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Distinct Mechanisms of STAT Phosphorylation via the Interferon-α/β Receptor

SELECTIVE INHIBITION OF STAT3 AND STAT5 BY PICEATANNOL*

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Leon Su and Michael David‡

From the Department of Biology and UCSD Cancer Center, University of California San Diego, La Jolla, California 92093-0322

Interferon-α (IFNα) can activate several members of the signal transducers and activator of transcription (STAT) transcription factor family, a process that requires the tyrosine kinases Jak1 and Tyk2. Here we provide evidence that IFNα-mediated activation of various STAT proteins is regulated by distinct mechanisms. Piceatannol, previously reported as a Syk/ZAP70-specific kinase inhibitor, selectively inhibits the tyrosine phosphorylation of STAT3 and STAT5, but not of STAT1 and STAT2. This inhibition is paralleled by the loss of Jak1 and IFNAR1 tyrosine phosphorylation in response to IFNα, whereas Tyk2 and IFNAR2 tyrosine phosphorylation is unaffected. Last, the IFNα-induced serine phosphorylation of STAT1 and STAT3 is not inhibited by piceatannol but is sensitive to the Src kinase-specific inhibitor PP2. Thus, our results not only demonstrate that the IFNα/β receptor utilizes distinct mechanisms to trigger the tyrosine phosphorylation of specific STAT proteins, but they also indicate a diverging pathway that leads to the serine phosphorylation of STAT1 and STAT3.

Signal transducers and activators of transcription (STATs)[1] comprise a family of transcription factors that link activation of the interferon receptor to the induction of immediate early response genes (ISGs) (1, 2). Seven genetically distinct mammalian STAT proteins have been described thus far (3–9), and related signaling molecules have been found in *Drosophila* (10, 11) and *Dictystelium* (12). A distinct characteristic of all STAT family members is the primary regulation of their activity through rapid tyrosine phosphorylation (13, 14), which is required for dimerization (15), nuclear translocation (16), and DNA binding (3, 17). Specificity of STAT activation is believed to be determined by the SH2 domain present in all STAT proteins (13, 14, 18). In the case of STAT1 and STAT3, phosphorylation on Ser^{727} in addition to phosphorylation on Tyr^{701} or Tyr^{705}, respectively, is essential to maximize their transactivation capabilities (19). Serine phosphorylation of STAT1 and STAT3 appears to require MAP kinase activity, and expression of dominant-negative extracellular signal-regulated kinase 2 suppresses STAT-mediated gene expression via the IFNα receptor (20). The tyrosine kinases required for IFNα/β-mediated STAT activation, Tyk2 and Jak1, were found to be associated with their substrate type I interferon receptor chains, IFNAR1 and IFNAR2, respectively (21, 22). Genetic deletion of Jak1 results in the inability to respond to IFNα or IFNβ (23). In contrast, deletion of Tyk2 causes a complete lack of IFNα responsiveness (24), whereas IFNβ can still elicit a limited signaling response in the absence of Tyk2 (25), albeit both interferons utilize the same receptor. Studies aimed at elucidating the function of these kinases as well as their transphosphorylation and their role in receptor phosphorylation have been complicated by the fact that the absence of Tyk2 causes a significant decrease in the expression levels and in the function of the type I interferon receptor (26). Interestingly, a kinase-defective Tyk2 mutant is able to partially restore sensitivity toward IFNα (27, 28), whereas a recently identified IFNβ-induced gene requires the kinase activity of Tyk2 (29). Much of the work on type I interferon receptor signaling focused on the activation of STAT1 and -2, which comprise the ISGF3 transactivation complex. However, IFNα/β are also able to induce the tyrosine phosphorylation of STAT3 and -5, albeit the target genes of these STAT proteins remain to be elucidated.

Here we report that the tyrosine kinase inhibitor piceatannol selectively prevents the IFNα-induced tyrosine phosphorylation of STAT3 and -5, as well as of Jak1 and IFNAR1. In contrast, the tyrosine phosphorylation of STAT1 and -2 and of Tyk2 and IFNAR2 was unaffected by the inhibitor, as was IFNα-induced serine phosphorylation of STAT1 and -3 and subsequent ISG induction. Furthermore, we show that the serine phosphorylation of STAT1 and -3 requires extracellular signal-regulated kinase 1/2 as well as the activity of a Src-like tyrosine kinase.

