine was washed away; the cells were then cultured in the absence of cytokine, and subcellular fractionation was performed to obtain the nuclear fraction of the cells. The level of tyrosine-phosphorylated Stat6 was assessed at various time points following cytokine removal and subcellular fractionation (Fig. 3B). As explained previously, the scans of the Western blots were used to determine the ratio of tyrosine-phosphorylated Stat6 to total Stat6 protein. The nuclear lysate was probed with Sp1, a nuclear protein, to confirm proper subcellular fractionation. After removal of IL-4, the level of detectable Stat6 tyrosine phosphorylation declined over time in the nuclear compartment in a similar fashion to the cytoplasmic compartment (compare Figs. 2 and 3). However, the half-life of nuclear tyrosine-phosphorylated Stat6 after a 15-min or 2-h stimulation with IL-4 was similar ($t_{1/2} = 1.2$). These results suggest that the difference in the half-lives of tyrosine-phosphorylated Stat6 between the 15-min ($t_{1/2} = 1.8$) and 2-h ($t_{1/2} = 1.2$) stimulation observed in Fig. 2 corresponds to a mechanism specific to the cytoplasmic compartment.

The decay of tyrosine-phosphorylated Stat6 was similar in the presence or absence of cycloheximide. Subsequently, IL-4 was removed, and the cells were cultured in the absence of cytokine but in the presence or absence of cycloheximide. As shown in Fig. 4, we found that the decay of tyrosine-phosphorylated Stat6 was similar in the presence or absence of cycloheximide. These results indicate that induction of new protein synthesis is not required for the decay of tyrosine-phosphorylated Stat6. Furthermore, cycloheximide did not alter the changes in the half-life of cytoplasmic tyrosine-phosphorylated Stat6 induced by longer IL-4 treatment.

We also found that SOCS-1 mRNA was induced by IL-4 stimulation in M12.4.1 cells and that this induction was blocked by actinomycin D (Fig. 5A). Similar results were obt-
Cycloheximide (0, 10, or 20 μg/ml) was added to cultures in the absence of cytokine, and then subcellular fractionation was performed to obtain cytoplasmic and nuclear compartments. The loss of tyrosine-phosphorylated Stat6 was prevented to the same degree as WT huIL-4Rα in IL-3-dependent 32D cells when human IL-4 was continuously present. To test the contribution of this tyrosine residue to the loss of tyrosine-phosphorylated Stat6, we stimulated 32D WT and Y5F cells with human IL-4 for 2 h; the cytokine was removed; the cells were cultured in the absence of cytokine, and then subcellular fractionation was performed to obtain the cytoplasmic and nuclear compartments. Strikingly, the rate of loss of tyrosine-phosphorylated Stat6 in the Y5F mutant cells was dramatically decreased compared with that of the WT cells in both the cytoplasmic and nuclear compartments (Fig. 7). These results indicate that the ITIM-tyrosine, Tyr-5, in the IL-4Rα is important in regulating the loss of tyrosine-phosphorylated Stat6.

It has been suggested that SHP-1 may negatively regulate the Jak family tyrosine kinases (35). It is possible that the results presented so far could be accounted for by continual Jak kinase activity and concomitant new Stat6 phosphorylation. Therefore, to verify that we are analyzing only the decay of tyrosine-phosphorylated Stat6 and not residual Jak activity, SHP-1 me(me) cells and 32D Y5F cells were stimulated with murine or human IL-4 as appropriate for 2 h; the cytokine was removed, and the cells were cultured in the absence of cytokine but in the presence or absence of 50 μg/ml AG490. The level of tyrosine-phosphorylated Stat6 was assessed at various time points following cytokine removal. The rate of loss of tyrosine-phosphorylated Stat6 in each of the mutant cell lines was equal in the presence or absence of AG490 (Fig. 8). Similarly, AG490 had no effect on the loss of tyrosine-phosphorylated Stat6 in the WT cell lines.3 The differences observed in Figs. 6 and 7 between WT and mutant cells are not simply due to residual Jak activity.

