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I IFN receptor controls activated TYK2 in the nucleus: Implications for EAE therapy

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Abstract

Recent studies have suggested that activated wild-type and mutant Janus kinase JAK2 play a role in the epigenetics of histone modification, where it phosphorylates histone H3 on tyrosine 41(H3pY41). We showed that type I IFN signaling involves activated TYK2 in the nucleus. ChIP-PCR demonstrated the presence of receptor subunits IFNAR1 and IFNAR2 along with TYK2, STAT1, and H3pY41 specifically at the promoter of the OAS1 gene in IFN treated cells. A complex of IFNAR1, TYK2, and STAT1 α was also shown in the nucleus by immunoprecipitation. IFN treatment was required for TYK2 activation in the nucleus. The presence of IFNAR1, IFNAR2, and activated STAT1 and STAT2, as well as the type I IFN in the nucleus of treated cells was confirmed by the combination of Western blotting and confocal microscopy. Trimethylated histone H3 lysine 9 underwent demethylation and subsequent acetylation specifically in the region of the OAS1 promoter. Resultant N-terminal truncated IFN mimetics functioned intracellularly as antivirals as well as therapeutics against experimental allergic encephalomyelitis without the undesirable side effects that limit the therapeutic efficacy of IFN β in treatment of multiple sclerosis. The findings indicate that IFN signaling is complex like that of steroid signaling.

Keywords

interferon alpha; interferon tau; nuclear TYK2; histone modification; steroid-like signaling;
multiple sclerosis

1. Introduction

The classical model of cytokine signaling dominates our view of specific gene activation by cytokines such as the interferons (IFNs) (Levi and Darnell, 2002). In this model, ligand activates the cell solely via interaction with the extracellular domain of the receptor complex. This in turn results in the activation of receptor or receptor-associated tyrosine kinases, primarily of the Janus or JAK kinase family, leading to phosphorylation and dimerization of the STAT transcription factors, which then disassociate from the receptor

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Disclosures

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cytoplasmic domain and translocate to the nucleus. This view ascribes no further role to the ligand, JAKs, or the receptor in the signaling process. Further, there is the suggestion that the STAT transcription factors possess intrinsic nuclear localization sequences (NLSs) that are responsible for nuclear translocation of STATs and specific gene activation (reviewed in Johnson et al., 2004).

It has recently been acknowledged that the classical model of JAK/STAT signaling was over-simplified in its original form, and that other ubiquitous pathways, including MAP kinase, PI3 kinase, CaM kinase II, and NF- κ B cooperate with or act in parallel to JAK/STAT signaling to regulate IFN γ effects on the cell (Gough et al., 2008). At the STAT level, there is evidence of a functional interaction between different STATs in gene activation/suppression, which provides more insight into STAT mediation of cytokine signaling (Yang et al., 2011). It is not clear, however, as to how these STAT interactions at the level of DNA binding translate into specific gene activation by the inducing cytokine.

There is evidence that JAK kinases, including the wild type and mutant JAK2V617F, play an important role in the epigenetics of gene activation in addition to STAT activation in the cytoplasm, where tyrosine 41 on histone H3 is phosphorylated (H3pY41) (Dawson et al., 2009). We have shown in the case of IFN γ that receptor subunit IFNGR1 is associated with activated JAK2, pJAK2, which phosphorylated histone H3Y41 at the promoter of the IRF1 gene, while the β -actin gene was unaffected, since it is not acted on by IFN γ (Noon-Song et al., 2011). In this report, we focus on activated TYK2, pTYK2, in the nucleus and at promoters of genes activated by type I IFNs. TYK2 is also activated by other cytokines such as IL-12 and IL-23, which have biological effects different from IFN (Jones et al., 2011; Duvallet et al., 2011). We were therefore particularly interested in whether there was an association between pTYK2 and type I IFN receptors at the promoters and chromatin of genes activated by these IFNs and whether such association provided insight into pTYK2 induced specific epigenetic events in genes activated by the IFNs. The findings provide insight into the mechanism of specific gene activation by type I IFNs, including the epigenetic events associated with JAK activation.

Multiple sclerosis is a T cell-mediated autoimmune disease that targets the myelin sheath of neurons of the central nervous system (Castro-Borrero et al., 2012, Burks et al., 2005; Kieseier, 2011). MS is a well-known example of dysregulation of the immune system where, for reasons not fully understood, the regulatory arms of the immune system fail in 250,000 to 400,000 Americans (2 million to 2.5 million world-wide) (Kieseier, 2011). Patients diagnosed with MS are classified into four types: relapsing-remitting, secondary-progressive, primary-progressive, and progressive-relapsing. More than 80% of MS patients begin with relapsing-remitting cycles, and 50% of patients within 10 years progress to having only partial remissions characterized as secondary progressive (Ebers et al., 2004). About 15% of patients have no periods of remission, referred to as primary-progressive (Trapp and Nave, 2008). Patients presenting with steadily worsening symptoms and attacks during remissions are characterized as the rare form of MS progressive-relapsing. There is no available cure for MS; however, the treatments for relapsing-remitting MS patients focus on expediting recovery from attacks, minimizing the number of attacks, reducing the number of brain lesions, and slowing disease progression (Trapp and Nave, 2008).