MATERIALS AND METHODS

Cells and Reagents—Ramos and Jurkat cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. ZAP70-deficient Jurkat cells were a kind gift from Dr. Robert Abraham. Piceatannol, PP2, and PD98059 were obtained from Calbiochem. Staurosporine was purchased from Sigma. IFNα was a generous gifts from Hoffman LaRoche.

Western Blot Analysis—Following treatment, cells were lysed in buffer containing 20 mM Hepes, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged, and protein concentration was determined by Bradford (Bio-Rad). Whole cell extracts or immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis and adsorbed to polyvinylidene difluoride membranes (Millipore Corp.). Proteins were detected with phosphospecific STAT1-Tyr^{701}, STAT3-Tyr^{705} and Tyk2-Tyr^{1054}/Tyr^{1055} from New England Biolabs, or with phosphospecific STAT1-Ser^{727}, phospho-

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‡ Recipient of the Sidney Kimmel Scholar Award. To whom correspondence should be addressed: Dept. of Biology, University of California, San Diego, Bonner Hall 3138, 9500 Gilman Dr., La Jolla, CA 92093-0322. Tel.: 619-822-1108; Fax: 619-822-1106; E-mail: midavid@ucsd.edu.

[1] The abbreviations used are: STAT, signal transducer and activator of transcription; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); ISG, interferon-stimulated gene; wb, Western blot; IFN, interferon; ISRE, interferon-stimulated response element; CTL, control; pY, phosphotyrosine.

