DNA BINDING ACTIVITY OF CYTOPLASMIC PHOSPHORYLATED STAT6
IS MASKED BY AN INTERACTION WITH A DETERGENT-SENSITIVE FACTOR

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*This work was supported by a grant from the American Heart Association #0060337B. M.D. was supported in part by a Procter Scholarship from Cincinnati Children’s Hospital Medical Center. S.E. was supported in part by a Summer Research Fellowship from the University of Cincinnati Medical School.
RUNNING TITLE: Detergent-Sensitive Inhibitor of Cytoplasmic Activated Stat6
**Summary**

Signal transducer and activator of transcription (Stat) 6 is vital to IL-4 and IL-13 responses and the generation of Th2 immunity. We investigated the cellular location of phosphorylated Stat6 and Stat6 DNA binding activity in A201.1 murine B cells and primary splenocytes. Phosphorylated Stat6 was present in cytoplasmic and nuclear extracts from IL-4 treated cells. Confocal microscopy confirmed the presence of phosphorylated Stat6 in the cytoplasm of IL-4-treated cells. In contrast, Stat6 DNA binding activity was present in nuclear extracts, but not in cytoplasmic extracts. Thus, cytoplasmic extracts from IL-4-stimulated cells were devoid of Stat6 DNA binding activity despite the presence of phosphorylated Stat6. Addition of cytoplasmic extracts to nuclear extracts did not inhibit Stat6 DNA binding present in the nuclear extracts. Detergent treatment restored Stat6 DNA binding activity in cytoplasmic extracts of IL-4-stimulated cells. Thus, DNA binding activity of cytoplasmic phosphorylated Stat6 is masked by a factor dissociable by detergent treatment.
Introduction

Signal transducers and activators of transcription (Stat) proteins are mediators of transcription of several cytokine-inducible genes. These proteins exist as latent cytoplasmic monomers until cytokine stimulation, whereupon they are phosphorylated by the Janus family of kinases (JAKs), dimerize, and translocate to the nucleus. Once in the nucleus, activated Stats bind specific canonical DNA elements and initiate cytokine-specific gene transcription (1). To date, six Stat proteins have been described (2) and of these, only Stat6 has been shown to be specifically activated by IL-4 and IL-13 (3-6).

It is clear from the characterization of Stat6-deficient mice that Stat6 is required for IL-4- and IL-13-dependent gene induction. Stat6-/- mice are deficient in their ability to mount a Th2 response following nematode infection, fail to produce IgE, and do not develop airway hypersensitivity following antigen challenge (3-9). The importance of Stat6 in human allergic disease is supported by the fact that Stat6 expression is increased in the bronchial epithelium of humans with severe asthma (10) and that polymorphic variants of Stat6 have been reported to associate with atopic asthma (11).

Activation of Stat6 following receptor engagement involves multiple steps and is dependent on critical structural elements within Stat6. First, association of Stat6 with tyrosine phosphorylated regions of cytokine receptors occurs via its SH2 domain. Subsequently, Stat6 is phosphorylated on Y641, a residue critical for Stat6 function (12), and phosphorylated Stat6 monomers dimerize via their respective amino terminal domains with Y641 of one monomer associating with the SH2 domain of the other (13). Activated Stat6 dimers then translocate to the nucleus, bind specific consensus sequences and promote transcription of downstream genes. The crystal structure of Stat1-DNA complexes revealed that dimeric interactions between the two
respective SH2 domains was critical for the formation of the DNA binding region (14). Nuclear translocation of Stat6 depends on its phosphorylation and dimerization (12,15), however, the elements responsible for nuclear localization of Stat6 have not been identified.

Although Stat6 activation in response to IL-4 and IL-13 has been well documented, the molecular mechanisms responsible for regulation of Stat signaling remain poorly understood. A number of negative regulators of the JAK-Stat signaling pathway have been described, including Suppressor Of Cytokine Signaling (SOCS) and Protein Inhibitors of Activated Stat (PIAS). SOCS proteins form a negative feedback loop whereby SOCS genes are induced following cytokine stimulation and, once produced, inhibit cytokine signaling (16,17). SOCS-1 has been shown to bind and inhibit JAK kinases, but this may not be true of all SOCS family members. A number of PIAS proteins have recently been described (18,19), which bind specifically to phosphorylated Stat dimers and prevent them from binding DNA (20). Recently, arginine methylation was shown to be another mechanism by which Stats can be regulated (21). Methylation of Stat1 prevented its association with PIAS1, thereby increasing the amount of Stat1 available for DNA binding and gene induction. No PIAS protein has yet been described for Stat6.