The first line treatment of relapsing-remitting MS is an interferon beta. There are four approved human recombinant IFN β therapies: Betaseron®, Extavia®, and Rebif® subcutaneously administered, and Avonex® intramuscularly delivered. Although not completely understood, the mechanisms of action of IFN β include an increase in anti-inflammatory IL-10 levels, reduction of proinflammatory IL-17 and osteopontin, modulation of matrix metalloproteinase activities that hinder leukocyte migration across the blood brain

barrier, promotion of repair factor secretion for traumatic brain injury, and an increase in CD56^{bright} natural killer cells (Kieseier, 2011). There is a growing body of literature indicating that increasing IFN β dose and frequency provides greater therapeutic benefit (Schwid et al., 2005). The most common side effects, flu-like symptoms and injection site reactions, result in tolerability issues and poor adherence to the prescribed dose (Giovannoni et al., 2012). The flu-like symptoms could be attributed to up-regulation of inflammatory cytokines and mediators, including interleukin-6, IFN γ , and prostaglandins (Brod et al., 1996; Dayal et al., 1995; Arnason and Reder, 1994). In addition, IFN β treatments often asymptotically alter hepatic functions, such as increasing alanine aminotransferase, in a dose related manner. Clinicians must weigh increased efficacy of higher more frequent doses with risk of hepatic complications, patient tolerance, and patient adherence to therapy (Francis et al., 2003).

N-terminal truncated type I IFNs (mimetics) were generated based on the resultant model that possessed potent therapeutic efficacy against experimental allergic encephalomyelitis (EAE) without the undesirable side effects associated with type I IFN β therapy of multiple sclerosis (MS). Type I IFN mimetics thus provide an approach to MS therapeutic where the dose is not limited by toxic side effects (Francis et al., 2003; Schwid et al., 2005).

Materials and methods

1.1. Cell culture and Reagents

WISH and Daudi cells were purchased from American Type Culture Collection (ATCC) and were grown in MEME and RPMI (Sigma-Aldrich), respectively, with 10% FBS and antibiotics. For all experiments, cells were serum starved for at least 4 hours, washed twice with PBS and then given serum free media with or without 1,000 U/ml IFN α 2 (Calbiochem) or IFN τ (Pepgen, Alameda, CA). The following polyclonal antisera were purchased from Santa Cruz Biotech: IFNAR1, STAT1, pSTAT1, STAT2, pSTAT2, TYK2, pTYK2, normal rabbit IgG, β -Tubulin, β -Lamin, and Histone H3. The following polyclonal antisera were purchased from Active Motif: H3K9ac and H3K9me3. Polyclonal antibodies to IFNAR2 were from PBL Interferon source. Additional Abs to TYK2 and IFNAR1 were also purchased from BD Bioscience and Epitomics, respectively. We produced the antibody to tyrosine phosphorylated histone H3 by immunization of rabbits with histone H3 peptide, ³³GGVKKPHRpYRPGTVALREIR, with a phosphate at tyrosine 41. We tested antibodies to some proteins from different sources to monitor the specificity.

1.2. Isolation of nuclei

IFN treated WISH cells were washed twice in cold PBS, removed by scraping in lysis buffer (10 mM HEPES pH 7.9, 100 mM KCl, 1% Triton X-100, 1 mM NaF, 1 mM Na₃VO₄, 2 mM MgCl₂, 1 mM DTT, and 1 mM PMSF), and pelleted by low speed centrifugation. The supernatant was saved as cytoplasmic fraction. The pellet containing intact nuclei, was gently resuspended in lysis buffer. The centrifugation, re-suspension, and decanting was then repeated twice more. Isolated nuclei were confirmed by trypan blue staining.

1.3. Western Blot analysis and immunoprecipitation

Western Blot analysis and Immunoprecipitation were carried out exactly as we previously described (Noon-Song et al., 2011).

1.4. GFP fusion constructs and microscopy

The coding sequence from IFN τ , IFNAR1, or IFNAR2 was used to generate a PCR product that was fused in frame with the C terminus of humanized rGFP in the plasmid phrGFPII-C (Stratagene). WISH cells that were grown on coverslips to near 30% confluency in a 35 mm

dish were transfected using lipofectamine (Invitrogen), with 3 µg of the empty vector or the IFN τ fused GFP vector followed by fixing with 2% paraformaldehyde in PBS, and confocal microscopy. IFNAR1 or IFNAR2 sequences fused to GFP were similarly transfected into cells. Where indicated, IFN τ was added at 1,000 U/ml. Next day, cells were fixed similarly and viewed in a Zeiss Axiovert Zoom confocal microscope using LSM Pascal software, as described before (Ahmed and Johnson, 2006).

1.5. Chromatin immunoprecipitation qPCR (ChIP-qPCR) assay

WISH cells were treated with type I IFN for 1 hr. Cells were then washed twice with cold PBS and treated with 1% formaldehyde for 10 min at 37°C (Ahmed and Johnson, 2006). The rest of the procedure was conducted using the ChIP kit from Millipore, as per the manufacturer's protocol. Control IgG, or different Abs, were used for each immunoprecipitation as indicated. DNA fragments eluted were used for qPCR using the iQ SYBR Green Supermix (Bio-Rad) with the following primers that spanned the ISRE element in their promoters. Human OAS1 promoter region was amplified with the primers 5'-CATTGACAGGAGAGAGAGTG-3' (-147 to -133) and 5'-TCAGGGGAGTGTCTGATTG-3' (-17 to +3). As a control, PCR was conducted with the primers from the human β -actin promoter 5'-CTCGCTCTCGCTCTTTTTTTTTTTC-3' (-967 to -941) and 5'-CTCGAGCCATAAAAGGCAACT-3' (-844 to -864). PCR was carried out with a DNA Engine thermocycler equipped with a Chromo4 Continuous Fluorescence Detection System and OpticonMonitor software. The PCR conditions were as follows: heating at 95°C for 3 min, followed by 50 cycles at 95°C for 15 sec, 55°C for 30 sec, and 72°C for 15 sec. Copy number was normalized for the level of β -actin in each sample. All samples were run in triplicate.

