PIASx Is a Transcriptional Co-repressor of Signal Transducer and Activator of Transcription 4*

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In response to interleukin 12 (IL-12) stimulation, a latent cytoplasmic transcription factor, Stat4 (signal transducer and activator of transcription 4), becomes tyrosine-phosphorylated and translocates into the nucleus where it binds to DNA to activate transcription. Co-factors that can directly bind and regulate Stat4 activity have not been described. We report here that PIASx, a member of the protein inhibitor of activated STAT (PIAS) family, is a negative regulator of Stat4. PIASx becomes associated with Stat4 following IL-12 stimulation in vivo. PIASx inhibits IL-12-stimulated and Stat4-dependent gene activation in human T cells. PIASx does not inhibit the DNA binding activity of Stat4. Instead PIASx is present in the Stat4-DNA binding complex. Finally the inhibitory activity of PIASx on Stat4-mediated gene activation is abolished by the histone deacetylase inhibitor trichostatin A. Our results suggest that PIASx may function as a co-repressor of Stat4.

STATS (signal transducers and activators of transcription) are a family of latent cytoplasmic transcription factors. Upon cytokine stimulation, STATs become tyrosine phosphorylated and translocate into the nucleus where they bind to DNA to activate transcription (1–3). The STAT signaling pathways can be negatively regulated at multiple steps. In the nucleus, two major mechanisms have been described that modulate the activity of STATs. First, STATs can be inactivated in the nucleus by protein tyrosine phosphatases. It has recently been documented that TCF4, the nuclear isoform of T cell protein tyrosine phosphatase (4), is responsible for the dephosphorylation of Stat1 and Stat3 in the nucleus (5). Second, the PIAS (protein inhibitor of activated STAT) family of proteins has been suggested to regulate the transcriptional activity of STATs in the nucleus. Four members of the PIAS family have been described including PIAS1, PIAS3, PIASy, and PIASx (6). It has been shown that PIAS1 and PIAS3 interact with Stat1 and Stat3, respectively, in response to cytokine stimulation. PIAS1 and PIAS3 inhibit Stat1- and Stat3-mediated transcription by blocking their DNA binding activity (7, 8). PIASy can also associate with Stat1, but it inhibits Stat1 transcription without affecting the DNA binding activity of Stat1 (9). The role of PIASx in STAT signaling has not been characterized.

IL-12, produced by activated macrophages or dendritic cells, plays an essential role in the development of Th1 cells. Stat4 is activated by IL-12 stimulation (10, 11). Consistent with the biochemical studies, gene targeting analysis has demonstrated an essential role of Stat4 in Th1 development (12, 13). In addition to IL-12, IFN-α/β has also been shown to induce the tyrosine phosphorylation of Stat4 (14, 15). Most recently, the activation of Stat4 by IFN-α/β has been shown to be critical for IFN-γ production during viral infection (16). Despite a clear role of Stat4 in IL-12 and IFN-α/β signaling, cofactors that can directly interact and modulate Stat4 activity have not been described.

We report here that PIASx interacts with Stat4 in vivo following IL-12 stimulation of T cells. PIASx forms a complex with activated Stat4 binding to DNA. The IL-12-stimulated Stat4-dependent transcription can be inhibited by PIASx. The inhibitory activity of PIASx on Stat4 is diminished by an inhibitor of histone deacetylase (HDAC). Our results suggest that PIASx is a transcriptional co-repressor of Stat4.

**EXPERIMENTAL PROCEDURES**

Plasmids, Antibodies, and Cell Lines—GST-PIASx was constructed as described previously (8). FLAG-PIASx was constructed by insertion of the human PIASx into the BamHI and SalI sites of pCMV-FLAG vector. Anti-PIASx antibody was raised against a recombinant fusion protein of GST with COOH-terminal residues 476–572 of human PIASx. Anti-PY20 was obtained from Santa Cruz Biotechnology, Inc. Anti-Stat4 was a gift from J. Darnell. Kit225/K6 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 10 units/ml recombinant human IL-2 at 5% CO2. Human Th1 clone 3F6 cells were maintained as described previously (17).

Immunofluorescence Analysis—The cellular localization of PIASx and PIASβ was analyzed by immunofluorescence as described previously (9).

