The role of IFNγ nuclear localization sequence in intracellular function

C. M. Iqbal Ahmed*, Marjorie A. Burkhart, Mustafa G. Mujtaba, Prem S. Subramaniam and Howard M. Johnson

Department of Microbiology and Cell Science, University of Florida, P.O. Box 110700, Gainesville, FL, 32611-0700, USA
*Author for correspondence (e-mail: ahmed1@ufl.edu)

Accepted 1 April 2003
Journal of Cell Science 116, 3089-3098 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00528

Summary

Intracellularly expressed interferon γ (IFNγ) has been reported to possess biological activity similar to that of IFNγ added to cells. This study addresses the mechanisms for such similar biological effects. Adenoviral vectors were used to express a non-secreted form of human IFNγ or a non-secreted mutant form in which a previously demonstrated nuclear localization sequence (NLS), ^{128}KTGKRKR^{134}, was replaced with alanines at K and R positions. With the vector expressing non-secreted wild-type IFNγ, biological responses normally associated with extracellular IFNγ, such as antiviral activity and MHC class I upregulation, were observed, although the mutant IFNγ did not possess biological activity. Intracellular human IFNγ possessed biological activity in mouse L cells, which do not recognize extracellularly added human IFNγ. Thus, the biological activity was not due to leakage of IFNγ to the surroundings and subsequent interaction with the receptor on the cell surface. Biological function was associated with activation of STAT1α and nuclear translocation of IFNγ, IFNGR1 and STAT1α. Immunoprecipitation of cellular extracts with antibody to the nuclear transporter NPI-1 showed the formation of a complex with IFNγ-IFNGR1-STAT1α. To provide the physiological basis for these effects we show that extracellularly added IFNγ possesses intracellular signaling activity that is NLS dependent, as suggested by our previous studies, and that this activity occurs via the receptor-mediated endocytosis of IFNγ. The data are consistent with previous observations that the NLS of extracellularly added IFNγ plays a role in IFNγ signaling.

Key words: Inteferon γ, Nuclear localization sequence (NLS), STAT1α, MHC

Introduction

The current view is that interferon γ (IFNγ) activates cells via interaction with the extracellular domain of the receptor complex (reviewed in Stark et al., 1998). This in turn results in the activation of the receptor-associated tyrosine kinases JAK1 and JAK2, leading to phosphorylation and dimerization of the transcription factor STAT1α, which dissociates from the receptor cytoplasmic domain and undergoes nuclear translocation. This current view ascribes no further role to IFNγ or the receptor chains IFNGR1 and IFNGR2 in IFNγ signaling. The above scenario ignores several events associated with IFNγ signaling. First, both IFNγ and one of the receptor chains, IFNGR1, undergo internalization or endocytosis and nuclear translocation (Larkin et al., 2000; Subramaniam et al., 2000). Second, at least three separate studies showed that intracellular IFNγ possessed biological activity that was qualitatively not unlike that of extracellular IFNγ. In one, microinjection of human IFNγ into mouse macrophage cells induced Ia expression (Smith et al., 1990). Extracellularly added human IFNγ did not have an effect, since it does not recognize the extracellular domain of the mouse IFNγ receptor complex. Another study involved internalizing human IFNγ into mouse macrophages via a liposomal delivery system, resulting in induction of an antiproliferative effect (Killion et al., 1994). Finally, intracellular expression of the human IFNγ gene in mouse fibroblasts in a non-secretory form resulted in induction of antiviral activity (Sanceau et al., 1987).

Recently, we have determined the structural basis for nuclear translocation of murine IFNγ by identification of a polycationic nuclear localization sequence in its C-terminus (Subramaniam et al., 1999). Further, internalized IFNγ binds to the cytoplasmic domain of the IFNGR1 receptor chain (Szente et al., 1994). Immunoprecipitation experiments showed that a cytoplasmic complex of IFNγ-IFNGR1-STAT1α complexed to the nuclear importin α protein, NPI-1, occurs in an NLS-dependent fashion (Subramaniam et al., 2000). Thus, the internalization of IFNγ and nuclear transport of IFNγ and IFNGR1 appear to be a mechanism for nuclear import of the IFNγ transcription factor STAT1α.

In the present study, we have expressed a non-secretable form of human IFNγ and found it to be biologically active. In order to demonstrate that the NLS of IFNγ was key to the intracellular events described above, positively charged amino acids in the NLS were replaced with alanines, such that the NLS sequence ^{128}KTGKRKR^{134} was mutated to ^{128}ATGAAAA^{134}. Non-secreted forms of IFNγ or its NLS-mutated version were tested in murine and human cells for their ability to induce IFNγ activity, to activate STAT1α and to carry out nuclear translocation of STAT1α. The data show that

intracellularly expressed IFNγ, like extracellularly added IFNγ, interacts with the cytoplasmic domain of IFNGR1 via its C-terminal NLS and that the IFNγ-IFNGR1-STAT1α complex is in turn complexed to NPI-1 for nuclear import of STAT1α.

Materials and Methods

Cell lines and recombinant adenoviruses

Human embryonic kidney cell line 293 (ATCC, Gaithersburg, MD) used for the propagation of recombinant adenoviruses was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Human WISH and mouse L929 cells (ATCC) were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum. P388D1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum.

AdEasy adenoviral vector system from Stratagene (La Jolla, CA) was used. Construction and propagation of adenoviral vectors was carried out according to the manufacturer's protocol. A plasmid containing human IFNγ (ATCC) was used to carry out PCR using the following primers: CGGTCGACGAACGATGAAATATACAAGT-TATATC (forward) and GCAAGCTTCATTACTGGGATGCTCT-TCGAC (reverse). To obtain the non-secreted IFNγ sequence, we used a forward primer that had the initiating methionine and the remainder of the coding sequence from the first amino acid in mature polypeptide, with the following sequence, CGGTCGACG-AACGATGTGTTACTGCCAGGACCCATA. The reverse primer was the same as above for secreted IFN. NLS-modified IFNγ sequence was obtained by using a reverse primer in which the coding sequence was changed to replace lysine or arginine with alanines, and the same forward primer as for the non-secreted IFN. PCR products were digested with *Sal*I (5′ end) and *Hin*dIII (3′ end), and the resulting fragments were cloned in the multiple cloning site in the plasmid, pShuttleCMV. For the control plasmid, pShuttle MCS, which does not have a transgene, was used. Linearized plasmids as above were cotransformed with pAdeasy plasmid in BJ5183 to obtain recombinant adenovirus sequence. Recombinant plasmids were used to infect human embryonic kidney 293 cells to obtain viruses. Purification of viruses was carried out by using two CsCl gradients. These viruses were characterized by restriction enzyme digestion and DNA sequencing across the coding sequence. Cells that were about 50% confluent were infected with different recombinant adenoviruses at a multiplicity of infection (m.o.i.) of 10 for 1 hour, followed by growth in EMEM medium.

Western blot analysis and immunoprecipitation

Cells were washed with phosphate-buffered saline (PBS) and harvested in lysis buffer [50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% NP-40, 50 mM NaF, 5 mM EDTA and protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN)]. Protein concentration was measured using a BCA kit from Pierce (Rockford, IL). Protein (10 μg each) was electrophoresed on an acrylamide gel, transferred to nylon membrane and probed with the antibodies indicated. Horseradish-peroxidase-conjugated secondary antibodies were used, and detection was carried out by chemiluminescence (Pierce). Immunoprecipitation was carried out by incubating specific antibodies with cell extracts followed by incubation with IgG-Sepharose (Sigma Chemicals, St. Louis, MO), followed by centrifugation and washing. The phospho-STAT 1 antibody was from Cell Signaling (Beverly, MA). The polyclonal antibody to STAT1α was from R&D chemicals (Minneapolis MN). Antibodies to NPI-1 and IFNGR1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody to IFNγ used to probe immunoprecipitate was obtained from PBL Biomedical (New Brunswick, NJ). The ELISA kit for IFNγ was obtained from Biosource International (Camarillo, CA).

