The Antiviral State Induced by Alpha Interferon and Gamma Interferon Requires Transcriptionally Active Stat1 Protein

CURT M. HORVATH AND JAMES E. DARNELL, JR.*

Laboratory of Molecular Cell Biology, The Rockefeller University, New York, New York 10021

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Both of the latent transcription factors activated by alpha interferon or gamma interferon contain the Stat1 protein. The role of Stat1 in virus interference mediated by interferons was directly examined by using cultured cells expressing Stat1 protein and its variants. In the absence of Stat1, no antiviral state is established. Full complementation of the antiviral state requires full-length Stat1 which is phosphorylated on both tyrosine 701 and serine 727. The closely related signal transducer and activator of transcription protein Stat3 cannot substitute for the antiviral properties of Stat1.

While it is clear that interferons (IFNs) have diverse effects, such as immune stimulation, tumor inhibition, and cell multiplication reduction, these extracellular signaling proteins were initially described as agents that interfere with virus replication (13). Both alpha and beta IFNs (IFN- α and IFN- β) and IFN- γ can cause diminished virus replication, presumably by initiating changes in gene expression that immediately follow the treatment of cells with IFN.

Although IFN-dependent virus interference relies on the failure of viral mRNA translation or of viral nucleic acid replication, the molecular basis for the antiviral state is still not completely understood. It is known that the establishment of the antiviral state requires cellular mRNA and protein synthesis after IFN- α or IFN- γ treatment (22, 29). IFN-induced proteins that may contribute to the antiviral state are known (17). For example, the double-stranded RNA-dependent enzyme 2',5'-oligoadenylate synthetase is synthesized in response to IFN and catalyzes the polymerization of ATP. These 2',5'-oligomers of ATP activate an RNase that can cleave cellular and viral RNAs (15, 16). A second induced activity that could have an antiviral effect involves the activation of a double-stranded RNA-dependent protein kinase (also known as protein kinase R [2]) that inhibits virus and host cell protein synthesis by directly phosphorylating the α subunit of the translation initiation factor eIF-2 (14, 28). A third possible mode of antiviral activity is mediated by the IFN-induced Mx protein. Mx homologs have been isolated from many species, and although the mechanism of their action is not clear, they have been associated with many activities, including GTP binding and protein sorting (8, 9, 20, 26). The effects of IFN in causing the antiviral state may depend on the synthesis of one or a combination of these and other proteins. For example, constitutive expression of the IFN-regulated transcription factor IRF 1 results in substantial resistance to virus infection, but this resistance is not as great as that provided by IFN itself (23).

Analysis of the promoters of many IFN-stimulated genes revealed response elements specific to either IFN- α (the IFN-stimulated gene response element) or IFN- γ (the gamma activation sequence). ISGF3 and GAF were identified as the protein factors that bind to these response elements and were found to contain two of the signal transducer and activator of

transcription family proteins. These factors have a common subunit, Stat1. The IFN- γ factor (GAF) is a homodimer of Stat1, while the IFN- α factor (ISGF3) is a heterodimer of Stat1 and Stat2 which binds to DNA in concert with a 48-kDa protein referred to as ISGF3 γ or p48 (5, 6, 30, 32; reviewed in reference 3).

Within minutes of the treatment of cells with IFN- γ , Stat1 protein is phosphorylated on tyrosine, homodimerizes via intermolecular SH2-phosphotyrosine interaction, and is translocated to the nucleus where it binds to the gamma activation sequence element in the promoters of target genes (31, 33, 34). The treatment of cells with IFN- α results in tyrosine phosphorylation of both Stat1 and Stat2. These factors heterodimerize, and the dimer is transported to the nucleus where it binds to the IFN-stimulated gene response element along with p48 (12, 25).

In these experiments, we examined the need for Stat1 as a fully competent transcription factor in the establishment of the antiviral state after IFN- α or IFN- γ treatment. Cells lacking Stat1 have no antiviral response to IFN, in agreement with earlier conclusions about IFN-induced transcription (19). Using a variety of cell lines carrying Stat1 or mutated versions of Stat1, we found that Stat1 is required for establishing the antiviral state in response to IFN and that Stat1 must be full length and phosphorylated on both tyrosine and serine residues for full IFN- γ antiviral activity. Full IFN- α antiviral activity requires only that Stat1 be phosphorylated on tyrosine, but either Stat1 α or Stat1 β (a form of Stat1 α lacking the 38-amino-acid transcriptional activation domain) suffices. Furthermore, the closely related Stat3 protein cannot substitute for Stat1 in mediating the antiviral effect.

