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Inhibition of Protein Phosphorylation Modulates Expression of the Jak Family Protein Tyrosine Kinases†

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Treatment of murine Friend cells with a dose of the protein kinase inhibitor staurosporine, which is able to block the response of the cells to interferons, appears to inhibit phosphorylation of Jak proteins and, interestingly, to specifically reduce tyk2 and Jak1 expression and to increase Jak2 both in the presence and in the absence of interferons. Therefore, a potential role for phosphorylation events in the regulation of expression of the Jak family members is suggested.

Interferons (IFNs) are able to induce transcription of several specific genes called the IFN-stimulated genes (ISGs). This event can occur within minutes of the binding of IFNs to their receptors and is responsible for the resulting physiological changes induced by IFNs in susceptible cells (6, 9). The specific induced transcription is mediated by the interaction of transcription factors with regulatory DNA sequences that lie in the ISG promoters (7, 10, 20, 29, 50). Although the molecular basis of transcriptional activation is understood in some detail, studies of the nature of the signal transduction pathways by which IFN receptors exert such rapid effects have just recently progressed through the identification and characterization of signalling components (for reviews, see references 8 and 24). Definitive evidence has been provided of the involvement of the Jak (Janus kinase) and Stat (signal transducer and activator of transcription) proteins. The rapid transcriptional response of cells exposed to IFNs relies on the activation of preexisting, cytoplasmic transcriptional activators or Stat proteins which, once activated, translocate to the nucleus and bind IFN-responsive DNA sequences. As in other pathways, regulated protein phosphorylation appears to provide a direct link for information transfer from the cytoplasmic compartment to the nucleus (3, 19, 28, 42). The lack of recognizable protein tyrosine kinase domains in the cloned alpha IFN (IFN-α)- and gamma IFN (IFN-γ)-binding subunits of the IFN receptors (1, 16, 23, 41, 45) suggested that additional receptor or nonreceptor proteins must provide the tyrosine kinase function(s) in these pathways.

Direct evidence for the involvement of the Jak family of nonreceptor protein tyrosine kinases in the IFN response pathways has been provided by genetic complementation of IFN-resistant mutant cell lines. The Jak family is composed of four known members, three of which are involved in IFN signal transduction: tyk2, which is essential for the IFN-α response (48); Jak2, which is essential for the IFN-γ response (51); and Jak1, which is essential for both the IFN-α and the -γ response pathways (21, 39). The activation of these proteins in response to IFN-α and -γ requires phosphorylation on tyrosine residues in the number of human and murine cell lines (4, 21, 34, 38, 48, 51). The current model of IFN-α-mediated transcriptional induction involves activation by Jak protein-mediated phosphorylation of the three components (Stat91, -84, and -113) of the α subunit of ISG factor 3 (ISGF3) (11, 14, 33, 34), whereas IFN-γ activates Stat91 (35–37). The activated proteins combine to form the specific transcription factor (ISGF-3 for IFN-α and the Stat91 homodimer for IFN-γ) (35, 46, 47) and, upon translocation to the nucleus, bind to *cis*-acting DNA response elements in the promoters of ISGs to initiate transcription. Phosphorylation of the Stat proteins is required for their association and translocation (18, 36, 38).

Furthermore, a subunit of IFN-α receptor has been shown to be phosphorylated on tyrosine residues upon ligand binding (27), and the IFN-γ receptor was reported to be phosphorylated on tyrosine in response to IFN-γ (13). Following ligand-receptor interaction, receptor dimerization is thought to mediate juxtaposition and activation of associated Jak proteins. Assembly of receptor chain and associated kinases is thought to form a multiprotein complex allowing recruitment and phosphorylation of Stat family members. It has been reported that a mutated Stat91 protein, which has a single tyrosine residue changed to phenylalanine, was not phosphorylated and failed to restore responsiveness to IFN-α and -γ in a p91-deficient mutant cell line (22, 36). The importance of phosphorylation events in the IFN signal transduction pathway appears to be clear.