Luciferase Assay—Kit225/K6 T cells (8 × 105) were transfected by electroporation at 270 V, 975 microfarads using a Bio-Rad electroporator. Cells were electroporated with endotoxin-free preparations of 2 μg of pCMV, 5 μg of 4xIRF1 (18), 10 μg of pCMV-Stat4 (10), and various amounts of FLAG-PIASx or FLAG-PIAS3 plasmids (7). Cells were incubated with or without IL-12 (60 ng/ml, R&D Systems) for 16 h and analyzed for luciferase activity as described previously (14). The relative luciferase units were corrected for the expression of β-galactosidase.
To examine the cellular localization of PIASx, human 2TGH fibroblasts were transfected with expression vectors encoding FLAG-PIASxα and FLAG-PIASxβ followed by immunofluorescence analysis. Both isoforms of PIASx are localized in the nucleus as distinct nuclear bodies (Fig. 1B). Interestingly the pattern of nuclear body formation of these two isoforms appears to be different. While PIASxα exists as small distinct nuclear dots, PIASxβ is expressed as larger but fewer nuclear bodies.

To examine whether PIASx interacts with a STAT protein in vivo, we performed a co-immunoprecipitation analysis. Protein extracts from human NK cells unstimulated or stimulated with IFN-α were analyzed by Western blot using antibodies that can specifically recognize tyrosine-phosphorylated Stat1 and Stat3 proteins. It has been shown previously that IFN-α can also induce the tyrosine phosphorylation of Stat4, and the activation of Stat4 by IFN-α plays an important role in the induction of IFN-γ during viral infection (14, 16). To examine the tyrosine phosphorylation of Stat4, the same protein extracts were immunoprecipitated by anti-Stat4 antibody followed by Western blot with anti-phospho-Stat4 antibody. Stat4 was found to be present in PIASx immunoprecipitates of IFN-α-stimulated NK cells (Fig. 1C, middle panel). Neither Stat1 nor Stat3 was present in the immunoprecipitates from cells treated with IFN-α, Stat1 and Stat3 antibodies (Fig. 1C, right panel). These results suggest that PIASx is associated with Stat4 but not Stat1 or Stat3. Similar to previously described PIASx-STAT interactions (6), the PIASx-Stat4 association occurs only in cells stimulated with cytokines.

Stat4 plays a critical role in IL-12 signaling pathway. To evaluate whether Stat4 associates with PIASx following IL-12 stimulation, human Kit225 T cells, which express functional IL-12 receptors, were used for analysis. Protein extracts of untreated or IL-12-treated Kit225 T cells were immunoprecipitated with anti-PIASx antibody followed by immunoblot analysis with anti-PIASx antibody. Stat4 was found to be present in PIASx immunoprecipitates of IFN-α-stimulated NK cells (Fig. 1C, middle panel). Neither Stat1 nor Stat3 was present in the immunoprecipitates from cells treated with IL-12-induced luciferase activity. In contrast, co-transfection of PIASx and Stat4 together with Stat4 and increases Stat4 expression about 20-fold. Co-transfection of PIASxα at various concentrations efficiently blocked IL-12-stimulated gene activation (Fig. 2A).

To examine the specificity of PIASx on IL-12 signaling, similar luciferase assays were performed in the presence of PIASxα or PIAS3 in Kit225 T cells. Consistently PIASx blocked IL-12-induced luciferase activity. In contrast, co-transfection of
various amounts of FLAG-PIASx T cells were transfected with Stat4 and 4xIRF1 reporter together with untreated. lactosidase was used to correct for differences in transfection efficiency. IL-12 (0.1, 0.5, and 1.0 μg) for 6 h and harvested for luciferase assays. Co-transfected /H9252/ 293T cells were transiently transfected with Stat1 and 3xLy6E reporter with various amounts of FLAG-PIAS1 (5, 15, and 50 ng), FLAG-PIASy (5 and 15 ng), or FLAG-PIASxα (5, 15, and 50 ng). 24 h post-transfection, cells were either untreated or treated with IFN-γ (5 ng/ml) for 6 h and harvested for luciferase assays. Co-transfected β-galactosidase was used to correct for differences in transfection efficiency.

PIAS3 at various concentrations had no effect on IL-12-induced gene activation (Fig. 2B). To further test the specificity of PIASx in STAT signaling, the effect of PIASx on Stat1-mediated gene activation was examined. 293T cells were transiently transfected with a Stat1 luciferase reporter (3xLy6E) and Stat1 in the presence of various amounts of FLAG-PIAS1, FLAG-PIASy, or FLAG-PIASxα. Consistent with the previous studies, PIAS1 and PIASy, two known inhibitors of Stat1 (8, 9), effectively repressed Stat1-dependent gene activation (Fig. 2C). In contrast, PIASxα had no significant effect on Stat1-mediated gene activation. These results suggest that PIASxα is a specific negative regulator of Stat4.

