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The COOH-terminal nuclear localization sequence of interferon γ regulates STAT1α nuclear translocation at an intracellular site

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SUMMARY

We have recently shown that the nuclear localization of IFNγ is mediated by a polybasic nuclear localization sequence (NLS) in its C terminus. This NLS is required for the full expression of biological activity of IFNγ, both extracellularly and intracellularly. We now show that this NLS plays an integral intracellular role in the nuclear translocation of the transcription factor STAT1α activated by IFNγ. Treatment of IFNγ with antibodies to the C-terminal region (95-133) containing the NLS blocked the induction of STAT1α nuclear translocation. The antibodies had no effect on nuclear translocation of STAT1α in IFNα treated cells. A deletion mutant of human IFNγ, IFNγ(1-123), which is devoid of the C-terminal NLS region was found to be biologically inactive, but was still able to bind to the IFNγ receptor complex on cells with a K_d similar to that of the wild-type protein. Deletion of the NLS specifically abolished the ability of IFNγ(1-123) to initiate the nuclear translocation of STAT1α, which is required for the biological activities of IFNγ following binding to the IFNγ receptor complex. Thus, the NLS region appears to contribute minimally to extracellular high-affinity receptor-ligand binding, yet exerts a strong functional role in STAT1α nuclear localization. A high-affinity site for the interaction of the C-terminal NLS domain of IFNγ with a K_d approx. 3×10^{-8} M^{-1} has been described by previous studies on the intracellular cytoplasmic domain of the IFNγ receptor α-chain. To examine the role of the NLS at the intracellular level, we microinjected neutralizing antibodies raised against the C-terminal NLS domain of IFNγ into the cytoplasm of cells before treatment of cells with IFNγ. These intracellular antibodies specifically blocked the nuclear translocation of STAT1α following the subsequent treatment of these cells extracellularly with IFNγ. These data show that the NLS domain of IFNγ interacts at an intracellular site to regulate STAT1α nuclear import. A C-terminal peptide of murine IFNγ, IFNγ(95-133), that contains the NLS motif, induced nuclear translocation of STAT1α when taken up intracellularly by a murine macrophage cell line. Deletion of the NLS motif specifically abrogated the ability of this intracellular peptide to cause STAT1α nuclear translocation. In cells activated with IFNγ, IFNγ was found to as part of a complex that contained STAT1α and the importin-α analog Npi-1, which mediates STAT1α nuclear import. The tyrosine phosphorylation of STAT1α, the formation of the complex IFNγ/Npi-1/STAT1α complex and the subsequent nuclear translocation of STAT1α were all found to be dependent on the presence of the IFNγ NLS. Thus, the NLS of IFNγ functions intracellularly to directly regulate the activation and ultimate nuclear translocation STAT1α.

Key words: Nuclear localization, NLS, Interferon, STAT

INTRODUCTION

Interferon-gamma (IFNγ) is thought of as predominantly an extracellular cytokine that transmits signals through the plasma membrane by binding the extracellular domain of its specific cell surface receptor complex. This interaction activates the JAK/STAT pathway for signaling to the nucleus. However, several independent studies have demonstrated that IFNγ can induce similar biological activities when it is expressed intracellularly, in effect bypassing the extracellular domain of the receptor (reviewed by Johnson et al., 1998a,b; see also Will et al., 1996). These effects include induction of MHC Class II antigens, induction of an antiviral state, expression of oligoadenylate synthetase and activation of STAT transcription factors (Johnson et al., 1998a,b).