In Friend leukemia cells (FLC), we observed that the dosage of staurosporine able to block the response of FLC to IFN-α is able to inhibit activation of Jak family members and/or phosphorylation of Stat proteins. Surprisingly, under these conditions, staurosporine also affects the expression of all three Jak kinases involved in the signal transduction of IFNs.

Protein kinase inhibitor staurosporine blocks antiviral response to IFN-α. A specific biologic effect of IFN is its ability to protect cells against a wide variety of RNA and DNA viruses. The effects of different dosages of the kinase inhibitor staurosporine (44) on the ability of IFN-α to protect cells against encephalomyocarditis virus infection were assessed. IFN-α treatment (500 U/ml) of the FLC results in a reduction of 3 $\log_{10}$ in virus yield (Table 1). Interestingly, the antiviral effect of IFN-α (as well as of IFN-β and -γ [data not shown]) was completely abolished only when 500 nM staurosporine was used. Accordingly, the induction of 2′-5′-oligoadenylate synthetase transcription was also inhibited (data not shown).

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† This paper is dedicated to the memory of the late Professor Giovanni B. Rossi.

TABLE 1. Effect of staurosporine on the induction of the antiviral state in FLC treated with IFN-α[a]

Inhibitor and concn (μM)	Virus titer	
	Without IFN-α	With IFN-α
None (0)	8.5	5.1
Staurosporine		
0.01	8.0	5.1
0.1	7.9	5.5
0.5	7.9	7.5

[a] FLC (10^6/ml) were treated with the protein kinase inhibitor staurosporine (Sigma) starting 15 min before the addition of IFN-α (500 U/ml). Amounts of IFNs are given in laboratory units, i.e., the amount of IFN that reduces the plaque titer of the vesicular stomatitis virus by 50%. This unit equals 4 reference units of the National Institutes of Health standard G-002-904-511. Murine IFN-α was prepared and purified as described elsewhere (5). The specific activities of the purified preparation ranged between 5×10^7 and 10^9 U/mg of protein. After overnight incubation, cells were infected with encephalomyocarditis virus (4 PFU per cell). Supernatants were collected 6 h later and were titrated by plaque assays. Results are expressed as $\log_{10}$ virus yield per 5×10^5 cells (standard errors are in the range of 0.5).

Effect of staurosporine on the expression of Jak mRNAs. Regulated protein phosphorylation has a central role in IFN signal transduction pathways (18, 30, 36, 38). The inhibitor of phosphorylation, staurosporine (44), has been utilized as the agent that perturbs signalling pathways in a variety of cell lines (17, 25, 26, 30). In FLC, we analyzed the steady-state levels of tyk2, Jak1, and Jak2 mRNAs. The RNA samples were extracted (12) after treatment with IFN-α and/or the dose of staurosporine (500 nM) that was able to block the response of FLC to IFN at the indicated times. RNase protection assaying was utilized to detect tyk2 mRNA (Fig. 1). ^{32}P-labeled RNA probes of high specific activity were prepared by in vitro transcription of the pBS-500 riboprobe containing 487 nucleotides (1130 to 1616) of tyk2 cDNA subcloned in the pBS-KS(+) vector (Stratagene). To generate a ^{32}P-labeled 487-nucleotide-long antisense RNA probe, pBS-500 was linearized by digestion with *Eco*RI and transcribed with T7 RNA polymerase (Promega). A pTRI-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-mouse antisense probe containing a 316-bp restriction fragment (Ambion) was included in each reaction mixture as an internal control. The plasmid was linearized by digestion with *Hin*dIII and transcribed with T3 RNA polymerase. After digestion of the unhybridized probes with RNases A and T_1 (Boehringer Mannheim), the protected fragments were separated on 8% denaturing polyacrylamide gels and subjected to autoradiography. A specific mRNA-protected band, which is shown in Fig. 1, was selected and densitometrically analyzed. IFN-α treatment did not affect the amount of the tyk2 mRNA in FLC, as already reported for other cell lines (48).