PIASx Does Not Inhibit the DNA Binding Activity of Stat4—To understand the molecular mechanism by which PIASx inhibits Stat4-mediated gene activation, we analyzed the effect of PIASx on the DNA binding activity of Stat4 by electrophoretic mobility shift assays. Nuclear extracts were prepared from Kit225 cells untreated or treated with IL-12 and analyzed by EMSA using the STAT-binding site from IRF1 gene as the probe. IL-12 treatment induced the formation of a specific shifted band on EMSA using the STAT binding sequence from Kit225 nuclear extracts (data not shown). These results indicate that the formation of the PIASx-Stat4 complex does not interfere with the DNA binding activity of Stat4.

PIASx Forms a DNA Binding Complex with Stat4—Certain protein complexes are unstable under EMSA conditions. Therefore, we investigated whether PIASx can still interact with Stat4 in the presence of DNA using oligonucleotide pull-down assays. The untreated and IL-12-treated extracts of human 3F6 Th1 clones were mixed with a biotin-tagged STAT-binding oligonucleotide. Proteins bound to DNA were pull down with streptavidin-agarose beads followed by immunoblot analysis with anti-Stat4 or anti-PIASx antibody. As expected, Stat4 was found to bind to DNA in IL-12-stimulated extracts. Interestingly PIASx was also detected in the IL-12-treated sample. In contrast, neither Stat4 nor PIASx was pulled down in untreated extracts. As a control, no binding was detected in the absence of DNA (Fig. 3B). Similar results were obtained using Kit225 T cell extracts (data not shown). These results indicate that the formation of the PIASx-Stat4 complex does not interfere with the DNA binding activity of Stat4.
Inhibition of Stat4 by PIASx

In this report, we provide evidence that PIASx is a transcriptional co-repressor of Stat4. By in vivo co-immunoprecipitation analysis, PIASx was found to be associated with Stat4 upon IL-12 stimulation. Oligonucleotide pull-down analysis indicated that PIASx is present in the Stat4-DNA binding complex. Consistently PIASx does not interfere with the DNA binding activity of Stat4. Using reporter assays, we showed that PIASx inhibited Stat4-mediated gene activation and that such an inhibitory activity of PIASx can be overcome by suppressing HDAC activity. Both PIASxα and PIASαβ can inhibit Stat4-mediated gene activation. The mode of action of PIASx is similar to that of PIASy in which both proteins repress the transcriptional activity of Stat without affecting its DNA binding activity (9). With the completion of this work, each member of the PIAS family has now been found to regulate STAT transcription.

In Drosophila, a PIAS homologue named dPIASzimp has been identified. Genetic studies suggest that dPIAS negatively regulates the activity of dSTAT (20). In addition to modulating dSTAT activity, dPIAS has also been shown to regulate chromosome stability (21). In yeast, two PIAS-related proteins named SIZ1 and SIZ2 have also been described (22). Interestingly SIZ1 and SIZ2 have been shown to possess SUMO (small ubiquitin-related modifier) E3 ligase activity. Subsequently members of the PIAS family have been demonstrated to participate in the SUMO modification of a number of transcription factors including p53, androgen receptor, and Lef-1 (23–29). These findings raise an interesting possibility that the SUMO ligase activity of a PIAS protein may be involved in its ability to regulate transcription. However, we have been unable to find any evidence to support that PIASx can promote the SUMO modification of Stat4. Instead our data presented in this report suggest the possible involvement of HDAC activity in the transcriptional repression of Stat4 by PIASx. Interestingly it has been suggested recently that PIASαβ is associated with HDAC3 (30). Thus, it is possible that PIASαβ is a component of a large transcriptional co-repressor complex, the identity of which remains to be uncovered.

PIASx has been suggested to participate in the regulation of several other transcription factors. ARIP3 (androgen receptor interaction protein 3), a rat homologue of PIASαβ, has been shown to regulate the activity of androgen receptor (26, 31, 32). Miz1 (Max-interacting zinc finger), which corresponds to the COOH-terminal portion of PIASαβ (amino acids 134–621), was suggested to modulate the transcriptional activity of a homeobox DNA-binding protein, Max2 (33). Thus, like many known transcriptional co-regulators, PIASx may participate in the regulation of various transcriptional responses.

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Fig. 4. The effect of TSA on the inhibitory activity of PIASx.

Kit225 cells were transfected with pcMV-Stat4 (10 μg) and 4xIRF1 (5 μg) together with or without FLAG-PIASx (1 μg) in the presence of absence of TSA (0.3 nm) as indicated. Transfected cells were untreated or treated with IL-12 for 16 h. TSA was added 4 h prior to IL-12 addition. The relative luciferase values are shown, untreated, untreated.
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