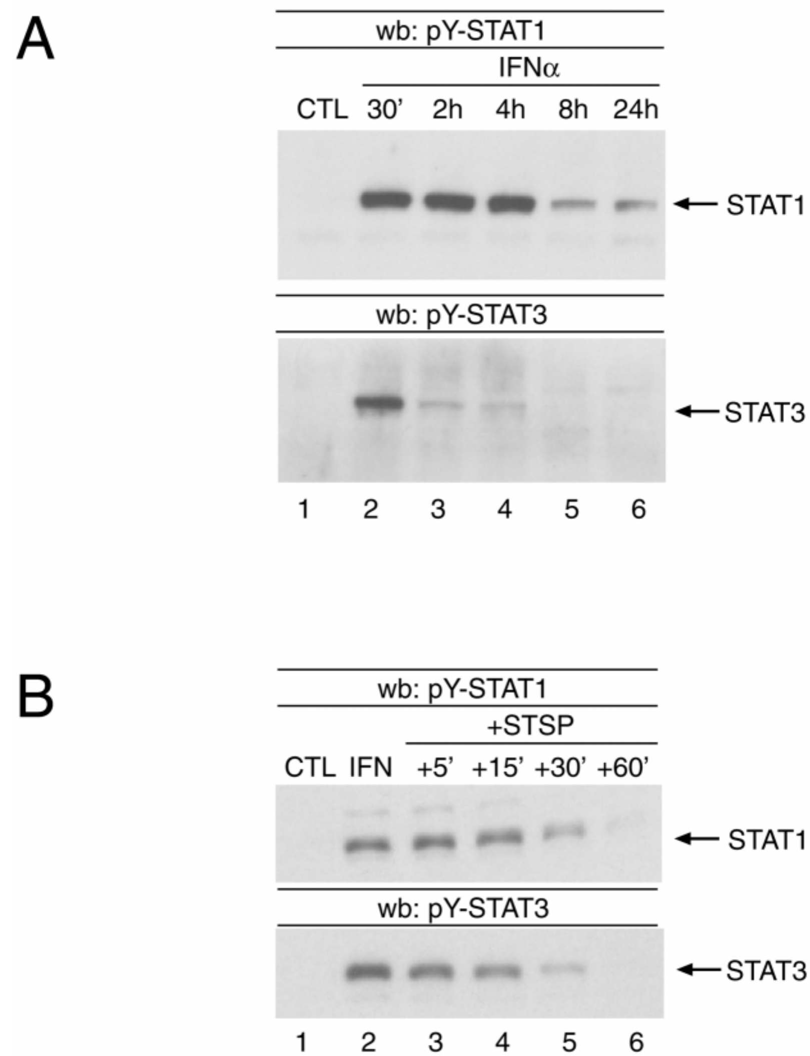

FIG. 1. **Differential regulation of IFN-α-mediated tyrosine phosphorylation of STAT1 and STAT3.** *A*, kinetics of STAT1 and STAT3 tyrosine phosphorylation after IFNα stimulation. RAMOS cells were left untreated (*lane 1*) or treated with 10,000 units/ml IFNα for the indicated times (*lanes 2–6*), and cell lysates were immunoblotted with phospho-(Tyr^{701})-specific STAT1 (*upper panel*) or phospho-(Tyr^{705})-specific STAT3 (*lower panel*) antibody. *B*, pulse-chase analysis of STAT1 and STAT3 tyrosine phosphorylation. RAMOS were left untreated (*lane 1*) or stimulated with 10,000 units/ml IFNα for 30 min, followed by the addition of 500 nM staurosporine for the indicated times (*lanes 3–6*). Immunoblots were probed with a phospho- (Tyr^{701})-specific STAT1 (*upper panel*) or phospho-(Tyr^{705})-specific STAT3 (*lower panel*) antibody.

specific STAT5A/B-Tyr^{694}/Tyr^{699}), or phosphotyrosine-specific (4G10-Biotin Conjugate) antisera purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibodies to IFNAR1 and IFNAR2 were generous gifts from Biogen, Inc. and Dr. O. Colamonici, respectively. Jak1 and phosphospecific (Tyr^{698}) STAT2 rabbit antiserum were generous gifts from Dr. Andrew Larner. Monoclonal Jak1 antibody from Transduction Laboratories was used for reprobing. All blots were developed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Pharmacia Biotech).

RNase Protection Assay Analysis—Jurkat cells were stimulated with IFNα in the absence or presence of piceatannol. Total cellular RNA was isolated with TRIzol Reagent (Life Technologies, Inc.). ^{32}P- Labeled antisense riboprobes were generated by *in vitro* transcription of the linearized plasmid using T7 or SP6 RNA polymerases (Promega). Labeled riboprobe and 10 μg of RNA were incubated in hybridization buffer (4:1 formamide and 5× stock; 5× stock was 200 mM PIPES, pH 6.4, 2 M NaCl, 5 mM EDTA), overnight at 56 °C before digestion with T_1 RNase (Life Technologies, Inc.) for 1 h at 37 °C. After phenol extraction and ethanol precipitation, protected fragments were solubilized in RNA loading buffer (98% formamide, 10 mM EDTA (pH 8), bromphenol blue, xylene cyanol), boiled for 2 min, and subjected to electrophoresis on a 4.5% polyacrylamide-urea gel.

RESULTS AND DISCUSSION

Differential Regulation of IFNα-mediated Tyrosine Phosphorylation of STAT1 and STAT3—IFNα can activate several members of the STAT family of transcription factors. This poses the interesting question of whether all STAT proteins are activated by the same mechanism, particularly since the activation kinetics differs between distinct STAT proteins. As such, IFNα induces in RAMOS cells, a Burkitt lymphoma B cell line, maximal phosphorylation of STAT1 Tyr^{701} and STAT3 Tyr^{705} within 30 min after stimulation. However, whereas Tyr^{705}-phosphorylated STAT3 is subjected to a rapid decrease within 2 h, Tyr^{701}-phosphorylated STAT1 can still be observed after 24 h (Fig. 1*A*, *upper versus lower panel*). This differential regulation of STAT tyrosine phosphorylation could be due to different kinase activity or could be based on selective dephosphorylation and/or degradation. To address this question, cells were stimulated with IFNα for 30 min prior to exposure to staurosporine, a general kinase inhibitor, for the indicated times. This pulse-chase type stimulation revealed that the tyrosine phosphorylation state of STAT1 is similar to that of STAT3 (Fig. 1*B*, *upper versus lower panel*), suggesting that different tyrosine kinases rather than phosphatases are responsible for the observed differences in the phosphorylation profile.