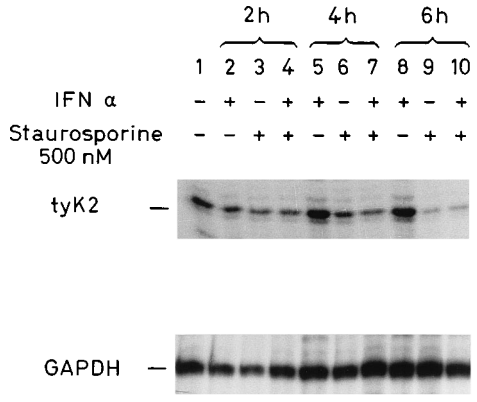

FIG. 1. tyk2 mRNA expression. tyk2 mRNA expression in response to IFN-α (500 U/ml) and/or staurosporine (500 nM) was monitored by RNase protection assays using a ^{32}P-labeled, 487-nucleotide-long antisense RNA probe for human tyk2. The protection of GAPDH mRNA serves as an internal loading control. Treatments were as described in footnote *a* to Table 1 at the indicated times. A total of 2×10^5 to 3×10^5 cpm of each probe and 10 μg of total RNA were used in each assay mixture.

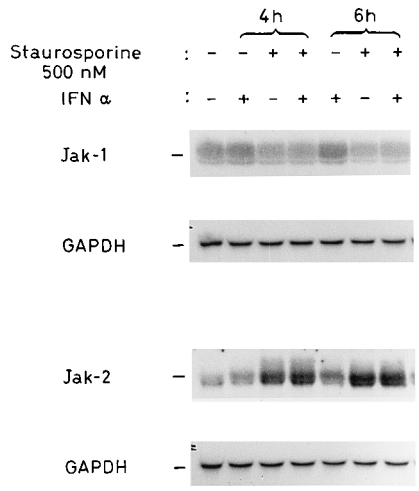

FIG. 2. Jak1 and Jak2 mRNA expression; Northern blot assay of total RNA from FLC treated as described in footnote *a* to Table 1 with IFN-α (500 U/ml) and/or staurosporine (500 nM). RNA samples were electrophoresed, transferred onto nylon filters, and hybridized to the labeled Jak1, Jak2, and GAPDH cDNAs (see Materials and Methods).

Interestingly, treatment of the cells with 500 nM staurosporine appeared to reduce the specific tyk2 mRNA level at 2 h of treatment, both in the presence and in the absence of IFN. The densitometric analysis of the specific band, normalized with respect to the GAPDH control, shows a major reduction (between 2- and 10-fold) after 6 h of treatment. In addition, the RNA samples were analyzed by Northern (RNA) blotting for Jak1 and Jak2 gene expression by using randomly primed, ^{32}P-labeled (1.5×10^6 cpm/ml) murine Jak1 (54) and Jak2 (40, 52) (Fig. 2). We analyzed the expression of Jak1 mRNA after 4 and 6 h of treatment with IFN-α and/or 500 nM staurosporine. The steady-state level of Jak1 mRNA appeared downregulated under these experimental conditions (Fig. 2) mainly after 6 h of treatment, as observed for tyk2. Surprisingly, the same staurosporine treatment appeared to significantly increase the level of Jak2 mRNA (Fig. 2) at 4 and 6 h. The specific bands were densitometrically analyzed with respect to the corresponding GAPDH mRNA, which was used as an internal loading control. In addition, no significant stimulation of either Jak1 or Jak2 mRNA was observed in response to IFN-α. The relative amounts of RNA extracted were found to be constant under each experimental condition, thus showing that the high dose of staurosporine used did not randomly affect RNA synthesis. In addition, the staurosporine treatment did not affect cell viability.