2.6. Expression and purification of type I IFN mimetics

Type I IFN mimetics were expressed as follows. The coding sequence for human IFN α 1, IFN α 1(69–189), preceded by nine arginine (R9) residues (for cell penetration) was inserted in the bacterial expression vector, pET30a+. The coding sequence for human IFN β , IFN β (100–187), preceded by R9 was similarly inserted into pET30a+. As controls, the human IFN α 1(69–189) or IFN β (100–187) without the R9 were also inserted in pET30a+. *E. coli* BL21 (DE3) Rosetta strain was used to transform the expression sequence in pET30a+. After the bacterial growth had reached the mid-log phase, induction with 0.5 mM IPTG was carried out and growth continued for 4 hours. The proteins were purified by using the Ni-NTA His Bind Resin (Novagen). The His tag was removed by digesting with enterokinase. The purity of the protein was assessed by SDS-PAGE analysis and coomassie blue staining.

2.7. Antiviral assay

Antiviral assays were performed by using a cytopathic effect (CPE) reduction assay using encephalomyocarditis (EMC) virus. WISH cells (40,000 per well in a microtiter dish) were grown overnight. IFN α 1(69–189)R9, IFN β (100–187)R9, or their controls without the R9 sequence were added to cells at the concentrations indicated for 4 hr, followed by infection with EMCV (moi = 0.01). Virus was washed after one hr and cells were grown overnight. Cells were stained with crystal violet and read in a microtiter plate at 550 nm.

2.8. Induction of EAE, evaluation of clinical disease, and administration of peptides

Female SJL/J mice (6 to 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in standard SPF facilities. On day 1, SJL/J mice were injected with 300 µg/mouse bovine myelin basic protein (Invitrogen) emulsified in Complete Freund's Adjuvant with 8 mg/ml H37Ra *Mycobacterium tuberculosis* (Sigma-Aldrich) and injected subcutaneously into two sites at the base of the tail along with 400 ng/mouse pertussis toxin

(List Biological Laboratories Inc) in PBS i.p. On day 3, the pertussis toxin injection was repeated (Jager et al., 2011). Beginning on day 12 post-immunization, after lymphocyte infiltration of the CNS had begun, mice were administered the following treatments or peptides every other day via i.p. injection in 100 μ l final volume: PBS, IFN α 1(69–189)R9 (15 μ g/mouse), or IFN α 1(69–189) (15 μ g/mouse). The mice were monitored daily for signs of EAE and graded according to the following scale: 0, normal; 1, loss of tail tone; 2, hind limb weakness; 3, paraparesis; 4, paraplegia; 5, moribund; and 6, death. The Institutional Animal Care and Use Committee at the University of Florida approved all of the animal protocols mentioned here.

2.9 Apoptosis Assay

Apoptosis on IFN and IFN mimetic treated cells was performed as previously described (Subramanian et al., 1995). Briefly, WISH cells (150,000) were seeded in a 6 well plate and grown overnight. They were then treated with type I IFN mimetics (100 U/ml), or parent IFNs (100 U/ml) for 4 days. Cells were doubly stained with Annexin V and propidium iodide (PI), using the reagents from Invitrogen, and analyzed by flow cytometry to measure the extent of apoptosis. The data shown indicate the percentage of apoptosis based on cells staining for both Annexin V and PI from the analysis of 10,000 cells.

2.10 Measurement of IFN toxicity

To measure toxicity induced by IFN treatment in vivo, mice (C57BL/6, n=3) were injected i.p. with IFN β (10^3 U/mouse), IFN α (69–189)R9 (2×10^3 U/mouse), IFN β (100–187)R9 (2×10^3 U/mouse), or PBS on alternate days. Mice were weighed daily until day 11 to see the effects of treatment on body weight. On day 11, blood was drawn from facial vein and white blood cell (WBC) counts were enumerated using a hemacytometer. Differential WBC counts were performed on Wright-Giemsa-stained blood smears.

3. Results

3.1. Activated TYK2, JAK1, and receptor subunits IFNAR1 and IFNAR2 in the nucleus of type I IFN treated cells

We treated WISH cells with type I IFN IFN α 2. The focus was on the presence of activated TYK2, JAK1, and receptor subunits IFNAR1 and IFNAR2 in the nucleus of cells treated with a type I IFN. At a concentration of 1000 U/ml, IFN α 2 treatment resulted in the presence of both phosphorylated (activated) JAKs, pJAK1 and pTYK2, in the nucleus (Figure 1A). Non-phosphorylated TYK2 and JAK1 (data not shown) were constitutively present in the nucleus of untreated cells. Treatment with another type I IFN, IFN τ , similarly resulted in the presence of pTYK2 in the nucleus (data not shown). Both phosphorylated STAT1 α and STAT2 were detected in the nucleus only after treatment of cells with the IFNs (Figures 1A). To ascertain the purity of nuclear fractionations, β -tubulin and β -lamin were used as markers of nuclear and cytoplasmic fractions, respectively (Figures 1A, and 1B).