Specific Inhibition of STAT3 and -5 Tyrosine Phosphorylation by Piceatannol—We had observed previously that piceatannol, a reportedly Syk/ZAP70-specific tyrosine kinase inhibitor, is able to block tyrosine phosphorylation of STAT1 after B cell receptor stimulation (30). Interestingly, we found piceatannol to also selectively inhibit IFNα-mediated STAT3 tyrosine phosphorylation, whereas STAT1 tyrosine phosphorylation was not affected (Fig. 2*A*, *upper versus lower panel*). In contrast, PP2, an inhibitor of Src and Src-like kinases, did not affect IFNα-mediated tyrosine phosphorylation of either STAT1 or STAT3 (Fig. 2*B*, *lanes 4* and *5*). To further investi-

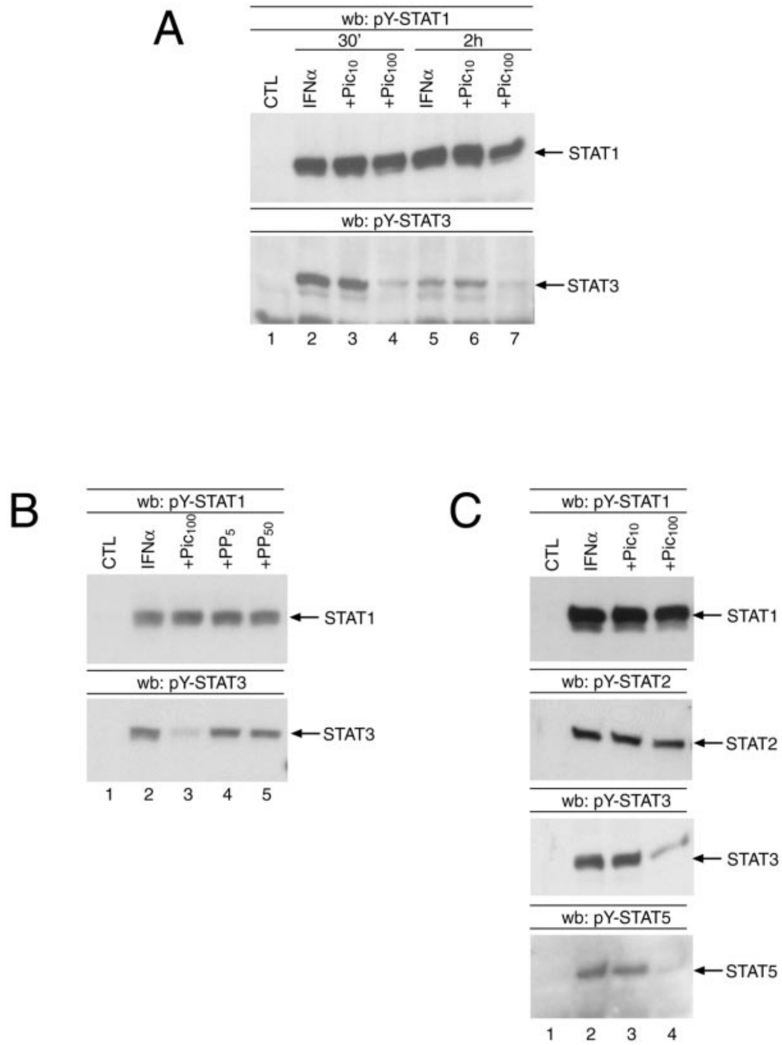

FIG. 2. **Selective inhibition of tyrosine phosphorylation on STAT3 and -5 by piceatannol.** *A*, effect of piceatannol on tyrosine phosphorylation of STAT1 and STAT3. RAMOS cells were exposed to Me_2SO, 10 μM or 100 μM piceatannol for 30 min before stimulation with 1000 units/ml IFNα for 30 min (*lanes 2–4*) or 2 h (*lanes 5–7*). Immunoblots were probed with phospho-(Tyr^{701})-specific STAT1 (*upper panel*) or phospho-(Tyr^{705})-specific STAT3 (*lower panel*) antibody. *B*, tyrosine phosphorylation of STAT1 and STAT3 does not involve Src-like kinases. Cells were exposed to Me_2SO (*lanes 1* and *2*), 100 μM piceatannol (*lane 3*), or 5 or 50 μM PP2 (*lanes 4* and *5*) for 30 min prior to stimulation with 1000 units/ml IFNα for 30 min. Immunoblots were probed with a phospho-(Tyr^{701})-specific STAT1 (*upper panel*) or phospho-(Tyr^{705})-specific STAT3 (*lower panel*) antibody. *C*, piceatannol inhibits tyrosine phosphorylation of STAT3 and STAT5, but not of STAT1 or STAT2. RAMOS cells were treated with Me_2SO (*lanes 1* and *2*) or 10 or 100 μM piceatannol (*lanes 3* and *4*) prior to stimulation with 1000 units/ml IFNα for 30 min. Immunoblots were probed with a phospho-(Tyr^{701})-specific STAT1 (*top panel*), phospho-(Tyr^{698})-STAT2 (*second panel*), phospho- (Tyr^{705})-specific STAT3 (*third panel*) antibody, or phospho-(Tyr^{694}/Tyr^{699})-specific STAT5A/B antibody (*bottom panel*).