Two intracellular sites of interaction for the C terminus of IFNγ have been identified (Johnson et al., 1998a,b; Subramaniam et al., 1999). We have shown that the C terminus of IFNγ encompassing residues 95-133 in mouse IFNγ and residues 95-134 in human IFNγ interact with high-affinity at a membrane proximal site on the cytoplasmic domain of the α-chain of the IFNγ receptor (Johnson et al., 1998a,b). This interaction contributes to the ligand-induced binding of JAK2 to this receptor subunit at an immediately adjacent site (Johnson et al., 1998a,b). A second site of interaction is at the level of the pathway for the nuclear transport. Extracellularly

added IFNγ is itself translocated to the nucleus (Bader and Wietzerbin, 1994; MacDonald et al., 1986). We have shown recently that this nuclear translocation of IFNγ is mediated by a polybasic nuclear localization sequence that is located in the C-terminal receptor binding domain described above, and this NLS utilizes components of the well-characterized Ran/importin pathway for nuclear import (Subramaniam et al., 1999). Both intracellular receptor binding and nuclear translocation are absolutely dependent on the presence of the NLS in the C-terminal domain, strongly suggesting that intracellular receptor binding and nuclear translocation may be linked.

The exact contributions of the C-terminal domain of IFNγ, in general, and the NLS, in particular, at the intracellular level to signal transduction events have not yet been elucidated. However, several lines of evidence suggest that this region is important for biological function, extracellularly and intracellularly. At the extracellular level, mutants of IFNγ that are modified or deleted in the NLS motif show poor biological activity (Arakawa et al., 1986, 1989; Dobeli et al., 1988; Wetzel et al., 1990; Lundell et al., 1991; Slodowski et al., 1991). At the intracellular level, we have shown that a peptide encompassing this C-terminal domain has agonist properties, and is sufficient to induce MHC Class II and an antiviral state when internalized by murine macrophages (Szente et al., 1994). The agonist properties of the internalized peptide were specifically dependent on the presence of the NLS (Szente et al., 1994). The agonist properties of the intracellularly delivered peptide have recently been confirmed by independent studies (Thiam et al., 1998). Using IFNGRα –/– cells, these studies, along with others, also showed that the intracellular properties of IFNγ and its agonist peptides were dependent on the presence of the α-chain of the IFNγ receptor subunit (Will et al., 1996; Thiam et al., 1998), strongly supporting our observation that one intracellular site of interaction of the C-terminal domain of IFNγ is at the cytoplasmic domain of the IFNγ receptor α-chain.

In the present report we show that the C-terminal domain of IFNγ specifically modulates STAT1α nuclear translocation, both intracellularly and extracellularly. Deletion of the NLS region in IFNγ or neutralization with C-terminal antibodies blocks the nuclear translocation of STAT1α induced by extracellularly added IFNγ. This deletion did not affect the affinity of the ligand for the receptor suggesting that this region does not contribute significantly to high-affinity binding. In contrast, at least one high-affinity site with a K_d of approx. 3×10^{-8} M^{-1} for the C-terminal domain encompassed by 95-133 has been demonstrated on the intracellular cytoplasmic domain of IFNGRα (Johnson et al., 1998a,b; Green et al., 1998). Cytoplasmic microinjection of anti-C-terminus antibodies is also able to inhibit the nuclear translocation of STAT1α initiated by extracellularly added IFNγ. IFNγ was found to be part of a complex with the STAT1α and the its nuclear transporter Npi-1. The activation, complexation of STAT1α and IFNγ to Npi-1 and the nuclear translocation of STAT1α was found to be dependent of the NLS of IFNγ. These data show that IFNγ interacts at an intracellular site to directly modulate the nuclear translocation of STAT1α, and suggest that the NLS of IFNγ plays a direct role in the nuclear translocation of STAT1α itself.

MATERIALS AND METHODS

Materials

Recombinant human IFNγ (≥1×10^7 units/mg) was purchased from BioSource International (Camarillo, CA). Recombinant mouse IFNγ (≥1×10^7 units/mg) was obtained from PeproTech (Rocky Hill, NJ). Mouse IFNα was purchased from BioSource International. Antibodies to STAT1α (developed in rabbit) were from Santa Cruz Biotechnology (Santa Cruz, CA) or from R&D Systems (Minneapolis, MN; developed in goat). Alexa-conjugated secondary antibodies for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Other secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA), and Santa Cruz Biotechnology (anti-goat IgG-HRP conjugate). Antisera to the C-terminal domain of murine IFNγ were developed in rabbits by immunizing with a synthetic peptide encompassing amino acids 95-133 of murine IFNγ, and have been described previously (Russell et al., 1986).