Stat1 is required for the IFN- γ - or IFN- α -induced antiviral response. The human cell line U3A was isolated during a somatic cell genetic analysis designed to discover any protein required for IFN signal transduction (18, 21). U3A cells lack Stat1 protein or mRNA and are incapable of responding to IFN- γ or IFN- α (19). To assess the ability of IFN- γ or IFN- α to establish the antiviral state in U3A cells, we used a standard cytopathic effect (CPE) assay with vesicular stomatitis virus (VSV) strain Indiana (the generous gift of Richard Peluso, Mount Sinai School of Medicine) (4). Monolayers of cells were pretreated for 12 h with human IFN- α (500 IU/ml) or IFN- γ (5 ng/ml), washed, and then infected with dilutions of VSV. These concentrations of IFN have been found to maximally activate ISGF3 and GAF (11). After 24 h of VSV infection, no significant differences in the numbers of methylene blue-positive

* Corresponding author. Mailing address: Laboratory of Molecular Cell Biology, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-8791. Fax: (212) 327-8801.

FIG. 1. Reductions in the CPE of VSV by IFNs require biologically active Stat1 protein. The results of CPE assays with U3A cells and derivatives containing Stat1 proteins, as indicated, are shown. UNT, untreated.

viable cells were observed, regardless of IFN treatment (Fig. 1).

The complementation of U3A cells by permanent transfection with a Stat1α expression vector reconstitutes the IFN signal transduction pathway (19). To determine if the antiviral response to both IFN-α and IFN-γ is also reconstituted by the complementation of U3A cells with Stat1α, CPE assays were performed with this cell line. Approximately 10- to 50-fold-more virus was needed to reach the same CPE endpoint when Stat1α-complemented cells were pretreated with IFN-γ for 12 h prior to infection with VSV compared with control untreated cells. More dramatically, when cells were pretreated with IFN-α, approximately 500-fold-more virus was needed to reach the CPE endpoint. Clearly, the ability to establish the antiviral state in response to IFN-γ and IFN-α is dependent on the presence of Stat1 protein. Another indication of the antiviral effect is the amount of virus produced after the infection of cells at a low multiplicity. U3A and Stat1α-complemented U3A cells were pretreated with IFN for 8 h and then infected with VSV at a multiplicity of 0.01 PFU per cell. At 1 h postinfection, cells were washed and fresh medium was added. Incubation was continued for 24 h, supernatants were harvested, and the titers of the virus were determined by plaque assays with untreated U3A cells. The presence of Stat1 in U3A cells correlates with a reduction in virus yield for cells pretreated with IFN-γ or IFN-α, and the level of reduction correlates with the intensity of CPE reduction by IFN-γ or IFN-α (Fig. 2).

Complementation of U3A with Stat1β reconstitutes the antiviral state for IFN-α but not for IFN-γ. Stat1β is derived from Stat1 mRNA by alternate splicing and lacks the 38 carboxyl-terminal amino acids found in Stat1α (30, 36). This truncated form of Stat1 is fully competent to form a heterodimer with Stat2 and reconstitute transcriptionally active ISGF3 after IFN-α treatment (19). Homodimers of Stat1β formed in response to IFN-γ are transported to the nucleus, where they bind DNA but lack the ability to activate transcription because of the absence of the carboxyl-terminal transcription activation domain (34). To assess the role of Stat1β in the antiviral response, U3A cell lines complemented with Stat1β were analyzed by the CPE assay. The virus titers needed for the maximal CPE were identical for untreated and IFN-γ-treated cells,

consistent with the lack of IFN-γ-dependent transcriptional activity supported by Stat1β. In contrast, pretreatment of Stat1β-expressing cells with IFN-α clearly protected these cells from VSV infection, reducing the CPE by 500-fold, similar to the reduction observed for cells containing full-length Stat1α (Fig. 1). The reduction in virus yield by IFN-α treatment agreed with the result of the CPE assay (Fig. 2). This result is consistent not only with the ability of Stat1β to participate in transcriptionally active ISGF3 but also with the conclusion that the transcriptional activation domain of the IFN-α-stimulated factor, ISGF3, is contributed by Stat2 (19, 24).