We also examined the movement of type I IFN receptor subunit IFNAR1 into the nucleus of WISH cells treated with the IFNs. For both IFN α 2 (Figure 1A) and IFN τ (data not shown), IFN treatment resulted in the presence of IFNAR1 in the nucleus. There were relatively low or trace amounts of IFNAR1 in the nucleus of untreated cells, which increased several fold after treatment with IFN α 2 (Figure 1A, 1C). Related to this, there was a dramatic increase in pTYK2 from undetectable levels in untreated cells. This is consistent with a low constitutive endogenous level of IFN β in untreated cells (Takoka et al., 2000; Taniguchi et al., 2008). We also determined that IFNAR2 similarly underwent nuclear translocation in IFN τ treated WISH cells (Figure 1B). The movement of IFNAR1 and IFNAR2 into the nucleus along

with the JAKs suggests an association of the two events. Consistent with these results, we have identified a functional nuclear localization sequence in IFNAR1 (Subramaniam and Johnson, 2004) and IFNAR2 (²⁸³RKKK; unpublished observation) and a putative NLS in TYK2 (Ragimbeau et al., 2001).

To further verify the movement of IFNAR1 and IFNAR2 into the nucleus of IFN treated cells as well as to determine if the type I IFN similarly underwent nuclear import, we carried out confocal microscopy with GFP fusion proteins. Specifically, WISH cells were transfected separately with CMV promoter driven constructs of IFN τ , IFNAR1, or IFNAR2 fused to GFP. As control, WISH cells were also transfected with vector containing only GFP. As shown in Figure 2 (top), IFN τ -GFP treated cells showed an increased presence of IFN τ -GFP in the nucleus, while control GFP was present throughout the cell. In IFNAR1-GFP transfected cells, treatment with IFN τ caused nuclear translocation, while untreated cells showed no preference of IFNAR1-GFP for the nucleus as shown in Figure 2 (middle). IFNAR2-GFP was similarly driven into the nucleus of cells treated with IFN τ as shown in Figure 2 (bottom). Thus, the type I IFN IFN τ and receptor subunits IFNAR1 and IFNAR2 all undergo increased nuclear translocation in cells treated with the IFN. These findings differ from the IFN γ system in that IFNGR1 translocated to the nucleus, while the IFNGR2 receptor subunit remained in the plasma membranes after IFN γ treatment of cells where it provided JAK2 to IFNGR1 (Ahmed and Johnson, 2006).

3.2. Recruitment of IFNAR1, IFNAR2, TYK2, and STAT1, along with phosphorylation of histone H3Y41 (H3pY41) at the ISRE in the promoter region of the OAS1 gene of cells treated with type I IFN

To determine if the type I IFN players of Figure 1 were specifically recruited to the promoter region of a gene activated by IFN α 2 in cells, we performed ChIP-qPCR assays. We picked the promoter region of oligoadenylate synthetase 1 (OAS1) spanning from nucleotides -147 to +3. This region contains an interferon sensitive response element (ISRE) that is activated by type I IFN, and does not contain the IFN γ activated sequence (GAS) within this region. This suggests that we are looking at elements that are associated with type I IFN induction. WISH cells were treated with 1000 U/ml of IFN τ for 1 hour and analyzed by ChIP of sonicated chromatin of approximately 500-bp fragments of DNA, followed by qPCR. Chromatin fragments were immunoprecipitated with antibodies to IFNAR1, IFNAR2, histone H3 tyrosine 41 (H3pY41), TYK2, and pSTAT1, followed by qPCR of the OAS1 promoter region extending from nucleotides -147 to 3. As a control, PCR product for the promoter of β -actin gene, -967 to -844, was chosen for ChIP analysis. IgG did not interact with the promoter containing complex as a control for non-specific binding. As shown in the ChIP-qPCR data of Figure 3, pSTAT1, IFNAR1, IFNAR2, TYK2, and H3pY41 were associated with the ISRE element of the OAS1 promoter in IFN α 2 treated cells. ChIP analysis followed by PCR and analysis of DNA on ethidium bromide stained gel did not show the association of pSTAT1, IFNAR1, IFNAR2, TYK2, and H3pY41 with β -actin promoter after IFN τ treatment (data not shown). The ChIP-qPCR data provide insight into the mechanism of specific gene activation as well as the associated H3pY41 epigenetic event of the type I IFN signaling and suggest that STAT is but one of the players in these complex events.

3.3. TYK2 associates with IFNAR1 in the nucleus of cells treated with type I IFN

We showed in Figure 3 above that TYK2 and H3pY41 were specifically associated with the OAS1 promoter in cells treated with a type I IFN. Since IFNAR1 is specific to type I IFN signaling and was present along with TYK2 at the OAS1 promoter, we asked the question as to whether TYK2 and IFNAR1 were associated in the nucleus of cells treated with a type I IFN, as this would suggest a basis of specificity. Accordingly, the human fibroblast cells

were treated with 1000 U/ml of IFN α 2 for 30 min, after which the cells were lysed and nuclear and cytosolic fractions were isolated. The nuclear and cytoplasmic fractions were immunoprecipitated with antibody to IFNAR1 and Western blotted with antibodies to IFNAR1, TYK2, activated STAT1 α (pSTAT1 α), and H3pY41. As can be seen in Figure 4A, nuclear IFNAR1, TYK2, pSTAT1 α , and H3pY41 showed increased binding to IFNAR1 in IFN α 2 treated cells. IgG treated control cells did not show similar association in whole cell extracts (data not shown). This is evidence that TYK2 as well as pSTAT1 α do not function alone or independently of the cytokine system whose function they are associated with in the nucleus at the level of gene activation.