Another important point of regulation of the Stat pathway that remains ill-defined is the control of Stat migration between the cytoplasm and the nucleus. Herein, we tracked the migration of phosphorylated Stat6 and Stat6 DNA binding activity between the cytoplasm and the nucleus in order to determine the kinetics of nuclear translocation and identify possible targets for regulation.
Experimental Procedures

Cells and reagents

A201.1 murine B cells, a gift from Dr. Gregg Milligan (Children's Hospital Medical Center, Cincinnati, Ohio), are derived from the parent line A20. The cells are B220⁺, IgG⁺, Ia⁺, IgA⁻, IgM⁻, and IgD⁻ and were originally derived from a BALB/c mouse. Cells were maintained in complete RPMI-1640 (cRPMI), consisting of RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Bio-Whittaker, Walkersville, MD), 100 u/ml penicillin and 100 μg/ml streptomycin (Bio-Whittaker), and 50 μM -mercaptoethanol (Sigma, St. Louis, MO). Murine splenocytes were obtained from BALB/c mice (Jackson Laboratory, Bar Harbor, ME) and were purified by filtration over nylon mesh followed by erythrocyte lysis. Recombinant mouse IL-4 was purchased from R&D Systems Inc, (Minneapolis, MN). Polyclonal anti-Stat6 antibody, S-20, was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and anti-phospho STAT6 was purchased from Cell Signaling Technology (Beverly, MA). The anti-phosphotyrosine monoclonal antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY).

Confocal Microscopy

A201.1 cells in culture were washed in cold PBS with 1% FBS, fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA), and then stained with anti-phospho-Stat6 (Tyr641) (Cell Signaling Technology, Beverly, MA) or rabbit IgG followed by FITC-Goat F(ab)2 anti-rabbit (Southern Biotechnology, Birmingham, AL) in a solution containing PBS, 1% FBS, 0.2% saponin, and 3 % cold fish gelatin (Sigma-Aldrich, St. Louis, MO). After the final wash, the cells were resuspended in a minimal volume of Vectashield (Vector Labs, Burlingame, CA), placed on a slide, covered with a coverslip, and the edges sealed
with nail polish. Cells were observed on a Leica DM IRBC confocal microscope (Leica Microsystems, Heidelberg, Germany).

**Electrophoretic Mobility Shift Assay (EMSA)**

Cells (2.5 x 10⁶) were stimulated with IL-4 (10 ng/ml) for 15 minutes, pelleted by centrifugation at 10,000 g and reconstituted in lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% NP-40, 1.0 mM DTT, 0.5 mM PMSF). Lysates were centrifuged at 10,000 g for 5 minutes at 4 °C and supernatants containing the cytoplasmic extracts were removed. Pelleted nuclei were reconstituted in nuclear extract buffer (20 mM Hepes pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 1.0 mM DTT, 0.5 mM PMSF). EMSA were performed as previously described (22,23). For the detergent treated samples, cytoplasmic and nuclear extracts were prepared for EMSA as described above. Ten percent deoxycholic acid (DOC) (Sigma) was added to achieve the noted final concentration and the samples were incubated on ice for 15 minutes. Radiolabeled Stat6 probe and nonionic detergent Igepal CA-630 (Sigma) were then added, to a final concentration of 1.2% Igepal. Samples prepared without DOC were incubated with the radiolabeled Stat6 probe only. Samples were incubated on ice for 15 minutes and then resolved by polyacrylamide gel electrophoresis as previously described (22,23)

**Immunoprecipitation and immunoblotting**

A201.1 cells (2 x 10⁷) were pelleted by centrifugation at 20,000 g at 4 °C and Stat6 was immunoprecipitated as previously described (23). Proteins were transferred to nitrocellulose membranes and blocked overnight in block solution (20 mM Tris pH 7.4, 150 mM NaCl, 3.1% bovine serum albumin, 0.1% polyethylene glycol 20,000). Membranes were probed with anti-phosphotyrosine monoclonal antibody PY20 (Transduction Labs, Lexington, KY) or anti-Stat6
polyclonal antibody. Bound antibodies were detected by incubation with anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Transduction Labs), followed by enhanced chemiluminescence using ECL substrate (Amersham, Arlington Heights, IL).
Results