Complementation of U3A with mutant Stat proteins restricts the antiviral response. The Stat pathway has been analyzed in some detail by expressing mutated Stat1 proteins in permanently transfected U3A cells and analyzing the abilities of these mutant proteins to form transcriptionally competent DNA binding complexes. The IFN-induced antiviral responses of two of these cell lines were examined. Changing the single

FIG. 2. Inhibition of VSV replication in response to IFNs requires biologically active Stat1 protein. The VSV titers determined by plaque assay with U3A cells from a typical experiment are presented. See the text for details. UNT, untreated.

target tyrosine residue that becomes phosphorylated in response to IFN treatment to a phenylalanine residue (Stat1 α Y₇₀₁F) results in a Stat1 protein which is not phosphorylated on tyrosine, does not dimerize, and remains in the cytoplasm after IFN stimulation (34). When U3A cells containing Stat1 α Y₇₀₁F were assayed for CPE inhibition, no differences between control untreated cells and IFN-treated cells were observed (Fig. 1). Furthermore, no differences in the amounts of virus released in these cells were observed (Fig. 2). This result indicates that virus interference depends on the presence of not merely Stat1 protein but a Stat1 protein that can be activated by phosphorylation.

Another mutant Stat1 α protein, which contains a single serine-to-alanine substitution at position 727 (Stat1 α S₇₂₇A), was examined. Recently, IFN-induced phosphorylation of this serine residue was shown to be required for maximal IFN- γ transcriptional induction. The loss of serine phosphorylation at this position reduces the accumulation of IFN- γ -responsive mRNA by approximately 80%, but the Stat1 α S₇₂₇A protein is still phosphorylated on tyrosine, dimerizes, and binds to DNA in response to IFN- γ treatment (35). Like Stat1 β , this mutant does not affect IFN- α induction of the IFN- α transcription factor, ISGF3 (10). In agreement with the transcription results, U3A cells complemented with Stat1 α S₇₂₇A exhibit only about a 5-fold reduction in CPE response to IFN- γ but have a normal reduction in CPE response to IFN- α pretreatment (approximately 500-fold [Fig. 1]). While the virus yield for IFN- α -pretreated Stat1 α S₇₂₇A cells was greatly reduced, no significant differences in virus yield were detected for IFN- γ pretreatment, despite the fivefold protection from CPE (Fig. 2). Although the CPE assay is carried out at a high virus multiplicity, the yield experiment is performed at a low multiplicity and a single time point was assayed. Perhaps the 20% activity of Stat1 α S₇₂₇A in response to IFN- γ was sufficient to allow a more slowly growing virus to reach the same output level during the experiment.

Expression of Stat3 does not confer antiviral effects. Currently, the Stat protein family is known to contain six unique members (Stat1 to Stat6), which are activated in response to various extracellular signals. The closest relative of Stat1 is Stat3, the interleukin 6- and platelet-derived growth factor-inducible factor also described as the acute-phase response factor or *sis*-inducible factor (1, 27, 37). Although Stat3 can be activated by IFN- α in many cell lines and U3A cells contain low levels of Stat3, this endogenous factor is inactive in response to IFN- α in these serially mutagenized cells (7). If endogenous Stat3 is supplemented by transfection of a Stat3 expression vector, cell lines in which the Stat3 homodimer is activatable by IFN- α treatment can be selected (11). These Stat3-complemented U3A cells failed to produce an antiviral state in response to IFN. Neither protection from CPE nor a reduction in viral replication was observed in response to the treatment of these cells with IFN- α or IFN- γ . This result indicates that these closely related Stat proteins (Stat1 and Stat3) are not functionally redundant in their ability to establish the antiviral effect.

These experiments clearly demonstrate that the antiviral effect produced by IFN- α and IFN- γ requires immediate signaling through a biologically active Stat1 protein. Furthermore, these data indicate that there is no simple functional redundancy of closely related Stat proteins for the antiviral effect. Perhaps most importantly, the activities of Stat1 β and Stat1 α S₇₂₇A which have previously been well characterized *in vitro* have been shown here to be of functional relevance *in vivo*.

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