Cell lines and cell culture

All cell lines were from ATCC. WISH and L 929 cells were grown in Eagle's minimal essential (EMEM) medium containing 10% fetal bovine serum (FBS) and antibiotics (penicillin: 10,000 units/ml, streptomycin: 10 mg/ml, amphotericin B: 25 μg/ml).

Purification of recombinant human IFNγ(1-123)

The expression vector for the C-terminal truncated mutant of human IFNγ, IFNγ(1-123) is to be described elsewhere (M. R. Walter, unpublished). The protein was expressed in *E. coli*, and IFNγ(1-123) and was purified from inclusion bodies. Inclusion bodies were washed extensively with extraction buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF) and treated with DNAse in extraction buffer. Following centrifugation and washing the inclusion bodies were solubilized into 6 M guanidine hydrochloride in extraction buffer. Insoluble material was removed by centrifugation, and the protein solution diluted slowly with extraction buffer to a final concentration of 0.1 M guanidine hydrochloride. The purity of the renatured protein was adjudged by a combination of SDS-PAGE analysis and western blotting with antibodies to the N terminus of IFNγ. In the assays described here the final concentration of the guanidine hydrochloride did not exceed 1 mM, which was not found to affect cell growth or viability.

Radiolabeling of IFN and binding assays

Recombinant human IFNγ, recombinant IFNγ(1-123) and recombinant murine IFNγ were labeled with $Na^{125}I$ as previously described (Green et al., 1998). Binding assays on WISH cells using recombinant human IFNγ and human IFNγ(1-123) were performed in triplicate using a standard 'cold' saturation method, as previously described (Green et al., 1998). Data from binding experiments were analyzed for binding constants using the LIGAND computer program, and values were replotted for presentation in Fig. 1.

Immunofluorescence staining

Cells were grown on tissue culture slides at approx. 2×10^5 cells per slide, and treated as described in the figure legends. For cells treated with antibodies, slides were washed with 4× 3 ml of pre-warmed growth medium, before fixation for staining. Cells were fixed in 95% methanol at –20°C for 4 minutes and allow to air dry. Following rehydration in TBS (100 mM Tris-HCl, pH 7.5, 0.9% NaCl), cells were permeabilized in TBS containing 0.5% Triton X-100 for 10-15 minutes. Permeabilized cells were then stained for STAT1α using anti-STAT1α antibodies followed by visualization with the appropriate fluorescently labeled secondary antibody. After each antibody staining, cells were washed with TBS containing 0.1% Triton X-100. Finally, cells were mounted between coverslips in Prolong Antifade (Molecular Probes, Eugene, OR) mounting medium

and observed using a deconvolution fluorescence microscope. Image acquisition and data analysis were performed at the Optical Microscopy Facility at the Center for Structural Biology, University of Florida, Gainesville, FL. Quantitation of fluorescence was performed on images using the NIH Image software. The mean fluorescence (f) intensity from approximately equal areas in the cytoplasm (fc) and the nucleus (fn) from each cell within a field was measured. The areas were chosen arbitrarily within cells and across fields. This was designed to give truly average values. The ratio fn/fc for each cell from treated samples was subtracted against the average fn/fc ratio from measurements on untreated cells, and the resulting values, Fn/Fc, were averaged for each field. Averaged Fn/Fc ratios were then plotted against time or dose of IFN treatment.