MG132 Enhances the Longevity of Tyrosine-phosphorylated Stat6—Another mechanism that could cause the decay of tyrosine-phosphorylated Stat6 is the degradation of proteins involved in the Jak/Stat pathway in the cytoplasm or nucleus. Inhibitors of proteasome activity have been shown to prolong the activation of Stat1 in response to interferon-γ (36, 37). One group found that in the continuous presence of cytokine, the down-regulation of phosphorylated Stat4, Stat5, and Stat6 was inhibitable by the proteasome inhibitors MG132 and lactacystin (38). To assess the contribution of the proteasome on the dephosphorylation of Stat6, M12.4.1 cells were treated with IL-4 for 2 h; the cytokine was removed, and the cells were cultured in the absence of cytokine but in the presence or absence of MG132 (Fig. 9A). The early rate (t ≤3 h) of decay was similar in the presence (slope = −0.21) or absence (slope = −0.20) of MG132. However, after 3 h, the levels of tyrosine-phosphorylated Stat6 were stabilized by MG132 (the slope of the line shifted from −0.21 to a slope of −0.04) (Fig. 9B). There was no effect of this inhibitor on the levels of total Stat6 protein. Similar results were obtained using other proteasome inhibitors including lactacystin (a specific proteasome inhibitor

4 H. Dickensheets and R. P. Donnelly, unpublished observations.
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Fig. 5. SOCS-1 mRNA induced by IL-4 but blocked by actinomycin D. A, M12.4.1 cells were treated with 10 ng/ml murine IL-4 in the presence or absence of 10 μg/ml actinomycin D (Act.D) for 3 h. Total RNA was isolated and analyzed by Northern blotting for SOCS-1. The blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, M12.4.1 cells were treated with 10 ng/ml murine IL-4 in the presence or absence of actinomycin D (0, 5, or 10 μg/ml) for 2 h. The cytokine was washed away, and the cells were cultured in the absence of cytokine but in the presence or absence of actinomycin for the indicated times. As in Fig. 2, the scans of the Western blots were used to determine the ratio of tyrosine-phosphorylated Stat6 to total Stat6 protein. Results are representative of three experiments.

Fig. 6. SHP-1 is important for the early loss of tyrosine-phosphorylated Stat6 in hematopoietic precursor cells. SHP-1 wild-type (SHP-1 WT) and mutant me/me' (SHP-1 MT) cells were treated with 10 ng/ml murine IL-4 for 2 h; the cytokine was washed away; the cells were cultured in the absence of IL-4 for the times indicated, and subcellular fractionation was performed using the Pierce NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer’s protocol. Cytoplasmic and nuclear lysates were immunoprecipitated and immunoblotted. As in Fig. 2, the scans of the Western blots were used to determine the ratio of tyrosine-phosphorylated Stat6 to total Stat6 protein.

Fig. 7. Mutation of tyrosine 5 in the human IL-4Rα chain decreases the rate of decay of tyrosine-phosphorylated Stat6 in 32D cells. 32D cells expressing WT huIL-4Ra and 32D cells expressing huIL-4Ra with a mutation in tyrosine 5 (Y5F) were treated with 10 ng/ml human IL-4 for 2 h; the cytokine was washed away; the cells were cultured in the absence of IL-4 for the times indicated, and subcellular fractionation was performed using the Pierce NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer’s protocol. Cytoplasmic and nuclear lysates were immunoprecipitated and immunoblotted. As in Fig. 2, the scans of the Western blots were used to determine the ratio of tyrosine-phosphorylated Stat6 to total Stat6 protein.

that does not inhibit any other proteases), clasto-lactacystin β-lactone (a more potent form of lactacystin), or Z-LLF-CHO (another potent and cell-permeable proteasome inhibitor) but not with specific inhibitors of calpain or cathepsins B or L such as Z-Val-Phe-CHO (inhibits calpain I and II) or ALLM (inhibits calpain I and II and cathepsins B and L).3 These results confirm that tyrosine-phosphorylated Stat6 is partially regulated by the action of proteasome-mediated protein degradation.

DISCUSSION

The IL-4-induced activation of Stat6 is clearly involved in many biologic responses related to allergy. Recent evidence has emphasized the importance of regulatory pathways that function to modulate intracellular signals initiated by IL-4 and other cytokines. In the last few years, the majority of research on the actions of cytokines has focused on the mechanisms by which they initiate signaling pathways. However, it is known that the actions of cytokines can be limited in both magnitude and duration. This could be especially important in the control of cytokine action in vivo. Even though it is apparent that tyrosine-phosphorylated Stat6 plays an important role in many IL-4-induced responses, there has not been much research on determining the mechanisms that regulate the longevity of Stat6 activation. Therefore, a clear understanding of the downregulation of tyrosine-phosphorylated Stat6 is essential.