Effect of staurosporine on tyrosine phosphorylation and expression of Jak proteins. The activation of Jak proteins in response to IFN-α, -β, and -γ requires hyperphosphorylation on tyrosine residues in a number of human and murine cell lines (4, 21, 38, 48, 51). Figure 3A shows tyrosine phosphorylation as a function of IFN-α or IFN-γ and/or staurosporine treatment. Proteins from whole-cell extracts were immunopre-

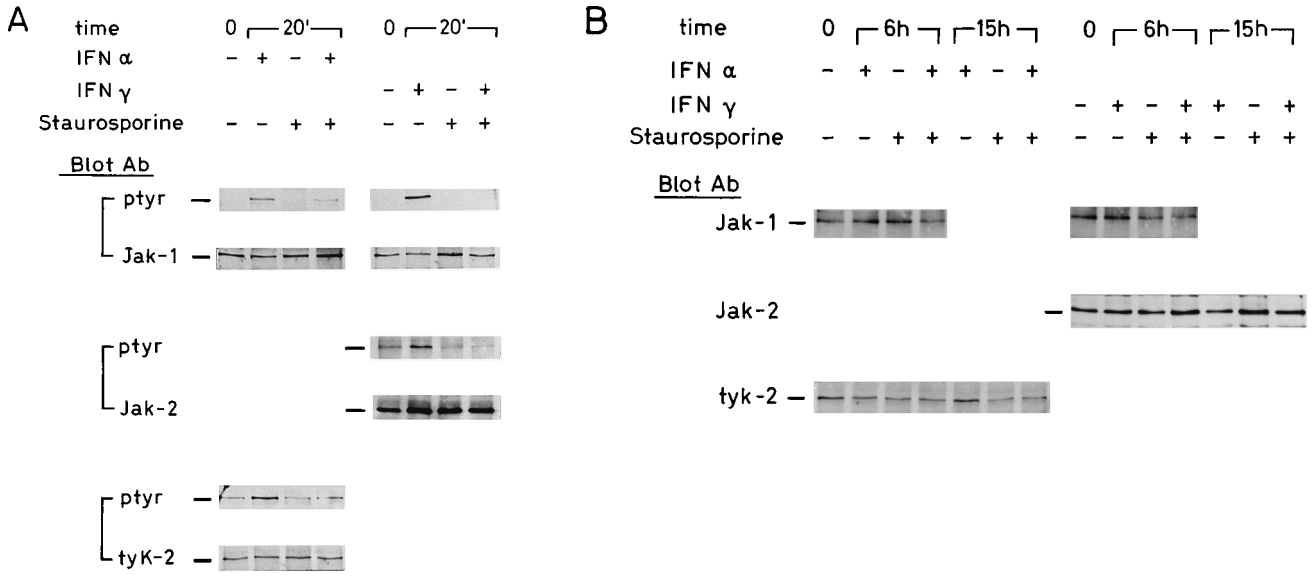

FIG. 3. Jak1, Jak2, and tyk2 immunoprecipitation-immunoblotting analysis; tyrosine phosphorylation (ptyr) and protein quantitation of Jak proteins in FLC treated with IFN-α or -γ (500 U/ml) and/or staurosporine (500 nM) for the indicated times. Recombinant IFN-γ (10^7 U/mg protein) was produced by Genentech and was kindly provided by Boehringer Ingelheim. (A) Proteins from whole-cell lysates immunoprecipitated with specific anti-Jak1, -tyk2, and -Jak2 antibodies (Ab) (UBI) and analyzed by sodium dodecyl sulfate-7% polyacrylamide gel electrophoresis and Western blotting with the 4G10 (UBI) antibody to phosphotyrosine and, after stripping, with the corresponding anti-Jak antibodies as indicated. (B) Proteins from whole-cell lysates immunoprecipitated with specific anti-Jak1, -tyk2, and -Jak2 antibodies (UBI) and analyzed by sodium dodecyl sulfate-7% polyacrylamide gel electrophoresis and Western blotting with the corresponding anti-Jak protein antibodies (Ab) as indicated. Densitometric analysis shows a 2-fold increase in Jak2 protein levels induced by staurosporine and a decrease in Jak1 and tyk2 levels of up to 2.7-fold.