For treatment with peptides, mouse P388D_1 macrophages (1×10^6 cells) in Dulbecco's modified essential medium containing 10% FBS were equilibrated at 37°C in 5% CO_2 before the peptides were added to a final concentration of 25 mM. Following further incubation for 30 minutes, cells were cytocentrifuged onto microscope slides and immediately fixed in 95% methanol at –20°C. The cells were then stained for STAT1α localization as above, and counterstained with DAPI to mark the nuclear volume.

Microinjection

Mouse L 929 cells grown on slides were microinjected on an Eppendorf automated microinjection system fitted on a Zeiss inverted microscope. Before use the growth medium was exchanged for Leibovitz's L-15 medium containing 10% medium that had been pre-warmed to 37°C. This medium is designed for growth in air and ensured the integrity of cells during microinjection. Cells were microinjected with a mixture of the test antibody - a 1:10 dilution of rabbit prebleed antisera as a control, or a 1:10 dilution of rabbit antisera to the C terminus of mouse IFNγ - and a 2.5 mg/ml solution of purified rabbit IgG. The latter was used to locate injected cells following immunofluorescence staining. Between 35-50 cells were routinely injected, following which cells were washed with 3× 3 ml of injection medium, and allowed to recover by incubation in regular growth medium (EMEM containing 10% FBS) at 37°C for 1 hour. The medium was then removed and replaced with pre-warmed medium containing mouse IFNγ (500 units/ml) or mouse IFNα (500 units/ml) that had been equilibrated in a 5% CO_2 atmosphere at 37°C. Cells were then treated as required and subjected to immunofluorescence staining for STAT1α as described above.

Immunoprecipitation and immunoblotting

To prepare cytoplasmic and nuclear extracts cells were washed twice with ice-cold PBS, dislodged by scraping into cold PBS, centrifuged at 4°C and flash-frozen in liquid N_2 for storage at –80°C until used. Nuclei were liberated from cells by lysis at 4°C in buffer A: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20, 50 mM NaF, 20 mM b-glyceroylphosphate, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 20 μg/ml each of leupeptin, pepstatin and aprotinin, 5 mg/ml benzamidine, 50 mM *p*-nitrophenylguanadinobenzoate, 1 mM PMSF. Nuclei were pelleted (500 ***g***) at 4°C and supernatants saved, and nuclei washed by agitation for 20 minutes with buffer A to which 0.2% NP-40 was added (buffer B). Following centrifugation (500 ***g***), the supernatant was combined with that from the previous step as cytoplasmic extract, and pelleted nuclei were further resuspended in buffer B. Efficiency of nuclei isolation was visualized by microscopic analysis of trypan blue stained aliquots of resuspended nuclei.

Nuclei were recovered by centrifugation and supernatant discarded. Nuclear extracts were prepared from the intact nuclei by lysis, for 30 minutes on ice, using a high salt buffer consisting of 20 mM Hepes (pH 7.9), 420 mM KCl, 1.5 mM $MgCl_2$, 50 mM NaF, 0.2 mM EDTA, 0.5 mM DTT, 2 mM sodium orthovanadate, 25% glycerol, 0.5 mM PMSF, 1 μg/ml each of pepstatin, aprotinin, and leupeptin, and centrifugation for 15 minutes at 14,000 rpm at 4°C to remove the insoluble fraction.

For co-immunoprecipitations using ^{125}I-labeled IFNs, mouse L 929 cells or human WISH cells (see legends) were incubated at 4°C with 0.33 μg/ml (3300 units/ml) of ^{125}I-labeled murine or human IFNs, as the case may be, for 1 hour. Cells were then shifted to 37°C for the appropriate time periods. Control cells were maintained at 4°C. Following incubation at 37°C, cells were washed three times with ice-cold growth medium and once with ice-cold PBS. Control cells were processed last. Cells were dislodged by scraping into cold PBS, and cell pellets flash-frozen in liquid N_2, for storage at –80°C till use. Cells were lysed at 4°C into lysis buffer (buffer A; see above), to provide cytoplasmic extracts.