In the continuous presence of IL-4, Stat6 is rapidly phosphorylated and remains that way for an extended period. However, after the IL-4 stimulus is removed, the levels of tyrosine-phosphorylated Stat6 decline over time. These results indicate that when IL-4 is present to signal there is equilibrium between the Jak-dependent forward reaction of tyrosine phosphorylation and the reverse reaction of tyrosine dephosphorylation (Fig. 10). By including AG490 in our experiments, we would inhibit any residual forward activity and specifically focus on the possible mechanisms of the tyrosine dephosphorylation of Stat6 (the reverse reaction).

A 2-h stimulation with IL-4 resulted in a surprisingly rapid loss of tyrosine-phosphorylated Stat6 in some cell types. This observation raised the question of whether IL-4 stimulation was causing the synthesis of regulatory proteins that could in turn act negatively upon IL-4 signaling. A natural candidate for a regulatory protein acting upon IL-4 signaling would be a member of the SOCS family. However, it was found that SOCS-1 does not play a major role in the turnover of tyrosine-phosphorylated Stat6 in M12.4.1 cells. At first glance, this may seem to contradict previous findings on the involvement of SOCS proteins in IL-4 signaling (15, 16, 39). Research thus far indicates that SOCS proteins are important for IL-4 signaling in the forward reaction of the Stat6 activation cycle and likely act to suppress directly Jak activity (Fig. 10). For example, it has been demonstrated that interferons inhibit IL-4-induced activation of Stat6 and Stat6-dependent gene expression at least in part by inducing expression of SOCS-1 (16). However,
our results suggest that SOCS-1 does not play a significant role in Stat6 tyrosine dephosphorylation, the reverse reaction. Several studies have indicated that tyrosine phosphatases play a role in regulating Stats. Whereas the IL-4R/H9251 does contain an ITIM consensus sequence (a conserved Tyr-713 surrounded by a proline-rich sequence) (23) shown to interact with SHP-1 in vitro, its role in regulating Stat6 phosphorylation remains unclear (33). One report stated that Stat6 activation by IL-4 is significantly enhanced in pre-B-cells derived from motheaten (SHP-1 mutant) mice, compared with normal pre-B-cells derived from control animals (13). However, hematopoiesis is greatly altered in the motheaten mice, which affects the cellular phenotype and activation state of the isolated cell populations (40). Therefore, it is not known whether the differences seen between the SHP-1 mutant mice and the wild-type mice are due to differences in SHP-1 activity or defects in the cells themselves. Another report (14) stated that SHP-1 is not a negative regulator of IL-4 signaling in bone marrow-derived mast cells. Their research revealed that mast cells derived from motheaten mice do not exhibit enhanced or prolonged Stat6 activation in the continuous presence of IL-4 when compared with wild-type cells. We found that sodium orthovanadate had an early effect in stabilizing the tyrosine-phosphorylated form of Stat6. Furthermore, in the absence of functional SHP-1, tyrosine-phosphorylated Stat6 was quite stable.
IL-4 during the forward reaction of the cycle of tyrosine phosphorylation of Stat6. These results suggest that SHP-1 may also play a role in the forward reaction in the cycle of Stat6 tyrosine phosphorylation.

To confirm SHP-1 as a player in the cycle of tyrosine phosphorylation of Stat6, cells expressing the huIL-4Rα with a mutated 5th tyrosine (ITIM site) were analyzed. It was possible that the mutation of this tyrosine would result in the loss of recruitment of tyrosine phosphatases, such as SHP-1, to the receptor because the site would no longer become phosphorylated upon ligand binding. Strikingly, the rate of loss of tyrosine phosphorylation of Stat6 in the Y5F mutant cells was dramatically decreased compared with that of the WT cells. Therefore, the 5th tyrosine residue in the IL-4Rα chain is important for regulation of Stat6. Our previous results (24) suggest that the 5th tyrosine does not play a role in the forward reaction. However, by specifically analyzing the dephosphorylation phase by treating cells with AG490, we revealed that this residue plays a dramatic role in the reverse reaction.