3.4. Specific epigenetic changes at the OAS1 promoter of cells treated with a type I IFN

We showed by ChIP analysis that IFN τ treatment of cells resulted in specific binding of IFNAR1, STAT1 α , and TYK2 to the ISRE of the promoter of the OAS1 gene. We examine here associated epigenetic changes by ChIP analysis at the OAS1 promoter. Figure 4B shows decreased trimethylated lysine 9 on histone H3, H3K9me3, in the OAS1 promoter region of cells treated with 1000 U/ml of IFN τ over 40 minutes. Acetylation of H3K9, H3K9ac, occurred concomitantly over the same time span. Demethylation/acetylation of H3K9 is associated with gene activation [14, 15]. Related to this, phosphorylation of H3 at Y41, H3pY41, increased as H3K9me3 decreased over the same time period. Phosphorylation of H3Y41 was confirmed by Western blot (Figure 4C). By comparison, the constitutively activated β -actin gene, which is not affected by IFN, showed constitutive H3K9ac, no H3pY41, and no H3 K9me3. The presence of activated JAKs at the OAS1 region of type I IFN treated cells may be related to H3Y41 phosphorylation which in turn could play a role in the demethylation and acetylation of H3K9 at the promoter region of the gene. These observations suggest that the receptor/transcription factor/JAK complex of type I IFN treated cells plays a key role in specific gene activation, including the associated events of heterochromatin modification.

3.5. N-terminal truncated type I IFNs lose extracellular activity while exhibiting antiviral activity when internalized

In development of the IFN γ mimetics, we found that N-terminal truncations of IFN γ to IFN γ (95–132) for mouse and IFN γ (95–134) for human IFN γ resulted in loss of recognition of extracellular receptor (Szente et al., 1995, 1996). These truncated IFNs were, however, active when introduced intracellularly via a palmitate group with full antiviral activity (Szente et al., 1995, 1996). The pattern of nuclear signaling by type I IFNs presented here is similar to that of IFN γ nuclear signaling (Johnson et al., 2012). Thus, in order to determine if IFN α 1 and IFN β possessed similar C-terminus function intracellularly while losing extracellular function, we expressed truncated IFN α 1(69–189)R9 and IFN β (100–187)R9 with nine arginines (R9) for cell penetration in a bacterial expression system and purified the polypeptides. As controls, we also expressed these truncations without R9. Both IFN α 1(69–189)R9 (Figure 5A) and IFN β (100–187)R9 (Figure 5B) possessed antiviral activity against EMC virus, while the same constructs without R9 for cell penetration lacked antiviral activity. R9 alone also lacked antiviral activity (data not shown). This is consistent with previous studies that showed that intracellularly expressed IFN α possessed antiproliferative and antiviral activity (Ahmed et al., 2001). The truncation studies, however, are not subject to the argument that somehow the intracellular IFN may have leaked out of the cell and interacted with the extracellular receptor domains, since the truncations were not functional in terms of extracellular induced antiviral activity.

3.6 N-terminal truncated type I IFNs possess therapeutic efficacy against relapsing/remitting experimental allergic encephalomyelitis (EAE), while lacking toxic side effects

There are over 20 different isoforms of type I IFNs and they all function through the same heterodimeric receptor complex (Stark et al., 1998). In addition to their similar antiviral activities, these IFNs vary with respect to anticellular and cytotoxic (apoptotic) effects. In this regard, IFN β is the treatment of choice for relapsing/remitting multiple sclerosis (Burks, 2005; Castro-Borrero et al., 2012). Further, it has been shown that higher doses of IFN β result in better therapeutic efficacy (Schwid et al., 2005), but undesirable toxic side effects of flu-like symptoms, liver damage, and bone marrow suppression limit the dose (Francis et al., 2003). We showed that type I IFN toxicity (apoptosis) was due to differential extracellular IFN receptor recognition, where greater receptor occupancy due to higher binding affinity contributed to the toxic effects (Subramaniam et al., 1995). This observation has been confirmed by others (Thomas et al., 2011).

We tested IFN α 1(69–189)R9 for its ability to therapeutically treat SJL/J mice in experimental allergic encephalomyelitis (EAE), a mouse model of MS, as well as for their toxicity relative to that of IFN β . Immunization of mice with bovine myelin basic protein (MBP) where cellular infiltration into the CNS has occurred by day 12 was used to test the truncated IFNs (13). SJL/J mice (n=5), were injected i.p. with PBS, IFN α 1(69–189)R9 (15 μ g/mouse), or the control peptide, IFN α 1(69–189), (15 μ g/mouse), every other day starting from day 12 post-immunization with MBP (Figure 6A). Mice were followed daily, and the mean severity of disease was graded as follows: 0, normal; 1, loss of tail tone; 2, hind leg weakness; 3, paraparesis; 4, paraplegia; 5, moribund; and 6, death. The IFN α mimetic with the R9 reduced paralysis essentially completely, while the mice treated with PBS or the mimetic lacking R9 developed paraplegia. Incidences of EAE in PBS or control peptide treatment were both 4 out of 5, with the severity increasing progressively. By day 56, 3 out of 5 PBS treated and 2 out of 5 control peptide treated mice were dead. In contrast, the IFN mimetic treated mice showed the disease severity of 0.5 and 2 in two of the mice, but they recovered quickly. One mouse in this group spiked to a severity of 4 on day 29, but fully recovered by day 35. On day 60, at study termination, all IFN peptide treated mice were healthy and there were no deaths. Thus, the IFN α R9 mimetic acted intracellularly to initiate the protection against EAE. Similar results were obtained with the IFN β mimetic (data not shown).

