Controlling Nuclear Jaks and Stats for Specific Gene Activation by Ifnγ and Other Cytokines: A Possible Steroid-like Connection

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Abstract

The mechanism of specific gene activation by cytokines that use JAK/STAT signalling pathway is unknown. There are four different types of JAKs and seven different types of STATs. In the classical model of signaling, ligand interacts solely with the receptor extracellular domain, which triggers JAK activation at the receptor cytoplasmic domain. Activated STATs are then said to carry out nuclear events of specific gene activation, including associated epigenetic changes that cause heterochromatin destabilization. Ligand, receptor, and JAKs play no further role in the classical model. Given the limited number of STATs and the activation of the same STATs by cytokines with different functions, the mechanism of the specificity of their signalling is not obvious. Focusing on gamma interferon (IFNγ), we have shown that ligand, receptor, and activated JAKs are involved in nuclear events that are associated with specific gene activation. In this model, receptor subunit IFNGR1 functions as a transcription/coregulation factor and the JAKs are involved in key epigenetic events that are required for specific gene activation. The model has implications for gene activation in cancer as well as stem cell differentiation.

Introduction

The STAT transcription factors have been shown to be essential for signaling by a host of proteins, including the interferons (IFNs), most of the interleukins, growth factors such as platelet derived growth factor, and hormones such as growth hormone [1]. The prevailing view is that the ligand activates the cell solely via interactions with the extracellular domain of the receptor complex [1]. This in turn results in the activation of receptor or receptor-associated tyrosine kinases primarily of the Janus or JAK kinase family [1], leading to phosphorylation and dimerization of the STAT transcription factors, which then dissociate from the receptor cytoplasmic domain and translocate to the nucleus. This view ascribes no further role to the ligand or the receptor in the signaling process. Further, there is the implicit assumption that the STAT transcription factors possess intrinsic nuclear localization sequences (NLSs) that are responsible for nuclear translocation of STATs in the dimerized, phosphorylated state [1].

The IFNγ receptor on cells consists of two chains, IFNGR1 and IFNGR2, that are noncovalently associated [2]. IFNγ in an asymmetric dimeric form binds predominantly to two IFNGR1 chains. The model contends that this cross-linking is responsible for the intracellular events that occur on the cytoplasmic domains of the receptor chains. Tyrosine kinase JAK1 is associated with IFNGR1, while JAK2 is associated with IFNGR2. IFNγ binding results in JAK2 moving from IFNGR2 to IFNGR1, where a sequence of events causes autophosphorylation of the JAK kinases, tyrosine phosphorylation of IFNGR1, followed by the recruitment of STAT1α and its subsequent tyrosine phosphorylation. Here, phospho-STAT1α forms a dimer, dissociates from the receptor complex, and goes to the nucleus, presumably via an intrinsic nuclear localization sequence (NLS). Structure studies have shown that dimeric STAT1α binds to the GAS element of IFNγ promoter [3], and this finding has been interpreted as validation of the above model. Recent studies have shown however that contrary to the original assumptions, monomeric IFNγ can also stimulate the activation of STAT1α [4,5]. This raises the question of whether cross-linking of IFNGR1 is the determining event in subsequent signal transduction of IFNγ. Further, there are several reports that STAT1α contains a novel intrinsic NLS but there is disagreement concerning its properties and nothing is presented as to how it functions in the complex low/high affinity binding nature of the nuclear import apparatus [6].

The classical model of JAK/STAT signaling with modifications

It has recently been acknowledged that the classical model of JAK/STAT signaling was over simplified in its original form (Figure 1A, ref. 7). In the case of IFNγ, complexity beyond simple JAK/STAT activation in signal transduction is indicated in the relatively recent demonstration that other pathways, including MAP kinase, PI3 kinase, Cam kinase II, NF-KB, and others cooperate with or act in parallel to JAK/STAT signaling to regulate IFNγ effects at the level of gene activation and cell phenotypes (Figure 1B, ref. 7). All of these pathways are generic in the sense that a plethora of cytokines with functions different from those of IFNγ also activate them. Thus, for IFNγ and other cytokines, uniqueness of function would seem to depend on cytokine control of complex and unique qualitative, quantitative, and kinetic aspects of the activation of these pathways. We are not aware that this has been demonstrated for any cytokine.