Antiviral assay

Antiviral assays were performed by using a cytopathic effect (CPE) reduction assay using Vesicular Somatitis Virus (VSV) (Familetti et al., 1981). Mouse L929 (4×10^3) cells were plated in a microtiter dish and allowed to grow overnight. These cells were then infected with different adenoviruses and incubated for various times followed by growth in EMEM medium for 24 hours. VSV was then added to these cells and incubated for 24 hours. Cells were stained with crystal violet. The dye retained was extracted in methylcellusolve, and absorption at 550 nM was measured.

Expression of MHC class I

Cells were transfected with different recombinant adenoviruses for 1 hour, followed by growth in EMEM medium for 48 hours. Cells were then washed and incubated with a monoclonal antibody to human MHC class I molecules conjugated with R-phycoerythrin (R-PE). Mouse IgG2a conjugated with R-PE was used as a control. Both of these R-PE-conjugated antibodies were from Ancell (Bayport, MN). Cells were analyzed for immunofluorescence (FL-2) in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data were collected in list-mode format and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems).

Immunofluorescence analysis

WISH cells (3×10^5) were grown overnight on tissue-culture-treated slides (Falcon, Becton Dickinson, Franklin Lakes, NJ) before infecting with adenovirus vector for 1 hour. This was followed by growth in EMEM medium for 7 hours. Cells were then fixed in methanol (–20°C) and dried. Cells were permeabilized using 0.5% Triton X-100 in 10 mM Tris-HCl, pH 8, 0.9% NaCl (TBS) for 10 minutes. Slides were washed in TBS and non-specific sites were blocked with 5% non-fat milk in TBS. Slides were then incubated for 1 hour in the same blocking buffer containing rabbit polyclonal antisera against IFNGR1 (Santa Cruz Biotechnology, Santa Cruz, CA) and goat polyclonal antisera to human STAT1α (R&D Systems). Cells were washed four times with TBS containing 0.1% Triton. This was followed by incubation with secondary antibodies, which were Cy-2-conjugated donkey anti-rabbit (Jackson Immunochemicals) and Alexa-Fluor-594-conjugated donkey anti-goat antisera (Molecular Probes, Eugene, OR) for 1 hour. After four washings in TBS with 0.1% Triton, slides were mounted in Prolong antifade solution (Molecular Probes), covered with a coverslip and sealed with nail varnish. To view the nuclear translocation of IFNγ, slides were incubated with a monoclonal antibody to human IFNγ (BD Pharmingen, San Diego, CA) as the primary antibody and Alexa-Fluor-488-conjugated anti-mouse antibody (Molecular Probes) as the secondary antibody. Images were recorded on epifluorescence microscope attached to a Macintosh computer running IP Lab software and deconvolution software (Scanylatics Corp). Images were recorded and a portion of out-of-focus haze from each image removed using the MicroTome software (Vaytek) to improve clarity. Quantitation of these images was done by measuring mean pixel intensity in cytoplasmic (Fc) and nuclear (Fn) regions in each cell using IP Lab software (Scanylatics Corp). The ratio Fn/Fc was determined for each cell and the average of the ratio Fn/Fc across at least seven different fields was measured and is presented as Fn/Fc for a given treatment.

Results

Expression vectors

The adenovirus vector used in these studies, pAdEasy, is a derivative of ad5, which is deleted in early regions I and III. A

cassette of the CMV-promoter-driven transgene replaces early region I. These vectors are replication deficient, owing to the deletion of early region I. The vector without the transgene is denoted as rAde. CMV-promoter-driven non-secreted IFNγ and NLS-modified non-secreted IFNγ were designated as rAdnI and rAdnIm, respectively. These vectors were first characterized by restriction enzyme digestion, followed by DNA sequencing across the transgene.

To follow the synthesis of IFNγ, WISH cells were transduced with control or IFNγ-expressing vectors. Proteins from cell extracts and supernatants, obtained two days after infection, were electrophoresed and probed with an antibody to IFNγ (Fig. 1A). Vectors expressing both the non-secreted IFNγ and NLS-modified non-secreted IFNγ showed the presence of IFNγ in cell extracts, and none was detected in the supernatants. There was no IFNγ expression in cells transduced with the empty control vector, suggesting that the infection with recombinant adenovirus in itself does not induce endogenous IFNγ expression. To test if murine L 929 cells were infectable with the recombinant adenovirus vectors, cell extracts and supernatants after infection with rAde, rAdnI and rAdnIm were assayed for IFNγ by ELISA (Fig. 1B). Synthesis of IFNγ was observed in cell extracts from cells treated with rAdnI and rAdnIm, whereas the supernatants from the same cells did not have any detectable IFNγ. Similarly, cell extracts or supernatants from L 929 cells infected with empty vector did not have any detectable IFNγ. In addition, purified virus preparations were found to be free of IFNγ. Therefore, the effects observed below were not from free contaminating IFNγ acting extracellularly. Thus, IFNγ produced from rAdnI or rAdnIm was expressed intracellularly and not secreted.

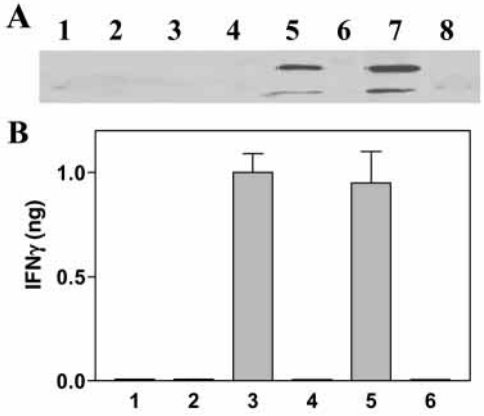

Fig. 1. Synthesis and intracellular retention of IFNγ. (A) WISH cells, untreated (lanes 1 and 2) or those transduced for two days with an empty vector control (lanes 3 and 4) or a vector expressing non-secreted IFNγ (lanes 5 and 6) or a vector expressing non-secreted IFNγ mutated in the NLS (lanes 7 and 8) were used. Proteins from cell extracts (odd-numbered lanes) or supernatants (even-numbered lanes) were separated by SDS-PAGE and probed with an antibody to IFNγ. Detection was carried out by using chemiluminescence. (B) Quantitation of IFNγ produced in L929 cells by ELISA. Cell extracts (odd numbers) and supernatants (even numbers) from L929 cells, transduced for two days with the empty adenoviral vector (column 1 and 2) or vector expressing non-secreted IFNγ (column 3 and 4) or vector expressing non-secreted IFNγ mutated in the NLS (column 5 and 6) were assayed for IFNγ by ELISA.

Biological activity of non-secreted IFNγ is dependent on the presence of NLS

We next determined if intracellularly expressed IFNγ possessed biological activity. Murine L cells were chosen for this study because these cells are not responsive to human IFNγ added extracellularly, so the effect observed would have to result from intracellular action of interferon. Although the extracellular recognition by the receptor is species specific, intracellular signaling events are not species specific because of extensive homology in the cytoplasmic region of IFNGR1 for human and mouse sequences that bind the C-terminus of both human and mouse IFNγ (Szente and Johnson, 1994). In fact, there is greater than 90% homology in this region of the cytoplasmic binding site of IFNGR1. Mouse L cells, untreated or transduced with an empty vector control, non-secreted IFNγ or NLS-modified non-secreted IFNγ expressing vector were allowed to grow for one day and then they were challenged with VSV. A day later, these cells were compared to discover their relative resistance to VSV-induced cytopathic effect (Fig. 2). With intracellular IFNγ expression, a threefold increase in cell survival was observed compared with untreated cells or cells treated with empty vector. With expression of NLS-modified IFNγ, cell survival was reduced to nearly the same level as with the empty vector control. Intracellularly expressed IFNγ thus induced antiviral activity in cells, and this activity was dependent on the presence of the NLS at its C-terminus. Given that mouse L cells do not recognize human IFNγ via the extracellular domain of the receptor, the data further support an intracellular effect of human IFNγ.