*Phosphorylated Stat6 is present in the cytoplasm A201.1 cells following IL-4 treatment but is incapable of binding DNA*

We first investigated the cellular distribution of phosphorylated Stat6 in A201.1 cells following stimulation with IL-4 (Figure 1A). Phosphorylated Stat6 was present in cytoplasmic and nuclear extracts as early as 5 minutes after IL-4 treatment, and the levels were maximal by 15 minutes. As expected, total Stat6 remained constant in the nuclear and cytoplasmic extracts (Figure 1A). The same extracts were then analyzed for the presence of Stat6 DNA binding activity (Figure 1B). Stat6 DNA binding activity was detected in the nuclear extracts after one minute of IL-4 stimulation and reached maximal levels by 15 minutes. Stat6 DNA binding activity was not detected in cytoplasmic extracts at any time point.

In order to further examine the subcellular localization of phosphorylated Stat6 in A201.1 cells and confirm the presence of phosphorylated Stat6 in the cytoplasm, confocal microscopy was performed on fixed, permeabilized cells following IL-4 stimulation (Figure 2). As depicted in Figure 2, phosphorylated Stat6 was not detectable in unstimulated cells, but was evident after 5 minutes of IL-4 stimulation. The majority of the phosphorylated Stat6 was in the cytoplasm, although in some cells, some nuclear staining was also detected. After 15 minutes of IL-4 stimulation, the phosphorylated Stat6 had migrated to the nucleus, in agreement with the observation that maximal Stat6 binding activity was detected in the nucleus after 15 minutes of IL-4 stimulation. When cells were stimulated for 15 minutes with IL-4, washed, and then incubated in media lacking IL-4 for 1 hour, the level of phosphorylated Stat6 staining was similar to that seen in unstimulated cells consistent a half-life of activated Stat6 of less than 1 hour. These data confirm the presence of active phosphorylated Stat6 in the cytoplasm of cells.
Cytoplasmic extracts do not inhibit Stat6 DNA binding activity

Our data established that although phosphorylated Stat6 was present in the cytoplasm, it was unable to bind cognate DNA sequences. One possibility for this was that cytoplasmic extracts contained an inhibitor capable of interfering with the DNA binding activity of phosphorylated Stat6. In order to address this, we examined the ability of cytoplasmic extracts to inhibit the Stat6 DNA binding present in nuclear extracts (Figure 3). Increasing amounts of cytoplasmic extracts were added to nuclear extracts of activated A201.1 cells and then the mixtures were assayed for DNA binding activity. As expected, DNA binding activity was present in the nuclear extracts, but not the cytoplasmic extracts. Addition of cytoplasmic extracts to the nuclear extracts had no effect on the DNA binding activity of the nuclear extracts even at a 10-fold excess of cytoplasmic extracts. Thus, cytoplasmic extracts do not contain a transferable inhibitor of Stat6 DNA binding activity.

Detergent treatment unmasks cytoplasmic Stat6 DNA binding activity

Our data demonstrates that phosphorylated Stat6 is present in the cytoplasm of IL-4 treated A201.1 cells, that this “activated” Stat6 is incapable of binding DNA, and that there is not a transferable inhibitor of Stat6 DNA binding in the cytoplasm. We next sought to determine if there is a tightly bound inhibitor of activated Stat6. To investigate this possibility, we treated cytoplasmic and nuclear extracts from A201.1 cells with the ionic detergent DOC to separate any dissociable inhibitor from the phosphorylated Stat6. The extracts were then treated with a non-ionic detergent, NP-40, to inactivate the DOC and EMSA analysis was performed (Figure 4A). Cytoplasmic or nuclear extracts derived from cells that were not treated with IL-4 did not display Stat6 DNA binding activity in the presence or absence of detergent. As expected, nuclear extracts derived from IL-4 treated cells demonstrated Stat6 DNA binding in the absence and
presence of detergent treatment. There was a slight change in mobility of Stat6 in the presence of detergent. Cytoplasmic extracts showed no significant Stat6 DNA binding in the absence of detergent. However, following detergent treatment, robust Stat6 DNA binding was observed in the cytoplasmic extracts. This demonstrates that in the cytoplasm there is a tightly bound inhibitor of activated Stat6 that can be removed or is denatured or degraded by detergent treatment.