There is evidence of a functional interaction between STATs in gene activation/suppression, which provides more insight into STAT mediation of cytokine signaling. The induction of IL-17 by activated STAT3, for example, was countered by IL-2 activation of STAT5 [8]. It was demonstrated by chromatin immunoprecipitation (ChIP)
sequencing that STAT3 and STAT5 bound to multiple common sites across the IL-17 gene locus, including non-coding sequences. Nothing was presented as to the activation state of these STATs. Activation of STAT5 by IL-2 resulted in more binding of STAT5 and less binding of STAT3 at these sites, whereas activation of STAT3 by IL-6 induced the opposite; the combination of the two STATs resulted in dynamic regulation of the IL-17 gene locus by the opposing effects of IL-2 (STAT5) and IL-6 (STAT3) [8]. A similar complementarity was observed with STAT4 and STAT6 with respect to Th1 and Th2 cell development, but with much less competition for binding sites at coding and non-coding regions of the gene [9]. These Yin-Yang interactions of STAT transcription factors are referred to as specification with respect to lymphocyte phenotypes. Important questions, however, are not addressed with respect to claims of specification and signaling specificity. For example, IL-6, IL-23, and IL-27 all activate STAT3 and are all involved in Th17 induction/differentiation and function [10-12]. Additionally, it has been shown that IL-23 receptor is required for terminal differentiation of IL-17-producing effector T helper cells [13]. Thus, STAT3 does not seem to be the only factor required for activation and generation of Th17 cells. Rather, the requirements of IL-6 and IL-23 for Th17 induction/differentiation and IL-27 for suppression all involve activated STAT3 mediation through multiple unique ligand/receptor interactions. Interestingly and contrary to the above report, it has been demonstrated that IL-2 participates in expansion of Th17 cells in uveitis and scleritis [14].

It is difficult to deal with disparate specificities of highly similar cytokines such as IL-12 and IL-23 in the restricted context of the STATs. IL-12 and IL-23 are members of the IL-12 family of cytokines. They are both produced primarily by macrophages/dendritic cells. Both IL-12 and IL-23 are heterodimers. The IL-12 ligand is composed of p40/p35 subunits, while IL-23 is composed of p40/p19 [15,16]. Thus, the cytokines share the p40 subunit. The heterodimeric receptor for IL-12 is IL-12Rβ1/IL-12Rβ2, while that for IL-23 is IL-12Rβ1/IL-23R. Thus, the cytokines also share a receptor subunit. At the level of JAK/STAT signaling, both IL-12 and IL-23 use JAK2 and TYK2, and both activate STAT1, STAT3, STAT4, and STAT5. It is stated that activated STAT4 is key to IL-12 specific signaling while activated STAT3 is key to IL-23 signaling [17,18]. IL-12 is key to T helper 1 (Th1) cell phenotype, while IL-23 is key to T helper 17 (Th17) cell phenotype [18]. Th17 cells are of high interest in autoimmune neuropathies such as multiple sclerosis and their EAE animal models [13]. IL-23R has been shown to be absolutely key to Th17 cell function [13]. As indicated, STAT3 has been proposed to be key to IL-23 signaling, but other players in Th17 signaling such as IL-6 and IL-21, also activate STAT3 [19].

The retinoic acid orphan nuclear receptor RORγt is critical for T cell development and mice with RORγt deficiency have reduced Th17 cell differentiation [20,21]. RORγt in conjunction with STAT3 has been proposed as a central player in Th17 cell differentiation [22]. If activation of STAT3 is the key function of IL-23, then why can’t IL-6, IL-21, or even IL-12 replace or compensate for the IL-23R requirement? Thus, the connection between IL-23 and the events of specific gene activation remain to be determined and are not addressed currently with this cytokine, notwithstanding recent results on ChIP sequencing in terms of STAT3 in Th17 cell specification [8]. One could come to a similar dichotomy with respect to IL-12 and Th1 cells.