Extracellularly added IFNγ induce MHC class I molecule expression on the cell surface. To test if non-secreted IFNγ also had a similar activity, WISH cells were transduced with an empty vector control, non-secreted IFNγ or NLS-modified non-secreted IFNγ expression vector. Two days later, these cells were stained with R-phycoerythrin (R-PE)-conjugated monoclonal antibody to human MHC class I molecules, and the cells were analyzed by flow cytometry. As an isotype

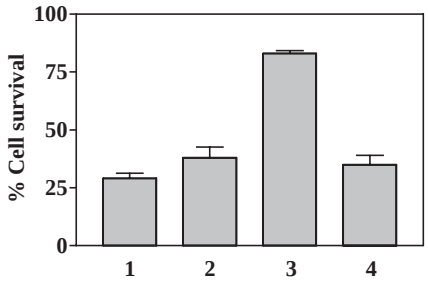

Fig. 2. Resistance to viral infection by intracellular human IFNγ is dependent on the presence of the NLS. Mouse L929 cells, untreated (column 1) or those transduced for 1 hour with an empty vector control (column 2), a vector expressing non-secreted human IFNγ (column 3) or a vector expressing non-secreted IFNγ with a mutation in the NLS (column 4) were allowed to grow for 24 hours, followed by infection with VSV for 24 hours. Cells were then stained with crystal violet, extracted with methylcellusolve, and the absorbance was measured, as described in Materials and Methods. Absorbance in cells that were not exposed to any virus was taken as 100%, and the percentage of cells surviving other treatments is presented. Results represent the mean of three independent determinations.

control, murine IgG2a antibodies conjugated with R-PE were used. Relative mean fluorescence profiles are presented in Fig. 3. Cells transduced with the empty vector control, non-secreted IFNγ or NLS-mutated IFNγ expression vectors showed 417±9, 778±10 and 362±29 units of mean fluorescence, respectively. Therefore, intracellular expression of IFNγ induced an approximately twofold increased expression of MHC I molecules. This induction was abolished with the removal of the NLS. Intracellularly expressed IFNγ therefore induces antiviral activity and upregulation of MHC class I molecules only with an intact NLS in its C-terminus.

Activation of STAT1α and its association with IFNγ, IFNGR1 and NPI-1

To determine if the biological activity observed with non-secreted IFNγ involved the activation of STAT1α, whole cell extracts from WISH cells transduced with an empty vector control, non-secreted IFNγ or NLS-modified non-secreted IFNγ expression vector were analyzed (Fig. 4). Phosphorylation of STAT1α was observed in response to intracellular expression of both wild-type IFNγ and its NLS mutant as seen by probing with Tyr^{701}phospho-STAT1-specific antibody. Cells transduced with empty vector control did not show STAT1α Tyr^{701} phosphorylation. Re-probing this filter to look for STAT1α showed similar amounts of STAT1α in all cell extracts. Thus, the intracellular expression of NLS-mutated IFNγ induced STAT1α tyrosine phosphorylation similarly to wild-type IFNγ.

We have previously shown that IFNγ addition to cells resulted in the formation of a complex of IFNγ-IFNGR1-STAT1α in the cytoplasm and that the nuclear importin α homolog, NPI-1, binds to the complex via the NLS in the C-terminus of IFNγ (Larkin et al., 2000; Subramaniam et al., 2000). Further, we have previously provided evidence that the

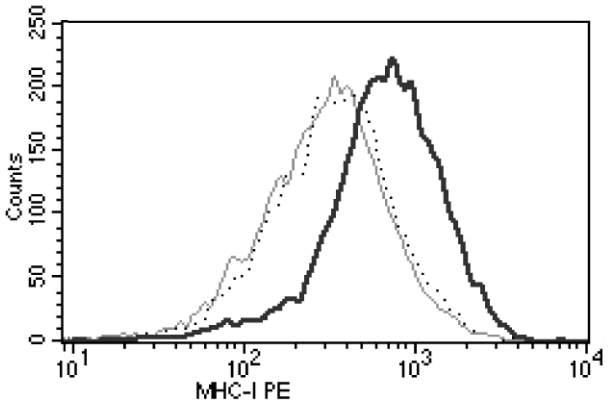

Fig. 3. Induction of MHC class I by intracellular expression of IFNγ is abolished by removal of the NLS. WISH cells were transduced for 1 hour with an empty vector control (dotted line), a vector expressing non-secreted IFNγ (dark grey) or a vector expressing non-secreted IFNγ mutated in NLS (light grey). Cells were then allowed to grow for 48 hours in regular medium followed by staining with R-PE-conjugated monoclonal antibody to human MHC class I and analysis by flow cytometry. R-PE-conjugated murine IgG2a was used as a control. A similar profile was noted in three independent experiments.

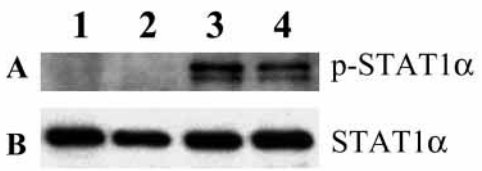

Fig. 4. Phosphorylation of STAT1α by intracellular IFNγ is independent of the IFNγ C-terminal NLS. WISH cells, untreated (lane 1) or treated with an empty vector control (lanes 2), a vector expressing non-secreted IFNγ (lanes 3) or a vector expressing non-secreted IFNγ with a mutated NLS (lanes 4) were allowed to grow for 8 hours. Proteins from whole cell extracts were electrophoresed and probed with an antibody to phospho-STAT1 (upper panel). Filter was stripped and re-probed with an antibody to STAT1α (lower panel). Detection was by chemiluminescence.

NLS of IFNγ is responsible for the nuclear transport of STAT1α (Subramaniam et al., 2000), which otherwise lacks an intrinsic NLS, which was demonstrated by the standard digitonin-based nuclear import assay (P. S. Subramaniam and H. M. Johnson, unpublished). Key to the chaperoning of STAT1α to the nucleus is the ability of IFNγ to bind to the cytoplasmic domain of IFNGR1 as well as to NPI-1 via its C-terminal NLS (Subramaniam et al., 2000). We therefore immunoprecipitated NPI-1 from extracts of cells expressing IFNγ intracellularly 18 hours after transduction with the empty vector or the vectors expressing wild-type IFNγ and NLS-mutated IFNγ. Immunoprecipitated proteins were then electrophoresed and probed individually with antibodies specific for IFNGR1, IFNγ, phosphorylated STAT1α (p-STAT1α) and NPI-1 (Fig. 5). Cells expressing wild-type IFNγ intracellularly contained IFNGR1, IFNγ and p-STAT1 in the anti-NPI-1 immunoprecipitate (Fig. 5, lane 3), whereas anti-NPI-1 precipitate from untreated cells (Fig. 5, lane1) and cells transduced with NLS-mutated IFNγ vector (Fig. 5, lane 2) were negative for IFNGR1, IFNγ and p-STAT1α. Similar concentrations of NPI-1 were present in the immunoprecipitates from all cell extracts. Thus, intracellular