For immunoprecipitation, equal protein amounts of lysates were immunoprecipitated using anti-STAT1α antibodies. Immunoprecipitates were washed once with 50 mM Tris-HCl, pH 6.8, containing 2 mM sodium orthovanadate, and immune complexes separated on SDS-PAGE. Following transfer of proteins to nitrocellulose, ^{125}I-IFNγ was detected by autoradiography. Immunoprecipitated STAT1α was detected by immunoblotting with anti-STAT1α antibodies. Phosphorylation of STAT1α was followed by immunoblotting with a polyclonal antibody directed specifically against Tyr^{701}-phopshorylated STAT1α (New England BioLabs, MA).

For the blots in Fig. 3B, L 929 cells were treated at 37°C with IFNγ as indicated with or without the addition of anti-C-terminus antibodies. Cells were lysed in the above lysis buffer, except that 1% NP-40 replaced 0.2% Tween-20, and 100 μg of total protein for each sample was directly resolved by SDS-PAGE before transfer to nitrocellulose and immunoblotting.

RESULTS

A number of earlier studies have shown that truncations in the C terminus of IFNγ that destroy the polybasic region, which we have identified as a NLS, lead to drastic loss in the biological properties of IFNγ (Arakawa et al., 1986, 1989; Dobeli et al., 1988; Wetzel et al., 1990; Lundell et al., 1991; Slodowski et al., 1991). These studies suggest that the NLS is critical for the biological activity of IFNγ. As a starting point to analyzing the role in signal transduction of the NLS in the C terminus of IFNγ, we re-examined one such mutant, IFNγ (1-123), that is deleted from residues 123 (see Table 1 for sequences) onwards including the IFNγ NLS. To determine the structural and functional properties of IFNγ(1-123), we

Table 1. Sequences referred to in this study

Hu IFNγ C terminus*	------^{91}KRDDFEKLTNYSVTDLNVQRKAIHELIQVMAELSPAA**KTGKRKR**SQMLFRGRRASQ
Hu IFNγ(1-123) C terminus*	------^{91}KRDDFEKLTNYSVTDLNVQRKAIHELIQVMAEL^{123}
IFNγ(95-134) (human)	FEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAKTG**KRKR**
IFNγ(95-133) (mouse)	MSIAKFEVNNPQVQRQAFNELIRVVHQLLPESSL**RKRKR**
IFNγ(95-125) (mouse)	MSIAKFEVNNPQVQRQAFNELIRVVHQLLPE

*The sequences are shown only for the relevant C-terminal portions of the intact IFNs. Sequences are derived from the mature form of IFNγ. NLS sequences are in bold.

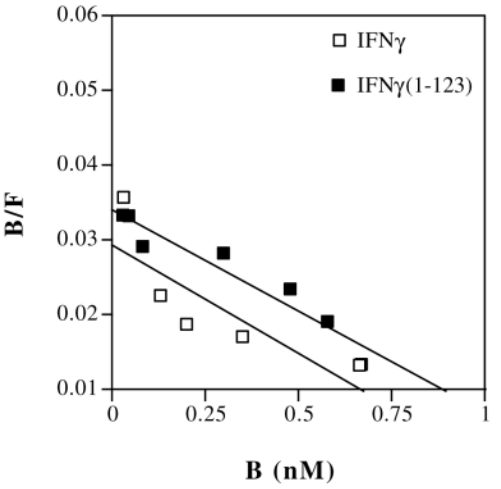


Fig. 1. Comparison of binding for IFNγ and IFNγ(1-123) on WISH cells. Binding was evaluated in a standard 'cold' saturation experiment using ^{125}I-IFNγ (5 nM). Data were analyzed using the LIGAND computer program, and data from Scatchard analysis of binding have been replotted here. Samples were run in triplicate. Specific binding of ^{125}I-IFNγ, determined in the presence of a 100-fold excess of unlabeled IFNγ, was found to be >80%.