**Figure 1**: Classical model of interferon signaling. A. Signaling through the transcription factors STATs. Binding of the cytokine to its cognate receptor begins a series of interactions that through the participation of the tyrosine kinases, JAK1 and JAK2, result in the phosphorylation of STATs. STATs are then translocated to the nucleus to activate specific genes. B. Alternate IFNγ signaling pathways. Multiple pathways through MAP kinase and IKK/NF-κB signaling can activate the genes involved. See ref. 7 for details.
Role of IFNγ, IFNGR1, STAT1α, and JAKs in IFNγ signaling: A more complex model

IFNγ has been known for some time to translocate to the nucleus of receptor-expressing cells with kinetics as rapid as those for the activation and nuclear translocation of the inducible transcription factor STAT1α that it activates [23,24]. More recently, IFNγ nuclear translocation has been shown to be driven by an NLS (RRKKRRSSR) in its C terminus that is similar to the prototypical SV40 T-ag NLS (PKKKRKV) [25]. Mutations of the IFNγ NLS destroy its biological activity [26], which can be restored by reconstitution with the T-ag NLS [27]. The Tag NLS is known to localize in the nucleus in an importin (IMP) α/B/Ran-dependent fashion; excess T-ag NLS peptide inhibits IFNγ NLS-dependent nuclear import, suggesting that the IFNγ NLS mediates nuclear import through the same pathway [25]. Results from immunoprecipitation experiments, which detected endocytosed IFNγ bound to IMPα5 (NPI-1) in cells actively transporting IFNγ to the nucleus, are consistent with this conclusion [28-30]. Subsequent experiments showed that the receptor α-subunit, IFNGR1-1, of the heterodimeric IFNγ receptor also translocates to the nucleus in IFNγ-treated cells, in contrast to the β-subunit (IFNGR2), which remains in the plasma membrane [26,29,31]. Uptake of IFNγ is a receptor-mediated endocytic process, recent studies indicating that plasma membrane lipid microdomains are primary sites for the endocytic events leading to nuclear translocation of IFNγ/IFNGR-1 as well as of STAT1α [32]. The trafficking of IFNγ, the role of its NLS and how this relates to signal transduction/function have been the subject of recent studies. The IFNγ NLS is known to contribute minimally to extracellular high-affinity receptor–ligand binding, but the C-terminal domain (including the NLS) appears to be able to interact with the intracellular cytoplasmic domain of IFNGR1 (residues 253–287) of the IFNγ–receptor complex [29]. This binding, which requires the NLS, also increases the affinity of the Janus family kinase JAK2 for IFNGR1 [33].

The IFNγ NLS has also been found to be required for internalization of IFNγ into the cell, even though, as indicated above, it contributes minimally to high affinity receptor binding. Intracellular expression of a full-length non-secreted form of IFNγ can also affect IFNGR1 nuclear translocation, activation and nuclear translocation of STAT1α, as well as induction of biological activities normally elicited by addition of extracellular IFNγ [26]. Intracellular expression of an IFNγ mutant where the basic residues of the NLS were replaced with alanines failed to induce nuclear translocation of IFNGR1 or STAT1α, and led to a loss of IFNγ activities [26]. This suggests that the IFNγ NLS functions intracellularly, mediating interaction with specific intracellular components critical for IFNγ activity [26]. Unlike the strict species specificity displayed by extracellular IFNγ, where mouse IFNγ will not act extracellularly on human cells and vice versa, there appears to be no species specificity in terms of the response to intracellularly expressed IFNγ [26,29]. In this regard, previous studies have shown that the cytoplasmic domains of human and mouse IFNGR1 are interchangeable with respect to extracellular IFNγ function [2]. This cross-species functionality of intracellular IFNγ further highlights the fact that the cytoplasmic domain of IFNGR1 is the key target of intracellular interaction in the cytosol.