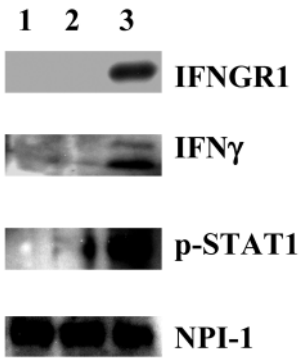

Fig. 5. Association of activated STAT1, IFNγ and IFNGR1 with nuclear importer, NPI-1. Cell extracts from WISH cells transduced for 8 hours with an empty vector (lane 1), NLS-mutated IFNγ expression vector (lane 2) or non-secreted IFNγ expression vector (lane 3) were used for immunoprecipitation with an antibody to NPI-1. Equal amounts of immunoprecipitates were electrophoresed and probed individually with antibodies to IFNGR1 (first row), IFNγ (second row), phospho-STAT1 (third row) or NPI-1 (fourth row).

wild-type IFNγ formed a complex of IFNγ-IFNGR1-STAT1α-NPI-1, but this complex was absent in cells expressing the IFNγ NLS mutant. Since, NLS mutant IFNγ also activated STAT1α phosphorylation similarly to wild-type IFNγ, these data suggest that the IFNγ NLS is required for the binding of phosphorylated STAT1α to NPI-1.

Nuclear translocation of STAT1α, IFNGR1 and IFNγ

Consistent with intracellular activation of STAT1α and the association of IFNGR1 with nuclear import machinery, cells expressing IFNγ intracellularly also showed the movement of STAT1α and IFNGR1 to the nucleus by immunofluorescence analysis. Simultaneous staining of WISH cells with antibodies to STAT1α and IFNGR1 showed the translocation of these molecules into the nucleus with the intracellular expression of IFNγ, wheras the NLS-modified IFNγ or the empty vector failed to induce similar translocation of either of these (Fig. 6A). Further, consistent with our previous studies (Larkin et al., 2000), cells that were simultaneously stained with IFNGR2 and STAT1α showed translocation of only STAT1α, whereas IFNGR2 was not translocated with the expression of intracellular IFNγ, and neither of these was translocated with the NLS-modified IFNγ or the control vector (data not shown). Fluorescence images from at least seven different fields were then used to quantitate the ratio of fluorescence in nuclei (Fn) to the fluorescence in cytoplasmic (Fc) fractions. Thus, cells expressing non-secreted IFNγ showed translocation of both STAT1 and IFNGR1 into the nucleus, whereas the cells treated with NLS-modified IFNγ or the empty vector did not show nuclear translocation (Fig. 6B).

Since IFNγ is also translocated into the nucleus, we determined the effects of removal of the NLS on such translocation by using immunofluorescence analysis (Fig. 7A). Intracellular expression of IFNγ resulted in its nuclear translocation, whereas removal of the NLS resulted in lack of nuclear tanslocation of IFNγ. With the empty vector control, no IFNγ signal was seen (Fig. 7A). Mean fluorescence intensities for the non-secreted and NLS-mutated forms of IFNγ were compared by measuring mean pixel intensity across several lines drawn through the cells as shown in Fig. 7B. The results show approximately 40% more fluorescence in nuclei treated with non-secreted IFNγ compared with the NLS-mutated IFNγ. Quantitation of the images (Fn/Fc ratios) is shown in Fig. 7B. Association of IFNγ together with IFNGR1 and STAT1α points to a role for this ligand and one of its receptor subunits in chaperoning STAT1α into the nucleus.

Evidence for intracellular function of extracellularly added IFNγ

We have shown above that intracellularly expressed IFNγ activates STAT1α similarly to IFNγ added extracellularly to cells. Our previous studies have shown that the C-terminus of human and murine IFNγ, defined by peptides huIFNγ (95-134) and muIFNγ (95-133), bind to the cytoplasmic domain of soluble recombinant human and murine receptor chain

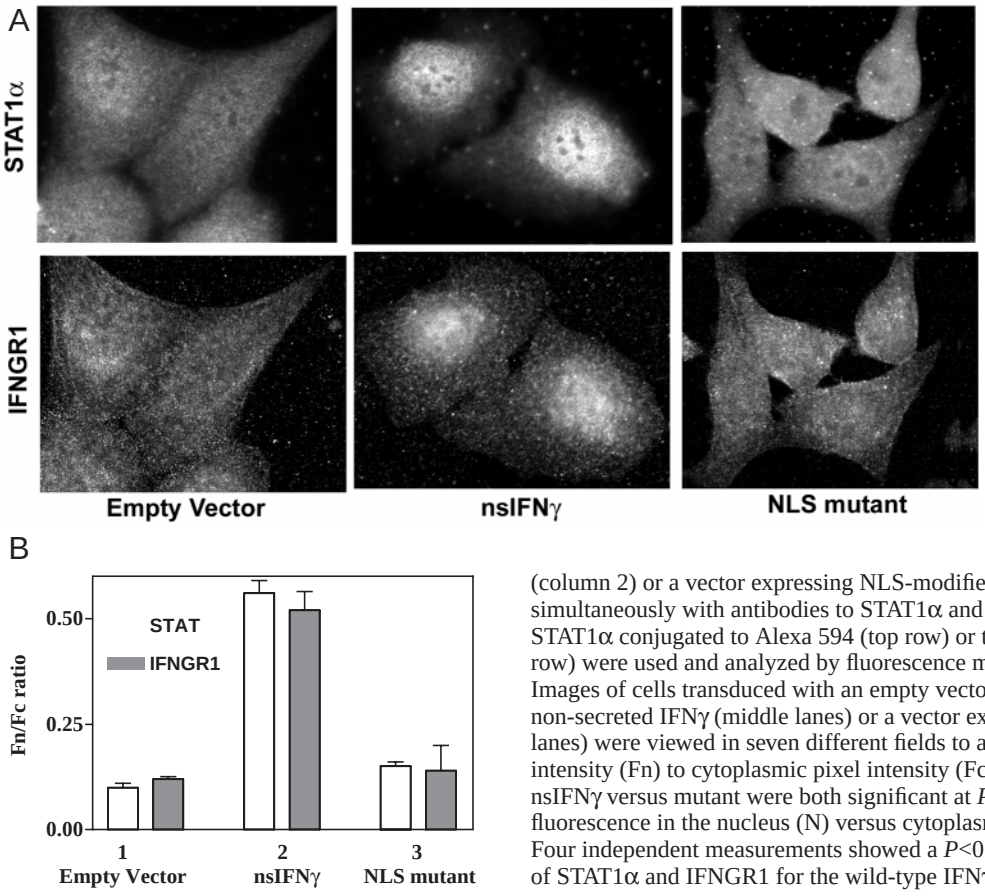

Fig. 6. Nuclear translocation of STAT1α and IFNGR1 by intracellular IFNγ requires the NLS of IFNγ. (A) WISH cells were transfected for 8 hours with an empty vector (column 1), a vector expressing non-secreted IFNγ (column 2) or a vector expressing NLS-modified IFNγ (column 3) and stained simultaneously with antibodies to STAT1α and IFNGR1. Secondary antibodies to STAT1α conjugated to Alexa 594 (top row) or to IFNGR1 conjugated to Cy-2 (bottom row) were used and analyzed by fluorescence microscopy. (B) Quantitation of images. Images of cells transduced with an empty vector control (left lanes), a vector expressing non-secreted IFNγ (middle lanes) or a vector expressing NLS-mutated IFNγ (right lanes) were viewed in seven different fields to a obtain mean ratio of nuclear pixel intensity (Fn) to cytoplasmic pixel intensity (Fc). STAT1α and IFNGR1 Fn/Fc for nsIFNγ versus mutant were both significant at $P<0.002$ by *t*-test. Calculations for fluorescence in the nucleus (N) versus cytoplasm (C) were also done by using N/N+C. Four independent measurements showed a $P<0.03$ by *t*-test for the nuclear translocation of STAT1α and IFNGR1 for the wild-type IFNγ versus the NLS-mutated IFNγ.