compared it with wild-type IFNγ in in vitro binding assays and antiviral assays. Fig. 1 shows the Scatchard analysis of a standard 'cold' saturation experiment on binding of IFNγ(1-123) to WISH cells and its comparison with wild-type IFNγ (Green et al., 1998). Scatchard analysis of the binding showed that IFNγ(1-123) bound to receptors on WISH cells with a K_d that was very similar to that of wild-type IFNγ. Thus, IFNγ(1-123) was just as competent as wild-type IFNγ in binding to receptors on intact cells. Deletion of the C-terminal amino acids, including the NLS, from IFNγ did not significantly affect the affinity of IFNγ(1-123) for the receptor, showing that the C-terminal amino acids in general, and the NLS in particular, do not contribute significantly to high-affinity binding to the receptor complex. This conclusion is further supported by recent studies using surface plasmon resonance to address the binding of IFNγ(1-124) to the extracellular domain of IFNGRα (Sadir et al., 1998), and by data from the X-ray crystal structure of the complex between IFNγ and the extracellular domain of IFNGRα (Walter et al., 1995).

When compared in antiviral assays, however, IFNγ(1-123) was found to be drastically reduced in its biological activity. IFNγ(1-123) had less than 0.5% of the specific antiviral activity of wild-type IFNγ when compared on the same WISH cells (data not shown). This is consistent with the earlier functional studies on C-terminal deletion mutants (Arakawa et al., 1986, 1989; Dobeli et al., 1988; Wetzel et al., 1990; Lundell et al., 1991; Slodowski et al., 1991). Thus, while the NLS-containing C-terminal region does not seem to contribute significantly to high-affinity interactions for binding to the receptor complex on WISH cells, it appears to play a crucial role in the ability of IFNγ to induce a biological response in these cells. The possibility, however, that this region is involved in low-affinity interactions with the extracellular domains of the receptor cannot be ruled out.

Since nuclear translocation of STAT1α is dependent on phosphorylation of STAT1α by JAK1 and JAK2, we used nuclear translocation of STAT1α as a marker of receptor activation. Cells were treated with IFNγ(1-123) and nuclear localization of STAT1α followed by immunofluorescence, and compared with wild-type IFNγ. As can be seen in Fig. 2, IFNγ(1-123) was impaired in its ability to induce STAT1α nuclear translocation, compared to IFNγ. Thus, the deletion of the NLS in IFNγ is coincident with the loss of ability to induce the activation and nuclear translocation of STAT1α. Since STAT1α presence in the nucleus is required for biological activity this is consistent with the poor biological activity of IFNγ(1-123). In this regard, preliminary studies have revealed that reconstitution of the NLS function of IFNγ using the heterologous NLS of the SV40 large T-antigen (IFNγ-SV) restored biological activity to wild-type levels (M. M. Green, P. S. Subramaniam, B. A. Torres and H. M. Johnson, unpublished). Consistent with its biological properties, IFNγ-SV is able to induce the nuclear translocation of STAT1α similar to wild-type IFNγ by inducing the phosphorylation of STAT1α on Tyr^{701} that is required for its dimerization, nuclear translocation and gene-induction properties.