An intracellular excess of a peptide representing the cytoplasmic binding site on IFNGR1 for the C terminus of IFNγ, IFNGR1 (253–287), prevented the complexation of internalized IFNγ with the cytoplasmic domain of cell surface IFNGR1 in cells that were actively internalizing IFNγ [28,29]. Moreover, such cells were also blocked with respect to the tyrosine phosphorylation of STAT1α. Thus, internalized IFNγ appears to be able to interact with the cytoplasmic domain of IFNGR1 in intact cells as part of the signal transduction events leading to STAT1α tyrosine phosphorylation.

The IFNGR1 cytoplasmic domain would be on the outer surface of the endocytic vesicle following endocytosis, which suggests that IFNγ can traverse the membrane of the endocytic vesicle during internalization to contact the cytoplasmic domain of IFNGR1. Cytosolic injection of antibodies to IFNγ amino acids 95–132 blocks STAT1α nuclear translocation in response to extracellular IFNγ [28], consistent with these observations. This further supports the idea that the C terminus of endocytosed IFNγ accesses the cytosol, although the mechanism is as yet undetermined. The requirement of the IFNγ NLS for internalization, binding to the cytoplasmic domain of IFNGR1, activation of JAK2 and STAT1α, and nuclear translocation of activated STAT1α and IFNGR–1 suggests that some or all of these processes may be coupled, presumably through the NLS. Consistent with this, it has been observed that, after internalization, extracellular IFNγ could be recovered directly associated with IMPα5 in a cytosolic complex of IFNγ/IFNGR1/phosphorylated STAT1α [26]. The formation of the complex was dependent on the IFNγ NLS. Similar results have been obtained with intracellular expression of nonsecreted full-length IFNγ, which, as outlined above, induces nuclear translocation of IFNGR1 and STAT1α [26]. Intracellular expression of a non-secreted, NLS-mutated IFNγ fails to induce complexation of IFNγ, IFNGR1 or STAT1α with IMPα5 and nuclear transport of STAT1α and IFNGR1 [26].

Others have reported the direct association of phosphorylated STAT1α in complexes with IMPα5 [34-36]. All of these NLSs were non-classical and there was no agreement with respect to the fact that they were different for all three reports. These differences have not been addressed, nor has the low binding affinity when compared to the classical polycationic NLS [6].

The clear implication of the above is that the IFNγ NLS plays a direct role in STAT1α nuclear transport at least in the context of specific gene activation. Thus, a complex of IFNγ/IFNGR1/STAT1α with IMPα5, mediated by the IFNγ NLS, provides the link between the nuclear translocation of IFNGR1 and STAT1α and that of IFNγ, implying that one important function of nuclear transport of IFNγ may be to chaperone the nuclear transport of activated STAT1α to specific genes. A model representing the events in direct involvement of IFNγ and IFNGR1 in signal transduction is presented in (Figure 2). Epigenetic aspects of this model are discussed in the following section.

Gene activation and associated epigenetic events

We have identified the nuclear targets of IFNγ and IFNGR1. By ChIP followed by PCR, IFNγ, its receptor subunit IFNGR1, and STAT1α were found to be associated with the IFNγ-activated sequence (GAS) in the promoter of two of the genes stimulated by IFNγ [37]. Immunoprecipitated chromatin also showed the association of the IFNγ, IFNGR1, and STAT1α on the same DNA sequence. Examination of nuclear extracts from WISH cells treated with IFNγ revealed the specific binding of IFNγ, IFNGR1, and STAT1α to biotinylated GAS nucleotide sequence. Association of IFNγ, IFNGR1, and STAT1α with the GAS promoter was also demonstrated by EMSA. Transfection with a GAS-luciferase gene together with the IFNGR1 and nonsecreted IFNγ resulted in enhanced reporter activity. In addition, IFNGR1

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fused to the yeast GAL4 DNA binding domain resulted in enhanced transcription from a GAL4 response element, suggesting the presence of a trans activation domain in IFNGR1. Our observations put IFNγ and its receptor subunit, IFNGR1, in direct contact with the promoter region of IFNγ-activated genes with associated increased activity, thus suggesting a transcriptional/cotranscriptional role for IFNγ/IFNGR1 as well as a possible role in determining the specificity of IFNγ action [37].