For toxicity studies, mice were injected i.p. on alternate days with IFN β (10^3 U/mouse), IFN β (100–179)R9 (2×10^3 U, 200 μ g), or IFN α 1(69–189)R9 (2×10^3 U, 200 μ g). Activity refers to the antiviral activity assessed by the cytopathic effect of EMC virus on L cells. Body weight was measured daily. The average body weight is presented as a percentage of initial weight, which is shown in Figure 6B. The weight loss seen in the IFN β treated mice was not seen with either mimetic. Injection of mice with IFN β resulted in approximately 15% weight loss by day 10, while mice injected with the IFN mimetics gained weight, which is expected under normal growth conditions. On day 11, the weight difference between the IFN β and the mimetic treated mice showed significance at $p < 0.05$.

For the effect on lymphocytes, mice (C57BL/6, n=3) were injected i.p. with PBS, IFN mimetics (2×10^3 U in 200 μ g/mouse), or IFN β (10^3 U/mouse) on alternate days for 10 days. On day 11, mice were bled. Blood smears were stained and lymphocytes were counted. Lymphocyte counts showed a similar pattern of toxicity with 24% reduction in IFN β injected mice and 4–9% loss in IFN mimetic treated mice (Table 1).

For apoptosis study, WISH cells (150,000) were seeded in a 6 well plate and grown overnight. They were treated with type I IFN mimetics (100 U/ml), IFN β 1 (100 U/ml), or IFN α 2 (100 U/ml) for 4 days. Cells were doubly stained with Annexin V and propidium

iodide and analyzed by flow cytometry to measure the extent of apoptosis. The data, shown in Figure 6C, indicate the percentage of apoptosis from the analysis of 10,000 cells. The human IFN β 1 and IFN α 2 had toxicity of approximately 14% above controls, while the mimetics showed toxicity at the levels close to the untreated cells. Thus at the dosing producing the same antiviral activity, the type I IFN mimetics lacked toxicity of weight loss, lymphopenia, and cellular toxicity under conditions where the intact type I IFNs were toxic.

4. Discussion

Specific gene activation by cytokines such as the IFNs is attributed solely to the activated STATs (Levy and Darnell, 2002). In the case of IFN γ signaling, interaction of IFN γ with receptor results in autoactivation of JAK1 and JAK2, which in turn activate STAT1 α in conjunction with receptor subunit IFNGR1. Activated STAT1 α forms a homodimer, dissociates from IFNGR1, and undergoes active nuclear transport via an unconventional nuclear localization sequence (NLS) that associates with the importin α/β proteins (Johnson et al., 2004). The fact that STAT1 α is activated by other cytokines in addition to IFN γ would suggest that STATs do not intrinsically contain the mechanism for specific gene activation by a particular cytokine (Johnson et al., 2004; Johnson and Ahmed, 2006). This is reinforced by the fact that there are just seven STATs that function mostly as homodimeric transcription factors for over 60 different cytokines, growth factors, and hormones (Johnson et al., 2004).

Recently, nuclear JAK2 has been shown to play an important role at the epigenetic level in gene activation. Mutant activated JAK2, JAK2V617F, was shown to be constitutively present in the nucleus of effector cells of myeloproliferative disorders where it phosphorylated tyrosine 41 on histone H3 (H3pY41) (Dawson et al., 2009). It was also shown that wild-type JAK2 was constitutively present in the nucleus of nonmyeloproliferative cell lines, but was only activated after treatment of K562 cells with PDGF or LIF or treatment of BaF3 cells with IL-3 (Dawson et al., 2009). Phosphorylation of H3Y41 by activated nuclear JAK2 assigns a previously unknown function to a JAK kinase. The presence of JAKs in the nucleus, phosphorylated and unphosphorylated, was however previously known. JAK1, JAK2, and TYK2 have all previously been shown to be constitutively present in the nucleus (Lobie et al., 1996; Nilsson et al., 2006; Zouein et al., 2011).

We have previously shown that IFN γ and one of its receptor subunits, IFNGR1, are translocated to the nucleus together with activated STAT1 α and activated JAK2 as one macromolecular complex via the classical importin-dependent pathway (Ahmed et al., 2003; Ahmed and Johnson, 2006). The IFN γ studies of activated JAK2 in the nucleus suggest that it functions in the context of the IFN γ /IFNGR1/pSTAT1 α /pJAK2 complex. This in turn provides a mechanism for controlling or identifying specific chromatin regions for pJAK2 epigenetic effects.

Both IFNAR1 and IFNAR2 underwent nuclear translocation in type I IFN treated cells. This is in contrast to IFN γ where IFNGR2 remained associated with the cell membrane while IFNGR1 underwent nuclear translocation as part of a complex as indicated above (Ahmed et al., 2003; Ahmed and Johnson, 2006). For type I IFNs, TYK2 is associated with IFNAR1 while JAK1 is associated with IFNAR2 (Stark et al., 1998). After type I IFN treatment, pTYK2 and probably pJAK1 undergo nuclear translocation as a part of a macromolecular complex that contains IFNAR1 and IFNAR2. The events are associated with phosphorylation of H3Y41 specifically on genes such as OAS1. We also observed nuclear translocation of type I IFN, IFN τ , by confocal microscopic analysis. Nuclear translocation

of type I IFN has been known for some time (Kushnaryov et al., 1986). This observation cannot be explained by the classical model of JAK/STAT signaling.