IFNGR-1 via the region IFNGR-1 (253-287) (Szente et al., 1994). One would predict that if this domain of IFNGR-1 played an essential role in IFNγ signaling, an intracellular excess of the peptide IFNGR-1 (253-287) should compete for intracellular binding of IFNγ and thus interfere with IFNγ early signaling events. To test this we used the murine macrophage cell line P388D1, which internalizes peptides by pinocytosis as demonstrated previously in our earlier studies using peptides of the C-terminus of IFNγ (Szente and Johnson, 1994). Pinocytosis by P388D1 is an active process that requires cells

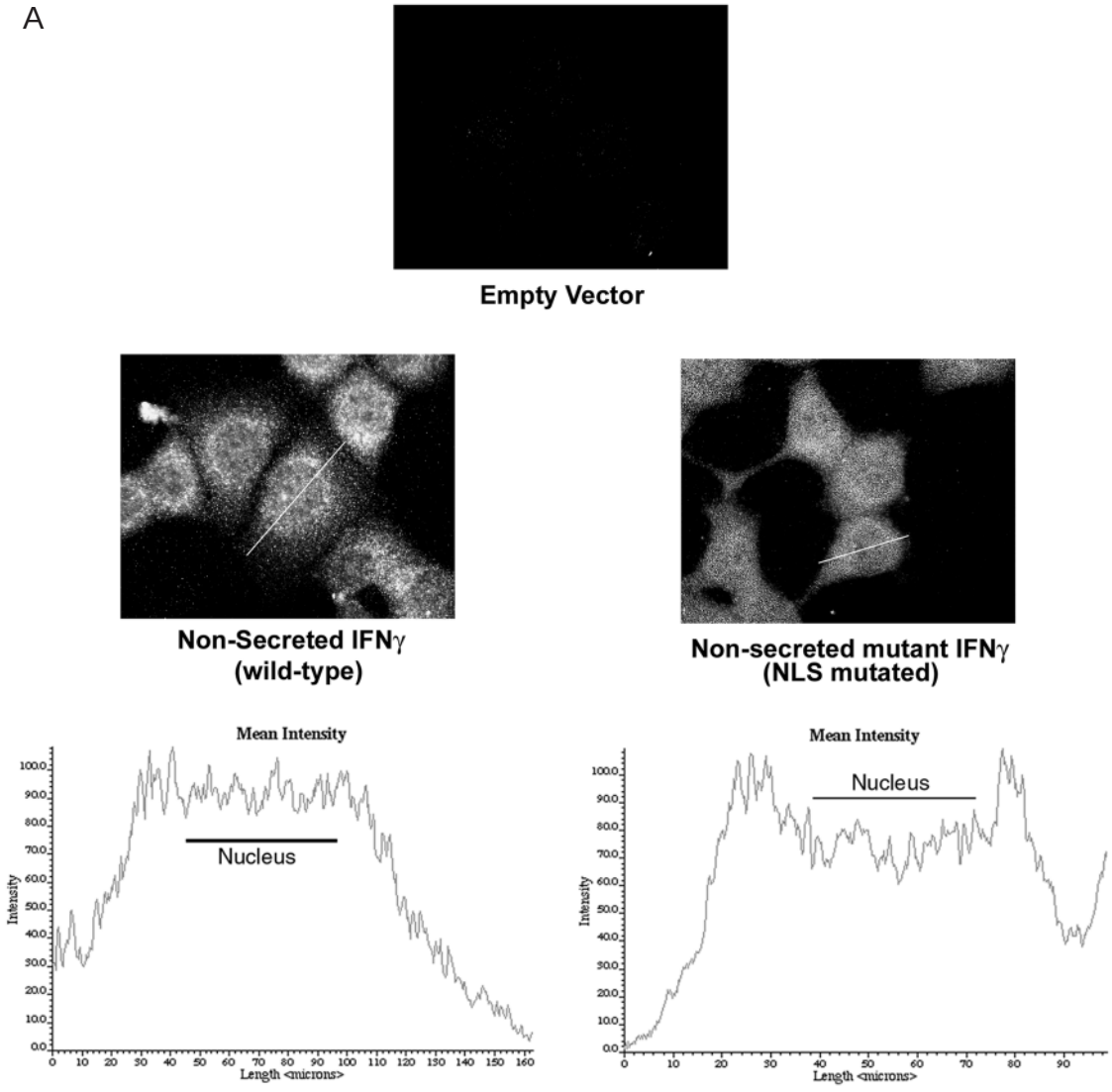

Fig. 7. Nuclear translocation of IFNγ requires the presence of the C-terminus NLS. (A) WISH cells, transduced for 8 hours with an empty vector control (top) or a vector expressing non-secreted IFNγ (middle left) or NLS-modified IFNγ (middle right) were probed with a monoclonal antibody to IFNγ and stained with a Alexa-Fluor-488-conjugated secondary antibody and analyzed by confocal microscopy. The lower graphs show mean fluorescence intensity comparisons for non-secreted IFNγ and NLS-mutated IFNγ. Measurements of fluorescence intensities were done with IP Lab (Scanalytics) using the MP-line measure tool. Mean pixel intensity was measured across a line drawn through the cells in the plane of the images shown. A representative measurement line is shown in white. The resultant graphs were generated by the software averaging the intensities of a total of 100 pixels on either side of the line at each point along the line. Triplicate determinations showed a significance of $P<0.25$ (*t*-test) for nuclear presence of IFNγ versus NLS mutant. (B) Quantitation of images. Images of cells transduced with non-secreted IFNγ or NLS-mutated nonsecreted IFNγ were used to determine Fn/Fc values, which are shown in columns 1 and 2, respectively. Seven fields were examined and the results were averaged±s.d. The significance was $P<0.02$ by the *t*-test. Calculations for fluorescence in the nucleus (N) versus cytoplasm (C) were also done by using N/N+C. Four independent measurements showed a $P<0.025$ for the nuclear translocation of IFNγ for the wild-type IFNγ versus the NLS-mutated IFNγ.

to be incubated at 37°C. Firstly, in binding assays at 4°C (no pinocytosis) we established that extracellularly added cytoplasmic IFNGR-1 (253-287) peptide does not interfere with binding of extracellular ^{125}I-IFNγ to the receptor at the concentrations to be used for functional studies at 37°C (Fig. 8A). Extracellular addition of IFNGR-1 cytoplasmic domain peptide did not inhibit the binding of ^{125}I-IFNγ to the receptor extracellular domain on these cells.