There have been many studies on the IL-4-induced activation of Stat6; however, each study has usually been performed with a single cell type (5, 6, 23, 24). The activation of Stat6 in monocytic THP-1 cells was examined after IL-4 treatment, and it was found that the activation was rapid and transient (5). Another group (13) noted that IL-4-dependent activation of Stat6 is sustained longer in fibroblast cells than in lymphoid cells. These reports suggest that tyrosine-phosphorylated Stat6 may have a short half-life in some cell types, although a direct comparison of different cell types has not been performed before. Potential differences may have direct relevance to the potency of in vivo effects of IL-4. The cells of hematopoietic lineage all seem to follow a similar trend, a long stimulation resulting in a rapid loss of tyrosine-phosphorylated Stat6 in the cytoplasm, although this is not evident in the nucleus. SHP-1, shown herein to be important in the loss of tyrosine-phosphorylated Stat6, is predominantly expressed in cells of hematopoietic lineage (41). However, all cell types did show a loss of tyrosine-phosphorylated Stat6 after removal of cytokine. Therefore, there must be other tyrosine phosphatases playing a role in the regulation of Stat6 in non-hematopoietic cells.

In contrast to the phosphatase inhibitors, the proteasome inhibitors had effects at later time points (t ≥ 3 h). MG132, lactacystin, and other inhibitors stabilized the tyrosine-phosphorylated form of Stat6 after an initial loss. It should be noted that there was no loss of total Stat6 protein in these experiments, so it is likely that the proteasome is not acting on Stat6 itself. It may be acting on other as yet uncharacterized proteins involved in the dephosphorylation phase. The data herein present the possibility that two independent phases contribute to the loss of tyrosine-phosphorylated Stat6. The protein-tyrosine phosphatases may play early roles (t ≤ 1 h) in the loss of tyrosine-phosphorylated Stat6, and the proteasome may play a more important role in the later loss (t ≥ 3 h) of tyrosine-phosphorylated Stat6. Therefore, proteasome-mediated protein degradation is another possible mechanism that could play a role in the reverse reaction of the cycle of tyrosine phosphorylation of Stat6.

Recent studies (28) have suggested that the amount of cytosolic tyrosine-phosphorylated Stat6 may influence the amount of Stat6 able to translocate into the nucleus to activate gene expression. Therefore, the regulation of the amount of cytosolic tyrosine-phosphorylated Stat6 would be important. If tyrosine-phosphorylated Stat6 is depleted from the cytoplasmic pool by phosphatases or by proteasome-mediated protein degradation, then the nuclear pool of tyrosine-phosphorylated Stat6 would be affected. It has been proposed that tyrosine-phosphorylated Stat6 is imported into the nucleus at the same rate it is exported (28). Therefore, a depletion of tyrosine-phosphorylated Stat6 in the cytoplasm would mean that little or no tyrosine-phosphorylated Stat6 could be imported into the nucleus. At the same time, the Stat6 in the nucleus would still be exported out to the cytoplasm at the same rate such that a depletion in the cytoplasm would eventually lead to a depletion in the nucleus. In addition, the action of nuclear tyrosine phosphatases, including SHP-1, would enhance the loss of nuclear tyrosine-phosphorylated Stat6 (43–46).

The importance of these Stat6-regulating mechanisms on the magnitude of in vivo responses is not yet clear. The production of cytokines is not similar to production of endocrine hormones, but rather more similar to the production of neurotransmitters (47). For example, when helper T-cells interact with B-cells, IL-4 is secreted in a directional manner in a small, confined space created by close apposition of the B- and T-cell membranes, the so-called immunological synapse (47, 48). This cellular interaction would in theory be of relatively short duration in vivo (49), perhaps making the exposure of target cells to IL-4 more akin to a pulse than to a continuous exposure. This would also be true for IL-4 released from mast cells or basophils in response to FcεRI cross-linkage. If, indeed, the in vivo situation is analogous to an in vitro IL-4 pulse, then any mutations or alterations in the dephosphorylation of Stat6 should have a potent effect on IL-4-mediated responses. Of special interest are the polymorphisms of the human IL-4Rs in the cytoplasmic domain that have been associated with allergy and asthma (33, 50). Analyses of the ability of these polymorphic receptors to signal in various transfusion systems have not yet revealed any major changes in signaling efficiency, although the previous studies (24) were done in the continuous presence of IL-4. In this study, we found that differences between the Y5F mutant and WT were not revealed until we utilized an IL-4 pulse treatment protocol. Therefore, it will be important to analyze specifically the impact of IL-4R polymorphisms on regulation of Stat6 dephosphorylation in normal cell types.

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