To further characterize the role of the IFNγ C terminus in

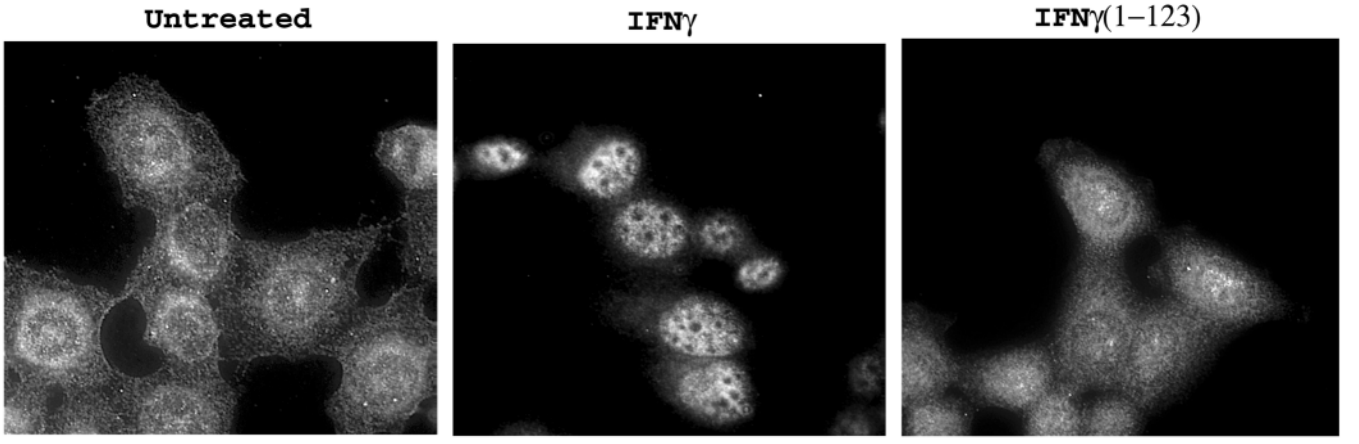


Fig. 2. Deletion of the NLS in IFNγ inhibits nuclear translocation of STAT1α. WISH cells were treated for 30 minutes at 37°C with equal amounts (2.5 ng/ml) of IFNγ or IFNγ(1-123), or left untreated, as indicated, before being fixed and subjected to immunofluorescence staining for STAT1α localization using rabbit anti-STAT1α antibodies.

STAT1α activation and nuclear translocation, we determined the ability of IFNγ-neutralizing polyclonal antibodies raised against the C-terminal domain to inhibit nuclear translocation of STAT1α. These polyclonal antisera to the amino acids 95-133 of murine IFNγ have been previously described (Russell et al., 1986). Consistent with results with truncated IFNγ, the antibodies, when incubated with IFNγ prior to addition to cells, blocked the ability of IFNγ to activate STAT1α nuclear translocation (Fig. 3A). As a specificity control, we also tested these antibodies against the type I IFN IFNα, which also activates STAT1α. These antibodies did not block the nuclear translocation activity of STAT1α by the IFNα, showing that they were specific for IFNγ. This inhibition of nuclear translocation of STAT1α by these antibodies coincided with their ability to block the tyrosine phosphorylation of STAT1α in IFNγ-treated cells (Fig. 3B). Thus, neutralization of the NLS-containing C terminus region of IFNγ with specific antibodies resulted in loss of STAT1α nuclear translocation function, similar to that of the C-terminal truncated IFNγ.

To directly examine the possible intracellular role of the IFNγ C-terminal NLS region in STAT1α nuclear translocation we microinjected the above-described polyclonal antibodies raised against the C terminus into to the cytoplasm of cells before treatment of cells with IFNγ. As seen in Fig. 4A, intracellularly injected C-terminal antibodies were able to inhibit the nuclear translocation of STAT1α induced by IFNγ that was added extracellularly (Fig. 4A, lowest panel). Note that this effect was specific for cells injected with the antibodies since uninjected cells in the immediate vicinity were unaffected with respect to their response to IFNγ. Further, normal rabbit serum (prebleed) did not block nuclear translocation of STAT1α (Fig. 4A, middle panel). Quantitiation