To determine functional effects, the following experiment was performed. Cells were preloaded with the peptide muIFNGR-1 (253-287) at 37°C, washed to remove excess peptide and then challenged with extracellular ^{125}I-labeled IFNγ at 37°C, in the presence of 1 μM of IFNGR-1 (253-287) that does not inhibit IFNγ extracellular binding as per Fig. 8A. After 5 minutes at 37°C, cells were washed at 4°C, and extracellularly bound ^{125}I-IFNγ was removed by acid washing at 4°C. Cell lysates were immunoprecipitated with polyclonal antibodies to IFNGR-1, and ^{125}I-IFNγ was detected by autoradiography to follow internalized IFNγ bound to IFNGR-1. As seen in Fig. 8B, cells that were not preloaded with peptide (lane 4) showed detectable levels of ^{125}I-IFNγ associated with IFNGR-1. By contrast, where cells were preloaded with 25 μM (lane 2) or 50 μM of IFNGR-1 (253-287) peptide (lane 3), 125I-IFNγ did not bind to the cytoplasmic domain of IFNGR-1. Lane 1 shows that at 4°C, where internalization is blocked, even in the absence of preloading of peptide no signal for 125I-IFNγ was detectable. Thus, the presence of free intracellular IFNGR-1 (253-287) peptide blocked the binding of internalized 125I-IFNγ to IFNGR-1. These data show that extracellularly added IFNγ that is internalized interacts with the cytoplasmic domain of IFNGR-1 in intact cells and that this interaction is blocked by IFNGR-1 cytoplasmic peptide IFNGR-1 (253-287), an IFNGR-1 cytoplasmic binding site for IFNγ (Szente and Johnson, 1994). The peptide did not block binding of IFNγ to the receptor extracellular domain (Szente et al., 1994) (Fig. 8A).

To determine the effect of the cytoplasmic binding on signaling, we performed the same experiment as above but this time followed STAT1α tyrosine phosphorylation (Fig. 8C). Binding of internalized IFNγ to IFNGR-1 resulted in STAT1α phosphorylation (lane 4). STAT1α tyrosine phosphorylation was inhibited by preloading cells with IFNGR-1(253-287) (lanes 2 and 3). Thus, the binding of internalized IFNγ to the IFNGR-1 cytoplasmic domain is linked to the activation and tyrosine phosphorylation of STAT1α. The data from Fig. 8 demonstrate that under normal physiological conditions there is an intracellular role in signaling for extracellularly added IFNγ. Our data presented earlier with intracellularly expressed IFNγ are also consistent with this conclusion, and together these data highlight for the first time a mechanism for the physiological function of intracellular IFNγ.

Presumably following endocytosis of IFNGR-1 after interaction with IFNγ, the IFNGR-1 cytoplasmic domain would be present on the surface of the endocytic vesicle. The

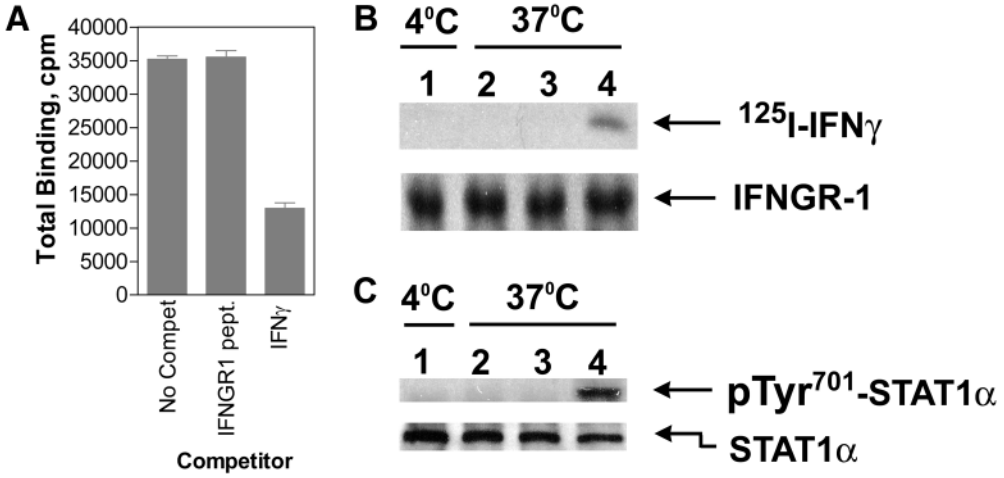

Fig. 8. Intracellular presence of peptide IFNGR-1(253-287) inhibits binding to IFNGR of extracellular IFNγ and subsequent activation of STAT1α. (A) Presence of extracellular peptide IFNGR-1(253-287) did not inhibit binding of ^{125}I-IFNγ to P388D1 cells at the concentrations to be used in subsequent experiments. Unlabeled murine IFNγ or peptide IFNGR-1(253-287), as indicated, was added at a final concentration of 1 μM to P388D1 cells at 4°C along with 10 nM of ^{125}I-IFNγ, and cells were incubated at 4°C for 30 minutes. Control cells were incubated with ^{125}I-IFNγ in the absence of any competitor. Cells were then washed and bound IFNγ determined. Samples were run in triplicate and values plotted as mean±s.d. (B) Intracellular accumulation of peptide IFNGR-1(253-287) in P388D1 cells by pinocytosis was accomplished by incubating cells with either 25 μM (lane 2) or 50 μM (lane 3) of peptide at 37°C for 1 hour. Cells used in lanes 1 and 4 did not receive any peptide. Cells were then washed at room temperature to remove extracellular peptide and then incubated with ^{125}I-IFNγ (10 nM) along with 1 μM of IFNGR-1 peptide for 5 minutes at 37°C. Control cells (lane 1) were washed in ice-cold medium and then incubated with ^{125}I-IFNγ at 4°C without peptide. After ^{125}I-IFNγ incubation, all cells were washed at 4°C and then acid-washed at 4°C to remove surface-bound ^{125}I-IFNγ. Cells were then lysed and immunoprecipitated with antibodies to IFNGR-1. After western transfer of immunoprecipitates to nitrocellulose membranes,^{125}I-IFNγ associated with IFNGR-1 was detected by autoradiography. Total IFNGR-1 immunoprecipitated was followed by immunodetection with IFNGR-1 antibodies (lower panel). (C) Conditions are the same as in (B), except that lysates were immunoprecipitated with STAT1α antibodies and tyrosine phosphorylation of immunoprecipitated STAT1α was followed by immunodetection with antibodies specific for Tyr701-phosphorylated STAT1α. Total immunoprecipitated STAT1α was followed by reprobing blots with antibodies to STAT1α (lower panel).

data suggest that IFNγ at some time during internalization traverses the membrane of the endocytic vesicle to interact with the cytoplasmic domain of IFNGR-1 at the site identified by peptide IFNGR-1 (253-287). This would suggest that there is a membrane penetration property associated with IFNγ.

In keeping with the implications of the above data, we next determined whether the C-terminal NLS domain itself was required for internalization of human IFNγ. We have previously characterized a C-terminal deletion mutant of huIFNγ, IFNγ (1-123) and have shown that this mutant binds to the extracellular domain of IFNGR on cells with the same affinity as intact IFNγ (Subramaniam et al., 2000). IFNγ (1-123) is deleted in the NLS region. We compared its internalization with that of intact IFNγ using ^{125}I-labeled IFNs. After binding of ^{125}I-IFNγ and ^{125}I-IFNγ (1-123) to WISH cells at 4°C, cells were washed to remove unbound ligands and incubated at 37°C for various times followed by acid washing to follow internalization of the bound ^{125}I-IFNγ and ^{125}IFNγ (1-123). As can be seen in Fig. 9, intact IFNγ was rapidly internalized, whereas IFNγ (1-123) did not undergo significant internalization. The small negative values seen at later times for IFNγ (1-123) most probably represent the slow dissociation of surface-bound ligand upon prolonged incubation at 37°C in the absence of its internalization. Thus, these data suggest that the C-terminal region of IFNγ containing the NLS is required for its internalization and may play a critical role in IFNγ traversing the endocytic vesicle membrane to bind to the cytoplasmic domain of IFNGR-1.