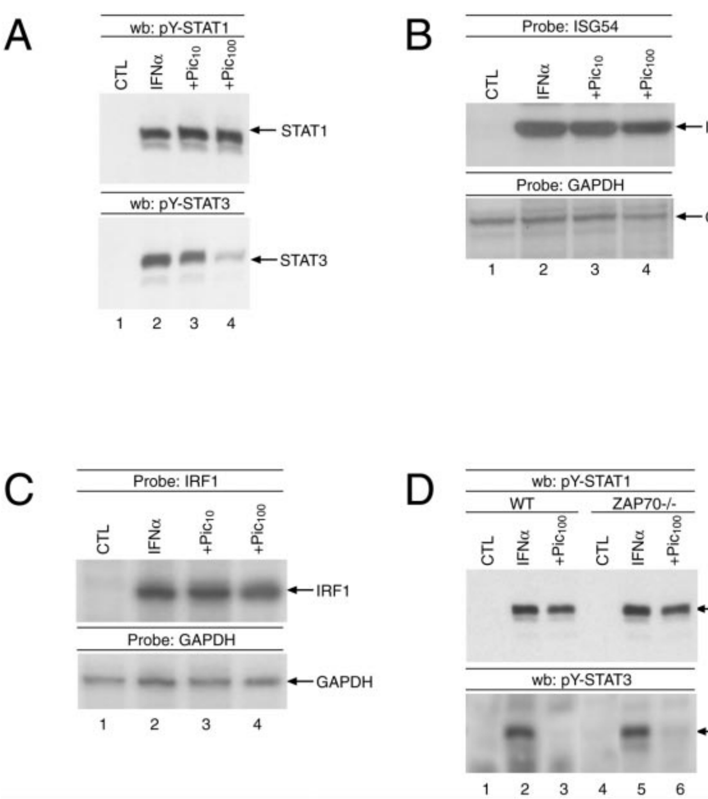

FIG. 3. **Piceatannol does not affect IFN-α mediated ISG54 expression.** *A*, differential STAT tyrosine phosphorylation in Jurkat cells. Jurkat cells were exposed to Me_2SO (*lanes 1* and *2*) or 10 or 100 μM piceatannol (*lanes 3* and *4*) for 30 min before stimulation with 1000 units/ml IFNα for 30 min. Immunoblots were probed with phospho-(Tyr^{701})-specific STAT1 (*upper panel*) or phospho-(Tyr^{705})-specific STAT3 (*lower panel*) antibody. *B*, Jurkat cells were exposed to Me_2SO (*lanes 1* and *2*) or 10 or 100 μM piceatannol (*lanes 3* and *4*) for 30 min before stimulation with 1000 units/ml IFNα for 3 h. Total cellular RNA was isolated, and ISG54 or IRF1 mRNA levels (*top panels*) were determined by RNase protection assay. GAPDH was used as an internal standard to ensure that equal amounts of RNA were loaded (*bottom panels*). *C*, Same as *B*, except that a probe against the *IRF1* gene was used for hybridization. *D*, effect of piceatannol on tyrosine phosphorylation of STAT1 and STAT3 in ZAP70−/− Jurkat cells. Wild type (*WT*) and ZAP70−/− Jurkat cells were exposed to Me_2SO (*lanes 1*, *2*, *4*, and *5*) or 100 μM piceatannol (*lanes 3* and *6*) for 30 min prior to stimulation with 1000 units/ml IFNα for 30 min. Immunoblots were probed with phospho-(Tyr^{701})-specific STAT1 (*upper panel*) or phospho-(Tyr^{705})-specific STAT3 (*lower panel*) antibodies.

gate the specificity of piceatannol, its inhibitory effect on tyrosine phosphorylation of the other STAT proteins was examined. As shown in Fig. 2*C*, IFNα-mediated tyrosine phosphorylation of STAT5A/B is also abrogated by piceatannol, while STAT2 parallels STAT1 in being unaffected by the inhibitor in its phosphorylation (*top panels versus bottom panels*). Similar results were obtained in HeLa cells or primary human foreskin fibroblasts, illustrating that the effects of piceatannol are not restricted to cells of hematopoietic origin (data not shown). These results demonstrate that STAT3 and -5 activation requires a piceatannol-sensitive kinase, whereas STAT1 and STAT2 activation occurs through a different mechanism.