Receptor tyrosine kinases (RTKs) such as EGF receptor (EGFR) and ErbB-2, also undergo nuclear translocation in association with their respective ligands (Wang et al., 2012). These EGFR family members traffic to the nucleus through a retrograde pathway from Golgi to the endoplasmic reticulum (ER), and finally to the inner nuclear membrane in association with Sec61 β translocon (Wang and Hung, 2010).

Focusing on trimethylated histone H3 lysine 9 (H3K9me₃), we observed that in type I IFN treated cells H3K9me₃ underwent demethylation in association with acetylation (H3K9ac) at the region of the OAS1 promoter. These changes in H3K9 are associated with gene activation (Berger, 2007; Mehta et al., 2011). The association of IFN receptors with pSTAT1 α , pTYK2, and probably other factors in the region of genes activated by IFN provides insight into the mechanism of specific gene activation, including associated phosphorylations, methylations, demethylations, and acetylations.

Steroid receptors (SRs) are a major subset of nuclear receptors (Stanisic et al., 2010). The current model of SH signaling is as follows. In the absence of hormone, cytoplasmic SR monomers are associated with heat shock proteins (HSPs) and usually possess some basal level of phosphorylation (Stanisic et al., 2010). Upon binding of hormone, SRs dissociate from HSPs, dimerize, and translocate to the nucleus where they bind to hormone receptor elements (HREs) at genes that are activated by SHs. The complex of SH/SR recruits a series of coactivators to both regulate target gene transcription as well as the associated epigenetic events that accompany gene expression.

Unlike SH/SR interaction, both type I and II IFN signaling initiates with ligand binding to the receptor extracellular domain. However, we have shown that IFN γ also binds to the cytoplasmic domain of receptor subunit IFNGR1 during the process of endocytosis (Szente et al., 1995). We showed that the N-terminus of IFN γ played the key role in recognition of IFNGR1 extracellular domain, while the C-terminus played the key role in binding to the cytoplasmic domain. This in turn led to development of IFN γ mimetics based on the C-terminus (Szente et al., 1996; Ahmed et al., 2007). We showed here that N-terminus truncations of IFN α and IFN β resulted in loss of signaling via extracellular receptor interaction, while the same truncated IFNs with R9 attached for cell penetration possessed antiviral activity and dramatic therapeutic efficacy in EAE, a model for MS. These results would suggest that type I IFNs also interact with receptor cytoplasmic domain. Type I IFN cytoplasmic receptor interaction is probably more complex than that of IFN γ where only the receptor subunit IFNGR1 undergoes endocytosis, while both IFNAR1 and IFNAR2 undergo endocytosis in type I IFN signaling. The demonstration of extracellular receptor interaction for IFNs is essentially the extra step in signaling compared to SHs, which interact directly with the cytoplasmic SR. In both systems we have ligand/receptor/coactivator complexes that undergo nuclear translocation to specific promoter sites. Thus, the results of this and previous studies with IFN γ suggest that signaling by cytokines such as the IFNs is but a variation of steroid/steroid receptor signaling.

The observation that the type I IFN mimetic was therapeutically effective against EAE, but lacked the toxicity associated with the intact IFN suggests that the two events are not associated. Further, the fact that the mimetic does not recognize the receptor extracellular domain would suggest that the undesirable side effects of IFN therapy for MS are due to signal transduction events activated extracellularly. There is evidence that higher IFN β doses result in better MS patient response (Schwid et al., 2005), but higher doses are limited by the undesirable effects (Francis et al., 2003). Thus, since the mimetics lack toxicity, they

should be ideal for high dose treatment of MS. This in turn should result in a better patient outcome.

There are interesting observations of IFN signaling that became both more interesting and potentially more relevant in the context of the intracellular aspects of our IFN signal transduction model (Johnson et al., 2012). Both type I and II IFNs possess species non-specific biological activity when expressed or introduced intracellularly (Ahmed et al., 2003; Rutherford et al., 1996). Additionally, it has been shown that treatment of mouse L cells with mouse IFN α/β in the presence of human WISH cells conveyed antiviral protection to the WISH cells (Blalock and Baron, 1979). This protection did not involve induction of human IFN or any direct effect of the mouse IFN on the cell surface of the WISH cells. The protection required close cell-cell association and it was possible that synaptical associations resulted in transfer of a signal from L cells to the WISH cells. The intracellular complexes of our model are not species specific and if transferred to the WISH cells could explain the observed protection.

The results presented here for induction of antiviral activity and EAE therapy by cell-penetrating truncated type I IFNs (mimetics) are inexplicable in the context of a model where the type I IFN exerts its effect solely by extracellular interaction with the receptor. The data are compatible with our IFN γ model where IFN after binding to receptor extracellular domain goes on to bind to the cytoplasmic domain of receptor in conjunction with endocytic events (Johnson et al., 2012). The complex formation and the functional cytoplasmic activity of IFN truncations thus show similarities in complexity to that of steroid signaling (Stanisic et al., 2010).

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Figure 1.