To address the possible epigenetic role of the JAKs that are activated, we carried out ChIP followed by PCR in IFNγ treated WISH cells and showed association of pJAK1, pJAK2, IFNGR1, and STAT1 with the same DNA sequence of the IFNγ promoter [38]. The β-actin gene, which is not activated by IFNγ, did not show this association. The movement of activated JAK to the nucleus and the IRF-1 promoter gene, which is not activated by IFNγ, did not show this association. The β-actin gene, which is not activated by IFNγ, did not show this association.

We have developed small peptide mimetics of IFNγ, based not on the classical model of IFNγ-initiated signaling by extracellular interaction but rather on direct intracellular signaling by IFNγ. IFNγ, its receptor subunit IFNGR1, and transcription factor STAT1α are transported to the nucleus of cells as a complex where IFNγ provides a classical polycationic NLS for such transport [26]. The C terminus of IFNγ, represented by the mouse IFNγ peptide, IFNγ(95-132), was capable of forming a complex with IFNGR1 and STAT1α when introduced intracellularly and provided the NLS signaling for nuclear transport [26]. Importantly, mouse IFNγ (95-132) and human IFNγ (95-134) mimetics both induced an antiviral state and upregulation of MHC class I molecules in cells similar to that of full length IFNγ [42,43]. Both IFNγ and its peptide mimetics bind to an intracellular site, IFNGR1(253-287), on the cytoplasmic domain of receptor subunit IFNGR1. This binding plays a role in tyrosine phosphorylation events, catalyzed by JAK1 and JAK2 kinases at both the cytoplasmic and nuclear levels that result in the phosphorylation and binding of STAT1α to the cytoplasmic domain of IFNGR1. Important structural requirements for IFNγ mimetic activity are a polycationic NLS and an α helix in the mimetics [44]. ChIP and reporter gene studies of IFNγ and IFNγ mimetic-treated cells indicate that they, along with IFNGR1 and STAT1α, bind to the IFNγ activation site element of IFNγ-activated genes and participate in STAT1α-mediated transcription [37]. IFNγ intracellular events played the key role in development of IFNγ mimetics [33,42]. In contrast to intact IFNγ, therefore, the mimetics do not bind to poxvirus B8R protein and can thus initiate an antiviral response in the presence of B8R protein in cell culture.
The fact that vaccinia virus vectors and vaccinia virus with inactivated B8R protein are attenuated in their virulence for mice, would suggest that the low affinity interaction is still biologically significant [48,49]. This assumes the production of relatively high concentrations of B8R protein in vivo by vaccinia virus, which would enhance the binding to and neutralization of mouse IFNγ involving the law of mass action [50]. Thus, by bypassing the virulence factor B8R, the IFNγ mimetic was able to convert an aggressive infection in mice into a rather benign infection. The data summarized here with IFNγ mimetic and a poxvirus provides specific evidence of an effective IFN mimetic against poxviruses. The fact that the IFN mimetic was developed based on our signal transduction studies with IFNγ is evidence of the dynamics of our model. Such dynamics are not obvious in the classical model of IFNγ signaling.

**Conclusion**

There is much insight into the mechanism of specific gene activation by steroid hormones. Steroid hormone receptors are intracellular proteins that are activated by internalized steroid hormones [51]. Receptor activation involves phosphorylation at sites that contain Ser/thr-Pro motifs. This results in nuclear localization and specific gene activation where the hormone/receptor complex and associated kinases function as transcription/co-transcription complexes. Our studies with IFNγ signaling as well as that of others with EGF receptor, growth hormone receptor, and prolactin receptor suggest that similar mechanisms of specific gene activation apply to transmembrane receptors that use the JAK/STAT pathway of signal transduction [6,52,53]. While it is difficult to approach the mechanism(s) of specific gene activation by JAK/STAT via the classical model, our model readily provides insight into such mechanism(s). It is but a variation of specific gene activation by steroid hormones.

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