Intact ISG Induction in the Presence of Piceatannol—To exclude the possibility that the observed effects of piceatannol were a peculiarity of the RAMOS cell line, we also subjected Jurkat T cells to IFNα stimulation in the absence or presence of piceatannol. Identical to RAMOS cells, piceatannol specifically blocked STAT3 but not STAT1 tyrosine phosphorylation after IFN stimulation (Fig. 3*A*, *upper versus lower panel*). Selective gene activation responsible for the different biological responses can be attributed to differential STAT dimer binding or dimer-complex formation on the DNA. IFNα-activated STAT1 and -2, which comprise the IFNα/β-induced interferon-stimulated response element (ISRE) binding complex, bind to ISREs to drive transcription of ISGs. Alternatively, STAT1 homodimers are able to stimulate transcription of genes controlled by the IFNγ-activated sequence. Since we had found that STAT1 and -2 were unaffected by piceatannol, we wanted to verify that the downstream transcriptional activation of ISRE-driven genes is indeed not inhibited by piceatannol. We therefore performed RNase protection assays using a probe corresponding to the human *ISG54* gene with RNA derived from Jurkat cells stimulated with IFNα in the absence and presence of piceatannol. As anticipated, IFNα-mediated transcription of ISG54 was not effected by the inhibitor (Fig. 3*B*). In addition, the induction of the IFNγ-activated sequence element-driven *IRF1* gene was also not affected by piceatannol (Fig. 3*C*). Thus, piceatannol is able to prevent IFNα-mediated tyrosine phosphorylation of STAT3 and -5 without affecting transcriptional activation of STAT1-controlled IFNγ-activated sequence-driven or STAT1/2-stimulated ISRE-driven genes.

Piceatannol has been reported to specifically inhibit the Syk/ZAP70 kinase family members (31, 32). To test whether Syk/ZAP70 are indeed the targets of piceatannol in the IFNα-activated STAT activation pathway, we utilized a ZAP70-deficient Jurkat variant. These cells, which also do not express detectable levels of Syk kinase (33), were still able to support STAT3 tyrosine phosphorylation; moreover, the selective sensitivity of STAT3 toward piceatannol was still preserved (Fig. 3*D*, *upper versus lower panel*). These results clearly demonstrate that Syk/ZAP70 are not involved in IFNα-mediated STAT1 or -3 activation.

Inhibition of JAK1 and IFNAR1, but Not Tyk2 and IFNAR2, Tyrosine Phosphorylation by Piceatannol—The current model of signaling through the IFNα/β receptor supports the notion that the tyrosine kinases Tyk2 and Jak1 associate with IFNAR1 and IFNAR2, respectively (21, 22). Upon IFN-induced receptor dimerization, transphosphorylation of the two associated tyrosine kinases occurs (27), and the kinases thus activated subsequently phosphorylate tyrosine residues on the cytoplasmic tails of the receptor chains. This receptor phosphorylation is thought to be required for STAT binding to the receptors via the STAT-SH2 domains (14). Since we had excluded Syk/ZAP70 as the target for piceatannol, we decided to investigate the effect of the compound on the tyrosine phosphorylation of Jak1 and Tyk2 tyrosine kinase. Intriguingly,