cipitated with the specific anti-Jak antibody and were analyzed by immunoblotting sequentially with the 4G10 antiphosphotyrosine monoclonal antibody and then by reprobing the membrane with the corresponding anti-Jak protein. Within a few minutes, treatment with IFN-α results in tyrosine phosphorylation of Jak1 and tyk2. Treatment with IFN-γ results in tyrosine phosphorylation of Jak1 and Jak2. The high dosage (500 nM) of staurosporine used inhibits phosphorylation of all three proteins.

Since staurosporine also appears able to affect mRNA levels of Jak kinases, in particular down-regulating Jak1 and tyk2 and increasing Jak2 mRNA, we analyzed the corresponding protein levels in the presence of staurosporine treatment. Immunoprecipitation and immunoblotting performed by using anti-Jak1, anti-Jak2, and anti-tyk2 antibodies showed that the protein levels essentially paralleled the specific mRNA modulation observed after staurosporine treatment, i.e., by showing an inhibition of Jak1 and tyk2 and an increase in Jak2 expression.

In summary, we observed that the antiviral effect induced by IFN-α was abolished when high doses of staurosporine were added to FLC. Staurosporine was originally described as an inhibitor of serine/threonine kinase; however, when used at high doses, it completely inhibits all protein kinase activities. The high dosage of staurosporine used is able to inhibit phosphorylation and thus activation of Jak proteins, blocking the IFN-induced antiviral effect. In fact, immunoprecipitation with the specific antibodies which was followed by Western blotting (immunoblotting) with antiphosphotyrosine and anti-Jak proteins antibodies positively identified the phosphorylated and dephosphorylated proteins as Jak kinases.

It is possible that the phosphorylation of each component of the IFN signal transduction pathway is the target of staurosporine treatment. It is noteworthy that staurosporine treatment also blocks activation of interferon responsive factor 1 (IRF-1) (49), which binds an upstream element in the 5′ promoter region of IFN-β and which was also shown to be involved in the activation of ISGs (15, 31). Thus, the block of posttranslational modification events of IRF-1 may also contribute to the inhibition of ISG transcription (data not shown) observed after staurosporine treatment.

Surprisingly, staurosporine also appears able to affect the mRNA levels of all three Jak proteins. tyk2 and Jak1 mRNA levels appear to be down-regulated by staurosporine. In contrast, the same treatment significantly increases the expression of Jak2 mRNA. These changes occurred independently of the presence of IFN. In fact, as previously reported for other systems, the levels of these transcripts did not change upon IFN treatment of FLC. The differential expression of the Jak mRNAs that we observed after staurosporine treatment indicates the existence of a regulatory mechanism involving phosphorylation and acting in the opposite way on the expression of Jak1 and tyk2 versus Jak2. The kinetics and magnitude of the protein levels essentially paralleled the corresponding mRNA modulation observed after staurosporine treatment.

The involvement of Jak proteins in response to other biological response modifiers such as growth hormone, leukemia inhibitory factor, ciliary neurotrophic factor, interleukin-3, and erythropoietin has been demonstrated elsewhere (2, 32, 40, 53). Other Jak family members may exist (recently, a rat Jak3 has been cloned and sequenced [43]), allowing for as many different kinases as there are different ligand-receptor-kinase complexes. Our experimental evidence indicates that a control of the expression of Jak family members exists and is mediated by phosphorylation events. However, it is too early to predict the role of such phosphorylation, since nothing is known about the regulation of these genes.

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