To verify that these findings were not peculiar to the murine A201.1 line, we repeated the detergent treatment experiments with freshly isolated murine splenocytes. The results obtained mirrored those observed with A201.1 cells. IL-4 stimulated cytoplasmic extracts derived from splenocytes lacked Stat6 DNA binding activity, while detergent treatment unmasked DNA binding activity in these extracts (Figure 4B).

Since DNA binding activity was restored in detergent-treated cytoplasmic extracts, we reasoned that the inhibitor might be unbound in these extracts. Thus, we investigated whether detergent-treated cytoplasmic extracts could inhibit nuclear DNA binding activity. We utilized varying ratios of the cytoplasmic:nuclear extracts prepared from A201.1 cells, and even with a 10-fold excess of the detergent-treated cytoplasmic extracts, no inhibition was observed (data not shown). Thus, the inhibitor is no longer able to inhibit Stat6 activity after detergent treatment, supporting that it is either degraded or denatured. Alternatively, residual detergent in the extracts may inhibit the interaction, although this is less likely since it is diluted in the EMSA buffer.
Discussion

Cytokine signaling involves many steps: the production of cytokine, the expression of its receptor, the recruitment of kinases, the activation of transcription factors followed by their translocation to the nucleus and binding to cognate DNA sequences. Each one of these layers of activation provides the potential for regulation. Herein, we describe the first report of a detergent-sensitive factor that blocks the DNA binding activity of activated Stat6 in the cytoplasm of IL-4 stimulated A201.1 cells. Our data establish the presence of phosphorylated Stat6 in the cytoplasm of activated cells by western blot and confocal microscopy. However, the cytoplasmic extracts failed to demonstrate DNA binding despite the presence of phosphorylated Stat6. There was no evidence of a transferable inhibitor of Stat6 in the cytoplasm of activated A201.1 cells, as the addition of cytoplasmic proteins did not inhibit DNA binding of nuclear extracts. Strikingly, Stat6 DNA binding activity was restored to cytoplasmic extracts of IL-4-stimulated A201.1 cells by detergent treatment. This observation was confirmed in primary and cultured cells, confirming that it is not restricted to one cell type.

These data support the presence of a factor that is tightly bound to activated Stat6 in the cytoplasm of cells and prevents its association with its cognate DNA sequence. The biologic function of this factor is not yet clear. It may act as an inhibitor or regulator of Stat6 function. Alternatively, the factor may act as a chaperone protein, which may be required for the nuclear localization of Stat6. The nuclear import requirements for Stat6 remain to be identified. Importin proteins have been shown to be involved in the nuclear localization of Stat1. Interestingly, in addition its role in nuclear localization, importin 5 has been reported to competitively block the Stat1 DNA binding site (24). Nuclear localization occurs both at the level of nuclear import and nuclear export (25). Although a leucine-rich nuclear export signal
(NES) has recently been implicated in the nuclear localization of Stat1 (26), no nuclear localization signals have been identified in the Stats. We have previously shown that the maintenance of Stat6 activity requires cycling of Stat6 with nuclear export (22).

Based on our observations, we propose the following model of the life cycle of activated Stat6 (Figure 5). Upon IL-4 binding, Stat6 is rapidly activated by tyrosine phosphorylation of Y641 in the cytoplasm. Site-specific phosphorylation of Stat6 activates reciprocal interactions between 2 Stat6 molecules, whereby the SH2 domain of one monomer couples with the phosphotyrosine of the other, and vice versa, leading to dimerization of Stat6 (2). The Stat6 dimers should then be capable of binding DNA. However, we found that phosphorylated Stat6 detected in the cytoplasm was incapable of DNA binding. It is unlikely that the phosphorylated monomers were unable to dimerize since the phosphotyrosines and SH2 domains, which have been shown to be critical for dimerization by mutational analyses (12,13) were intact. Thus, we propose that a chaperone-like molecule binds the phosphorylated Stat6 dimers in the cytoplasm, and either directly or indirectly masks their DNA binding domain. Once the activated dimers have translocated to the nucleus, this chaperone molecule likely dissociates from the activated Stat6.