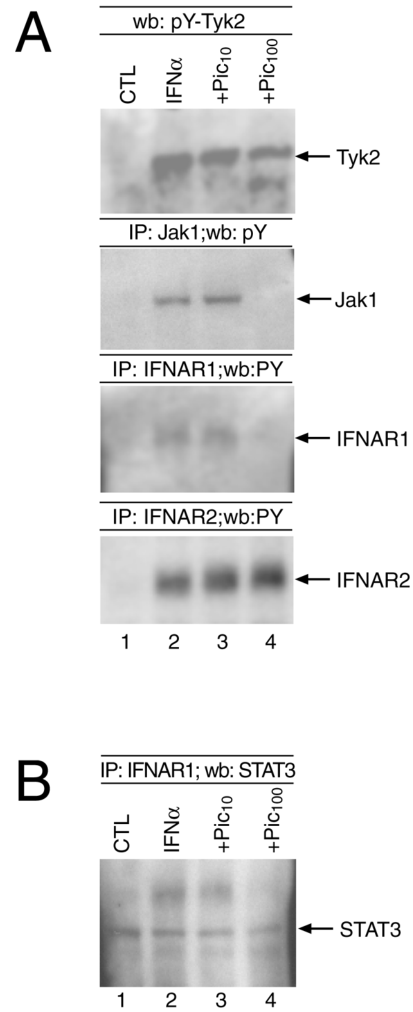

FIG. 4. **Selective inhibition of Jak1 and IFNAR1 tyrosine phosphorylation by piceatannol.** *A*, effect of piceatannol on tyrosine phosphorylation of Tyk2, Jak1, IFNAR1, and IFNAR2. Jurkat cells were exposed to Me_2SO (*lanes 1* and *2*) or 10 or 100 μM piceatannol (*lanes 3* and *4*) prior to stimulation with 1000 units/ml IFNα for 30 min. Lysates were prepared and subject to immunoprecipitation with Jak1, IFNAR1, or IFNAR2 antibodies. Immunoblots from whole cell lysates were probed with phospho-(Tyr^{1054}/Tyr^{1055}) Tyk2-specific antibodies. Immunoprecipitated, resolved Jak1, IFNAR1, and IFNAR2 proteins were probed for phosphotyrosine content (*middle* and *bottom panels*). *B*, constitutive association of STAT3 with the interferon receptor. The membrane of resolved IFNAR1 immunoprecipitates was reprobed with a monoclonal STAT3 antibody.

IFNα-mediated tyrosine phosphorylation of Jak1, but not the phosphorylation of the tyrosines in the activation loop of Tyk2, is blocked by piceatannol (Fig. 4*A*, *upper versus middle panel*). Immunoblots were reprobed for Jak1 and Tyk2 to verify that equal amounts of proteins were loaded (data not shown). Since Jak1 and Tyk2 have been shown to transphosphorylate each other for activation (23, 27), the lack of Jak1, but not Tyk2, phosphorylation is probably due to specific inhibition of Tyk2 kinase activity. Interestingly, Tyk2 has been reported to share a significant degree of homology with Syk (27), which offers a possible explanation for its sensitivity toward piceatannol. Indeed, Tyk2 displayed a higher sensitivity than Jak1 toward inhibition by piceatannol in *in vitro* kinase assays (data not shown). Tyk2 inhibition would be expected to be accompanied by the absence of IFNAR1 tyrosine phosphorylation, since this receptor chain functions as a substrate for Tyk2 (21). In contrast, intact phosphorylation of the Jak1 substrate IFNAR2

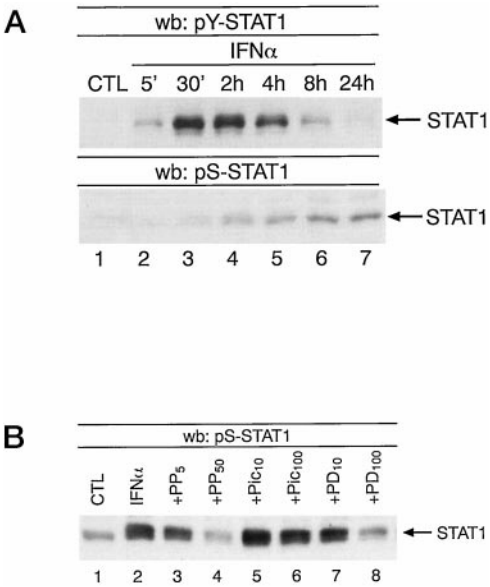

FIG. 5. **Serine phosphorylation of STAT1 requires a Src-like kinase.** *A*, differential kinetics of IFNα-mediated STAT1 tyrosine and serine phosphorylation. RAMOS cells were left untreated (*lane 1*) or treated with 1000 units/ml IFNα for the indicated times (*lanes 2–7*). Immunoblots were probed with phospho-(Tyr[701])-specific STAT1 (*upper panel*) or phospho-(Ser[727])-specific (*pS*) STAT1 (*lower panel*) antibody. *B*, inhibition of STAT1 serine phosphorylation by PP2 and PD98059, but not piceatannol. Cells were exposed to Me_2SO (*lanes 1* and *2*) or 5 or 50 μM PP2 (*lanes 3* and *4*), 10 or 100 μM piceatannol (*lanes 5* and *6*), or 10 or 100 μM PD98059 (*lanes 7* and *8*) for 30 min prior to stimulation with 1000 units/ml IFNα. Resolved protein were probed with phospho-(Ser[727])-specific STAT1 antibody.