Discussion

We have shown in this report that intracellularly expressed human IFNγ is biologically active. The intracellular function of human IFNγ required the presence of a previously identified NLS, ^{128}KTGKRKR^{134} (Subramaniam et al., 1999). Modification of this sequence to ^{128}ATGAAAA^{134} resulted in loss of intracellular human IFNγ antiviral activity in mouse cells and upregulation of MHC class I antigens in human WISH cells and was associated with loss of nuclear import of STAT1α function. We have also shown that extracellularly added IFNγ is endocytosed under conditions that requires the IFNγ NLS and that the internalized IFNγ binds to the cytoplasmic domain of receptor chain IFNGR-1. This binding is associated with STAT1α activation. Our data establish for the first time that intracellular IFNγ interactions are also physiologically necessary for the biological effects of extracellular IFNγ and provide a mechanism for how this occurs.

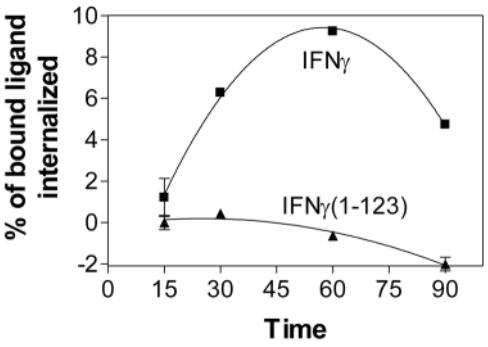

Fig. 9. The deletion mutant IFNγ(1-123) is not internalized compared to wild-type IFNγ. Internalization assays were run as described previously (Farrar et al., 1991). Briefly, 125I-labeled wild-type and mutant IFNγ (80-85 μCi/μg) were added to WISH cells at 5 nM. Cells were incubated at 4°C for 90 minutes, washed at 4°C and then incubated at 37°C for the indicated times. At the indicated times internalized cpm was counted from acid-washed cells lysed in 1% SDS. Data are plotted as a percentage of the total ligand bound that was internalized. Samples were run in triplicate and data are plotted as mean±s.d.

The data with intracellularly expressed IFNγ suggest that intracellular IFNγ can generate biological activity without the recognition of the extracellular domain of the receptor. The following cytokines and growth factors, when expressed with modifications that restricted their localization to the cytoplasm, were also found to be biologically active: human IFN α2b, IFN α2α1 (Ahmed et al., 2001), IFN α consensus (Rutherford et al., 1996), murine IFNγ (Will et al., 1996), IL-3 (Dunbar et al., 1989) and *v-sis* (Bejcek et al., 1989). It is possible that these ligands interact with a motif on the cytoplasmic side of their receptor(s) that initiates a cascade of events required for the nuclear import of STATs, as has been demonstrated for IFNγ (Szente et al., 1994; Szente et al., 1995). A feature common to these ligands and/or their receptors is the presence of an NLS or an NLS-like motif, which may have a role in directing the respective STATs to the nucleus (reviewed in Subramaniam et al., 2001b).

The IFNγ receptor consists of two subunits that are denoted as IFNGR1 and IFNGR2 (reviewed in Pestka et al., 1997). IFNGR1 contains the sites for binding of the ligand on the extracellular surface and the sites for Jak1 and STAT1 binding on the intracellular surface. We have shown earlier that a polypeptide from the C-terminus of murine or human IFNγ containing a nuclear localization signal (NLS) was capable of binding to a region of IFNGR1 sequence located on the cytoplasmic side of the plasma membrane (Szente et al., 1994; Szente et al., 1995). Internalization of this polypeptide was shown to be sufficient to induce an antiviral state and to cause upregulation of MHC class II molecules. Microinjection of antibodies raised against the murine NLS containing peptide resulted in loss of STAT1α nuclear translocation in cells treated extracellularly with IFNγ (Subramaniam et al., 1999). Replacement of the NLS in the C-terminus of IFNγ with the NLS from SV40 T antigen resulted in restoration of biological activity of IFNγ (Subramaniam et al., 2001a). This suggests a requirement for an interaction between the NLS-containing region of IFNγ and IFNGR1, which is supported by the observations that IFNGR1–/– cells do not respond to murine intracellular IFNγ (Will et al., 1996) or an agonist peptide (Thiam et al., 1998). An additional aspect of IFNγ and IFNγ receptor interaction is the nuclear translocation of IFNGR1, but not IFNGR2, in cells treated with IFNγ (Larkin et al., 2000). Nuclear translocation of a receptor subunit has been reported for a number of cytokines and growth factors (reviewed in Subramaniam et al., 2001b; Jans and Hassan, 1998). Demonstration of a transcription-factor-like activity in the EGF receptor subunit after nuclear uptake (Lin et al., 2001) suggests an important role for this translocation, since its association with STATs may allow the use of promoters specific to the ligand and/or receptor.

The NLS of human IFNγ was identified by the digitonin-

permeabilizion assay, which results in selective permeabilization of the plasma membrane of the cell (Subramaniam et al., 1999). Nuclear import of IFNγ was monitored via its coupling to the fluorescent protein allophycocyanin in the presence of reticulocyte lysate and ATP/GTP. Consistent with the above, we have recently shown that extracellularly added ^{125}I-IFNγ undergoes nuclear translocation (Subramaniam and Johnson, 2002). We have recently used the digitonin assay to test STAT1α for intrinsic NLS activity (P. S. Subramaniam and H. M. Johnson, unpublished). Fluorescently labeled STAT1α and IFNGR1 were tyrosine phosphorylated in vitro by recombinant Jak2. The phosphorylated STAT1α bound to its DNA response element, as shown by the electrophoretic mobility shift assay. Activated STAT1α failed to undergo nuclear translocation. However, in association with IFNGR1, it did undergo nuclear translocation in the presence of human IFNγ, as the IFNγ C-terminal peptide contains an NLS. The results are consistent with our demonstration here of the requirement of intracellular IFNγ with intact NLS for STAT1α activation and nuclear translocation in association with IFNGR1.

Nuclear translocation of STAT1α is carried out by the nuclear importer NPI-1 in a ran/importin-dependent pathway (Sekimoto et al., 1996). Mutational analysis of STAT1 did not reveal a clear nuclear localization sequence in this molecule (Sekimoto et al., 1997). Several recent studies have reported the nuclear translocation of STAT1α under conditions of overexpression of STAT1α fusion proteins in cells (Melen et al., 2001; McBride et al., 2002). Through the use of mutations it has been concluded that STAT1α contains a novel NLS that differs from the classical NLS such as that of SV40 T antigen and IFNγ (Melen et al., 2001; McBride et al., 2002). None of these studies tested STAT1α nuclear translocation via the digitonin assay, so it is not possible to directly compare our negative results above with STAT1α with those produced using overexpression.

That the phosphorylation of STAT1 in itself is not sufficient for its translocation into the nucleus has also been shown for angiotensin II receptor (Sayeski et al., 2001) and, more recently, with the use of a chimeric receptor, which had an extracellular domain of PDGF and an intracellular domain of gp130, the signaling subunit of Oncostatin M receptor. Addition of PDGF to cells expressing this chimeric receptor produced phosphorylated STAT1α, but no genes responsive to IFNγ were activated (Mahboubi and Pober, 2002). The following arguments suggest the requirement of additional factor(s) for the migration of STATs into the nucleus. The same STATs are utilized by different cytokines but give rise to different physiological responses (reviewed in Subramaniam et al., 2001b). IFNγ has a molecular mass that should allow its entry into the nucleus by simple diffusion, yet this molecule has a strong NLS. Association of IFNγ, IFNGR1 and STAT1α together with the nuclear importer NPI-1 strongly suggests that STAT1α may require a chaperoning function, which is provided by the ligand and its receptor subunit. In conclusion, nuclear translocation of STAT1α may not be unassisted, as the previous models have suggested. Association of IFNγ (containing a strong NLS), IFNGR1 and STAT1α with the nuclear importer, NPI-1, suggests a chaperoning role for IFNγ and IFNGR1 in nuclear translocation of STAT1α and may have a role in imparting specificity to STAT1α activity.