We have performed experiments attempting to co-precipitate the associated factor with Stat6, but these experiments have not yet been successful. Additional experiments aimed at identifying this factor are underway. One potential candidate molecule for this chaperone may be a PIAS-like protein. PIAS proteins exist in the cytoplasm and nucleus (18) and have been reported to bind exclusively to phosphorylated Stat dimers and block their DNA binding domain (20), which would be consistent with our model. Overexpression of PIAS3 and PIAS1 proteins has been reported to inhibit Stat3 (18) and Stat1 activation (19), respectively, as assayed by DNA
binding and gene activation. However, an alternative explanation is that the natural function of PIAS or a PIAS-like protein is to transport Stat dimers to the nucleus. In this case, overexpression of this protein might lead to inhibition of Stat function because an excess of PIAS relative to the Stat dimers would favor PIAS proteins remaining bound to the activated dimers. The generation of PIAS knockout mice will distinguish between these possibilities.

In summary, our data establishes that the DNA binding ability of phosphorylated Stat6 in the cytoplasm is inhibited by a detergent-sensitive factor. This is the first report of such a factor, and this represents a novel mechanism by which the JAK/Stat signaling pathway can be regulated.
Acknowledgements

We are grateful to Drs. Christopher Karp, Jeffrey Whitsett, and Marc Rothenberg for critical review of this manuscript; and to Dr. Timothy Weaver and Ledung Ray for help with the confocal microscopy. We thank Connie Petitt for excellent secretarial support.
References


Footnotes

The abbreviations used are: Stat, Signal transducer and activator of transcription; JAK, janus kinase; SOCS, Suppressor Of Cytokine Signaling; PIAS, Protein Inhibitors of Activated Stat; DOC, deoxycholic acid; EMSA, electrophoretic mobility shift assay; NES, nuclear export signal.
Figure Legends

Figure 1. Phosphorylated Stat6 is present in nuclear and cytoplasmic extracts, but only nuclear extracts demonstrate Stat6 DNA binding. **Panel A.** A201.1 cells were treated with IL-4 (10ng/ml) for the indicated times. Nuclear and cytoplasmic extracts were prepared and analyzed for the presence of phosphorylated Stat6 by immunoprecipitation using an anti-Stat6 antibody followed by western blotting with an antiphosphotyrosine antibody. In order to ensure equal loading of the lanes, the blot was then stripped and reprobed with anti-Stat6 antibody. **Panel B.** The same nuclear and cytoplasmic extracts prepared from A201.1 cells for the experiment above were analyzed for the presence of Stat6 DNA binding activity by EMSA.

Figure 2. Confocal microscopy of A201.1 cells demonstrates cytoplasmic phosphorylated Stat6. A201.1 cells were stimulated with IL-4 (10ng/ml) for the indicated times, fixed, permeabilized, and stained with an antibody specific to phosphorylated Stat6 followed by a FITC conjugated secondary antibody. Cells were then examined by confocal microscope, with FITC fluorescence indicated by yellow. Representative images of 3 independent experiments are shown.
**Figure 3.** Cytoplasmic extracts do not inhibit Stat6 DNA binding in nuclear extracts. A201.1 cells were treated with IL-4 (10ng/ml) for 15 minutes and then nuclear and cytoplasmic extracts were prepared. Increasing amounts of cytoplasmic extracts (0-50 µg protein) were added to 5 µg of nuclear extracts and Stat6 DNA binding was assayed by EMSA. As a negative control, the cytoplasmic extracts alone were also assayed for Stat6 DNA binding. This experiment is representative of 3 separate experiments.

**Figure 4.** Detergent treatment of IL-4 stimulated A201.1 cytoplasmic extracts unmasks Stat6 DNA binding activity. Cytoplasmic and nuclear extracts were prepared from IL-4 (10 ng/ml) treated and untreated A201.1 cells (Panel A), and murine splenocytes (Panel B). Extracts were treated with detergent as indicated and Stat6 DNA binding activity analyzed by EMSA. Stat6 mobility was slightly altered in the presence of detergent.

**Figure 5.** Proposed model of Stat6 regulation.
Figure 1

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Figure 2

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Figure 3

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Stat6

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Figure 4

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DNA binding activity of cytoplasmic phosphorylated stat6 is masked by an interaction with a detergent-sensitive factor

J. Biol. Chem. published online May 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301094200

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