Activated JAKs and receptor subunits are present in the nucleus of cells treated with type I IFNs. WISH cells were incubated with or without 1,000 U/ml of IFNα2 (**A**) for the indicated times and their nuclei were purified and solubilized. Nuclear and cytoplasmic samples were subjected to Western blotting against indicated antibodies. Time 0 is taken as untreated cells. (**B**) WISH cells were similarly treated with IFNτ and the nuclear fraction was Western blotted with antibodies to IFNAR2 and IFNAR1. β-tubulin (cytoplasm) and β-lamin (nucleus) blots were performed to confirm the purity of nuclear fraction. The presence of nuclear IFNAR1 as determined by densitometric measurements showed that treatment with IFN increased the intensity of IFNAR1 band by 4.5 ± 0.22 (s.d.) fold over 5 different determinations (**C**).

Figure 2.

Nuclear translocation of IFN τ , IFNAR1, and IFNAR2 as determined by confocal microscopy. GFP fusion constructs of IFN τ , IFNAR1, and IFNAR2 were used to separately transfect WISH cells. IFN τ -GFP transfected cells showed nuclear translocation of IFN τ at 4 hr (top). In the case of IFNAR1-GFP (middle) and IFNAR2-GFP (bottom), cells were treated with 1,000 U/ml of IFN τ and receptors were found to translocate to nuclei as seen by confocal microscopy.

Figure 3.

Type I IFN stimulation induces the association of IFNAR1, TYK2, STAT1 α , and H3pY41 with the ISRE at the OAS1 promoter by ChIP-qPCR assay. WISH cells were treated with 1,000 U/ml of IFN τ for 1 hr, then treated with 1% formaldehyde for 10 min. Details of ChIP assay are in MATERIALS and METHODS and described previously (Noon-Song et al., 2011).

Figure 4.

TYK2 complexes in the nucleus and epigenetic events. **A.** Association of TYK2, pSTAT1 α , and H3pY41 with IFNAR1 in the nucleus of cells treated with a type I IFN. WISH cells were treated with 1,000 U/ml of IFN α 2 for 1 hr, after which a solubilized extract from the isolated nuclei was immunoprecipitated with antibodies to IFNAR1 and Western blotted with the indicated antibodies. **B.** Type I IFN treatment induces histone H3K9 demethylation/acetylation at the ISRE of the promoter region of the OAS1 gene. WISH cells were treated with 1,000 U/ml of IFN τ for the indicated times and ChIP assays were performed using antibodies to H3K9ac, H3K9me3, and H3pY41. **C.** Type I IFN treatment induces H3Y41 phosphorylation. Western blot for H3pY41 in WISH cells treated with IFN τ . Abbrev: H3K9ac, acetylated lysine 9 in histone H3; H3pY41, phosphorylated tyrosine 41 in histone H3; H3K9me3, trimethylated lysine 9 in histone H3.

Figure 5.

IFNα1(69–189)R9 and IFNβ(100–187)R9, N-terminal truncations of human IFNα1 and IFNβ, possessed antiviral activity. IFNα1(69–189)R9 (**A**), or IFNβ(100–187)R9 (**B**), or the control peptides without the R9 plasma membrane penetration sequence were added to L929 cells (40,000 per well) and incubated for 4 hr. Washed cells were infected with EMC virus (EMCV) (moi = 0.01) for 24 hr, followed by staining with crystal violet and measurement of absorbance.

Figure 6.

N-terminal truncated IFN α 1(69–189)R9 and/or IFN β (100–187)R9 ameliorate symptoms of EAE and are less toxic than the parent IFN. **A.** SJL/J mice (n=5) were injected i.p. with PBS (●), IFN α mimetic IFN α 1(69–189)R9 (▲, 15 μ g/mouse), or the control peptide, IFN α 1(69–189) (□, 15 μ g/mouse), every other day starting from day 12 post-immunization with MBP. Mice were followed daily. The mean daily severity of disease was graded as follows. 0, normal; 1, loss of tail tone; 2, hind leg weakness; 3, paraparesis; 4, paraplegia; 5, moribund; and 6, death. A repeat of this experiment provided similar results. **B.** Weight loss comparison. Mice (C57BL/6, n=3) were injected i.p. with IFN β (▲, 10^3 U/mouse); IFN β (100–179)R9 (○), 2×10^3 U (200 μ g); or IFN α (69–189)R9 (□), 2×10^3 U (200 μ g) i.p. on alternate days. Activity refers to the antiviral activity assessed by cytopathic effect of EMCV on L cells. Body weight was measured daily. The average body weight is presented as a percentage of initial weight, and the standard deviation is shown. On day 11, the weight difference between IFN β and the mimetics showed a significance of $P < 0.05$. **C.** Increased apoptosis seen in parent IFN is not seen in IFN mimetics. WISH cells (150,000) were seeded in a 6 well plate and grown overnight. They were treated with type I IFN mimetics (100 U/ml), IFN β (100 U/ml), or IFN α 2 (100 U/ml) for 4 days. Cells were doubly stained with Annexin V and propidium iodide (PI) and analyzed by flow cytometry to measure the extent of apoptosis. The data shown indicate the percentage of apoptosis based on cells staining for both Annexin V and PI from the analysis of 10,000 cells.

Table 1

Lymphocyte suppression seen with IFN is not observed with IFN mimetics.

Treatment	Lymphocytes (%)	% Reduction	Significance
PBS	78 ± 4		
IFN α (69–189)R9	75 ± 3	4	NS
IFN β (100–187)R9	72 ± 5	9	NS
IFN β	59 ± 6	24	< 0.01

Mice (C57BL/6, n=3) were injected i.p. with PBS, IFN mimetics (2×10^3 U in 200 μ g per mouse), or IFN β (10^3 U/mouse) on alternate days for ten days. Blood was drawn on day 11.