We gratefully acknowledge Melissa Chen at the Flow Cytometry Core Facility, University of Florida, for assistance with flow cytometry. This work was supported by NIH grant CA38587 to H.M.J. This manuscript is Florida Experimental Station Journal Series no. R-09524.

References

Ahmed, C. M. I., Wills, K. N., Sugarman, B. J., Johnson, D. E., Ramachandra, M., Nagabhushan, T. L. and Howe, J. A. (2001). Selective expression of nonsecreted interferon by adenoviral vector confers antiproliferative and and antiviral properties and causes reduction of tumor growth in nude mice. *J. Interferon Cytokine Res.* **21**, 399-408.

Bejcek, B. E., Li, D. Y. and Deuel, T. F. (1989). Transformation by v-sis occurs by an internal autoactivation mechanism. *Science* **245**, 1496-1499.

Dunbar, C. E., Browder, T. M., Abrams, J. S. and Nienhuis, A. W. (1989). COOH-terminal modified interleukin-3 is retained intracellularly and stimulates autocrine growth. *Science* **245**, 1493-1496.

Familetti, P. C., Rubinstein, S. and Pestka, S. (1981). A conventional and rapid cytopathic effect inhibition assay for interferon. *Methods Enzymol.* **78**, 387-394.

Farrar, M. A., Fernandez-Luna, J. and Schreiber, R. D. (1991). Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. *J. Biol. Chem.* **266**, 19626-19635.

Jans, D. A. and Hassan, G. (1998). Nuclear targeting by growth factors, cytokines and their receptors: a role in signaling? *BioEssays* **20**, 400-411.

Killion, J. J., Fishbeck, R., Bar-Eli, M. and Chernajovsky, Y. (1994). Delivery of interferon to intracellular pathways by encapsulation of interferon into multilamellar liposomes is independent of the status if interferon receptors. *Cytokine* **6**, 443-449.

Larkin, J., Johnson, H. M. and Subramaniam, P. S. (2000). Differential nuclear localization of IFNGR1 and IFNGR2 subunits of the IFN-gamma receptor complex following activation by IFN-gamma. *J. Interferon Cytokine Res.* **20**, 565-576.

Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bourguignon, L. and Hung, M. C. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat. Cell Biol.* **3**, 802-808.

Mahboubi, K. and Pober, J. S. (2002). Activation of signal transducer and activator of transcription 1 (STAT1) is not sufficient for the induction of STAT1-dependent genes in endothelial cells. Comparison of interferon-gamma and oncostatin M. *J. Biol. Chem.* **277**, 801-821.

McBride, K. M., Banninger, G., McDonald, C. and Reich, N. C. (2002). Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-alpha. *EMBO J.* **21**, 1754-1763.

Melen, K., Kinnunen, L. and Julkunen, I. (2001). Arginine/lysine-rich structural element is involved in interferon-induced nuclear import of STATs. *J. Biol. Chem.* **276**, 16447-16455.

Pestka, S., Kotenko, S. V., Muthukumaran, G., Izotova, L. S., Cook, J. R. and Garotta, G. (1997). The interferon gamma (IFN-gamma) receptor: a paradigm for the multichain cytokine receptor. *Cytokine Growth Factor Rev.* **3**, 189-206.

Rutherford, M. N., Kumar, A., Coulombe, B., Skup, D., Carver, D. H. and Williams, B. R. (1996). Expression of intracellular interferon constitutively activates ISGF3 and confers resistance to EMC viral infection. *J. Interferon Cytokine Res.* **16**, 507-510.

Sanceau, J., Sondermeyer, P., Beranger, F., Falcoff, R. and Vaquero, C. (1987). Intracellular human gamma-interferon triggers an antiviral state in transformed murine L cells. *Proc. Natl. Acad. Sci. USA* **84**, 2906-2910.

Sayeski, P. P., Ali, M. S., Frank, S. J. and Bernstein, K. E. (2001). The angiotensin II-dependent nuclear translocation of STAT1 is mediated by the Jak2 protein motif 231 YRFRR. *J. Biol. Chem.* **276**, 10556-10563.

Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T. and Yoneda, Y. (1996). Interferon-gamma-dependent nuclear import of STAT1 is mediated by the GTPase activity of Ran/TC4. *J. Biol. Chem.* **271**, 31017-31020.

Sekimoto, T., Imamoto, N., Nakajima, K. Hirano, T. and Yoneda, Y. (1997). Extracellular signal-dependent nuclear import of STAT1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. *EMBO J.* **16**, 7067-7077.

Smith, M. R., Muegge, K., Keller, J. R., Kung, H. F., Young, H. A. and Durum, S. K. (1990). Direct evidence of an intracellular role for IFN-

gamma. Microinjection of human IFN-gamma induces Ia expression on murine macrophages. *J. Immunol.* **144**, 1777-1782.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. and Schreiber, R. D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227-264.

Subramaniam, P. S. and Johnson, H. M. (2002). Lipid microdomains are required sites for the selective endocytosis and nuclear translocation of IFNγ, its receptor chain IFNγ receptor-1, and the phosphorylation and nuclear translocation of STAT1α. *J. Immunol.* **169**, 1959-1969.

Subramaniam, P. S., Mujtaba, M. G., Paddy, M. R. and Johnson, H. M. (1999). The carboxyl terminal of interferon-gamma contains a functional polybasic nuclear localization sequence. *J. Biol. Chem.* **274**, 403-407.

Subramaniam, P. S., Larkin, J., Mujtaba, M. G., Walter, M. R. and Johnson, H. M. (2000). The COOH-terminal nuclear localization sequence of interferon gamma regulates STAT1 alpha nuclear translocation at an intracellular site. *J. Cell Sci.* **113**, 2771-2781.

Subramaniam, P. S., Green, M. M., Larkin, J., Torres, B. A. and Johnson, H. M. (2001a). Nuclear translocation of IFN-gamma is an intrinsic requirement for its biologic activity and can be driven by a heterologous nuclear localization sequence. *J. Interferon Cytokine Res.* **21**, 951-959.

Subramaniam, P. S., Torres, B. A. and Johnson, H. M. (2001b). So many ligands, so few transcription factors: a new paradigm for signaling through the STAT transcription factors. *Cytokine* **15**, 175-187.

Szente, B. E. and Johnson, H. M. (1994). Binding of IFN gamma and its C-terminal peptide to a cytoplasmic domain of its receptor that is essential for its function. *Biochem. Biophys. Res. Commun.* **201**, 215-221.

Szente, B. E., Soos, J. M. and Johnson, H. M. (1994). The C-terminus of IFN gamma is sufficient for its intracellular function. *Biochem. Biophys. Res. Commun.* **203**, 1645-1654.

Szente, B., Subramaniam, P. S. and Johnson, H. M. (1995). Identification of IFN-gamma receptor binding sites for JAK2 and enhancement of binding by IFN-gamma and its C-terminal peptide IFN-gamma (95-133). *J. Immunol.* **155**, 5617-5622.

Thiam, K., Loing, E., Delanoye, A., Diesis, E., Gras-Masse, H., Auriault, C. and Verwaerde, C. (1998). Unrestricted agonist activity on murine and human cells of a lipopeptide derived from IFN-gamma. *Biochem. Biophys. Res. Commun.* **253**, 639-647.

Will, A., Hemmann, U., Horn, F., Rollinghoff, M. and Gessner, A. (1996). Intracellular murine IFN-gamma mediates virus resistance, expression of oligoadenylate synthetase, and activation of STAT transcription factors. *J. Immunol.* **157**, 4576-4583.