would be anticipated if this kinase is not inhibited by piceatannol. Indeed, piceatannol prevented the IFNα-induced tyrosine phosphorylation of IFNAR1 (Fig. 4*A*, *second panel* from *bottom*) but not of IFNAR2 (*bottom panel*), as detected by receptor immunoprecipitation followed by phosphotyrosine blotting. The blot was reprobed for IFNAR1 and IFNAR2 to ensure equal amounts of immunoprecipitated receptors (data not shown). Interestingly, reprobing of the blot for STAT3 revealed that the inhibition of IFNAR1 tyrosine phosphorylation did not impair STAT3 binding to IFNAR1. Rather, STAT3 was found to be constitutively associated with this IFNα receptor chain (Fig. 4*B*), whereas probing of immunoprecipitates obtained with NRS revealed no STAT3 (data not shown). These results suggest that the catalytic kinase activity of Tyk2 is essential for the activation of STAT3 and STAT5 but is dispensable in the activation of STAT1 and STAT2. Furthermore, tyrosine phosphorylation of the cytoplasmic tail of IFNAR1 appears not to be essential for the recruitment of STAT3 to the receptor.

Serine Phosphorylation of STAT1 Requires a Src-like Kinase—In addition to tyrosine phosphorylation, which mediates nuclear translocation and DNA binding of STATs, additional serine phosphorylation of STAT1 and -3 is necessary for maximal transcriptional activation (19). Tyrosine and serine phosphorylation of STAT1 occur with different kinetics. IFNα-mediated STAT1 tyrosine phosphorylation is detectable within 5 min after stimulation, while observable serine phosphorylation does not occur until 30 min after treatment (Fig. 5*A*). Subsequently, serine phosphorylation of STAT1 is still maximal after a 24-h exposure to interferon, while STAT1 Tyr[701] phosphorylation is markedly diminished. As described previously, serine phosphorylation on STAT1 Ser[727] is dependent on extracellular signal-regulated kinase 1/2 activity, and PD98059, a potent mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor, abrogated STAT1 Ser[727] phosphorylation (Fig. 5, *lanes 7* and *8*). To investigate whether the serine phosphorylation of STAT1 and -3 was affected by either piceatannol or PP2, we analyzed the serine phosphorylation of STAT1 and -3 in response to IFNα in the absence or presence of these inhibitors. As shown in Fig. 5, piceatannol had no effect on the IFNα-induced serine phosphorylation of STAT1 (*lanes 5* and *6*); however, STAT1 Ser[727] phosphorylation was significantly diminished in the presence of the Src kinase inhibitor PP2 (*lanes 3* and *4*) at a concentration that did not affect STAT1 tyrosine phosphorylation (Fig. 2*B*). Identical results were obtained when STAT3 Ser[727] phosphorylation was analyzed (data not shown). Thus, distinct requirements exist that govern the processes that lead to STAT tyrosine or serine phosphorylation.

In summary, our data indicate that the tyrosine phosphorylation of different STAT proteins, although initiated through the same IFNα receptor complex, is regulated by distinct mechanisms. We have shown that STAT3 and -5 can be included in a piceatannol-sensitive group, while activation of STAT1 and -2 is unaltered by this tyrosine kinase inhibitor. We provide evidence suggesting that Tyk2 kinase activity is required for STAT3 activation but is dispensable for tyrosine phosphorylation of STAT1. Furthermore, the lack of tyrosine phosphorylation on the cytoplasmic tail of IFNAR1 does not preclude STAT3 recruitment, since the protein is found constitutively associated with the receptor chain.

Our data demonstrate the feasibility of the development of compounds that specifically abrogate the activation of selected members of the Jak/STAT pathway. STAT1 activation is essential for the antiproliferative effects of IFNα (34), whereas STAT3 has been recently described as an oncogene (35). Thus, selective inhibitors such as piceatannol might prove useful as pharmacological agents to specifically block STAT3 but not STAT1 activation and to modulate cellular responses to type I interferons.

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