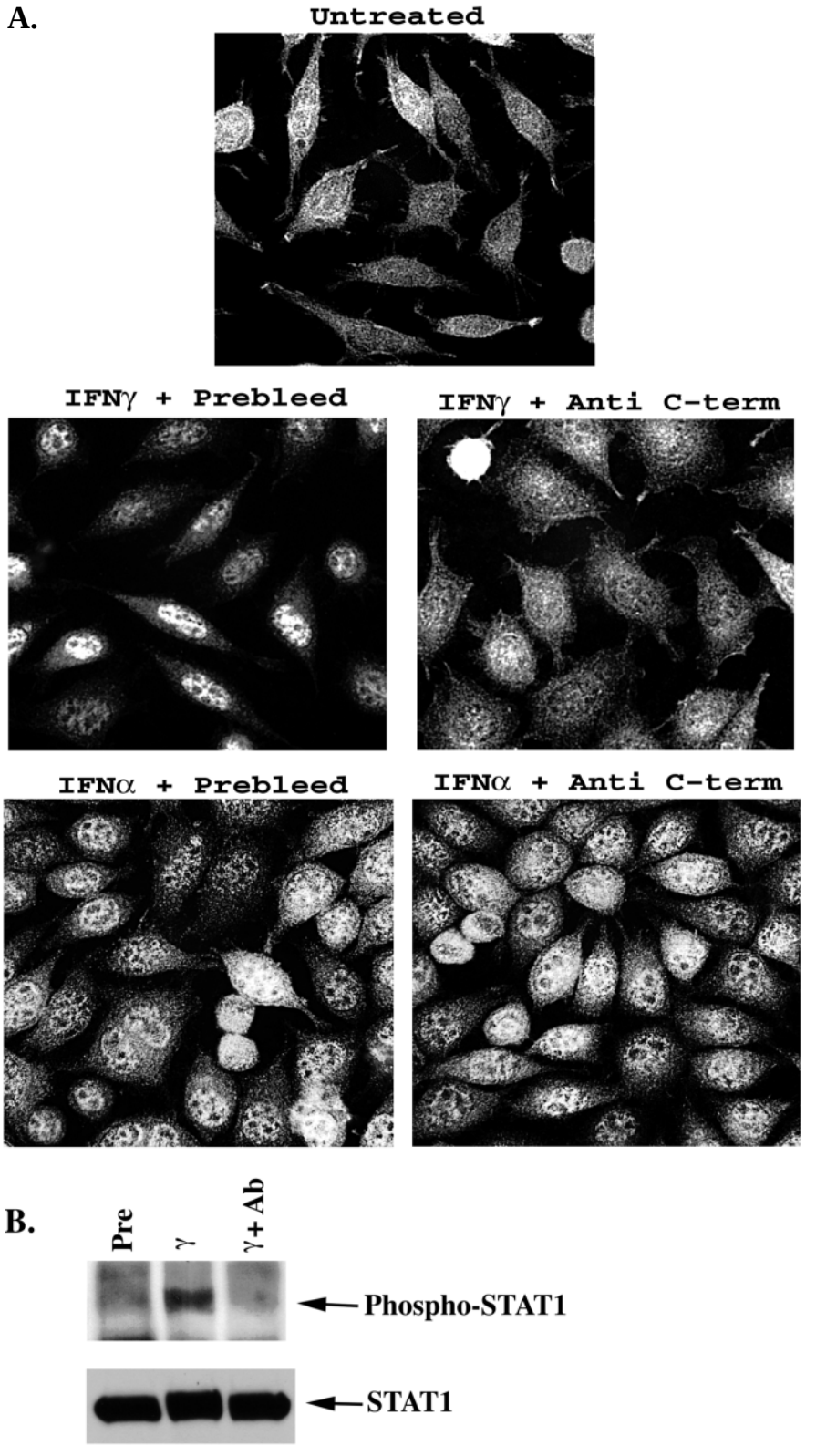


Fig. 3. Antibodies to the C terminus of IFNγ block the STAT1α activation and nuclear localization function of IFNγ. (A) Mouse L 929 cells were either treated with rabbit prebleed antibodies as a control (Untreated), or treated with 5000 units/ml each of IFNγ (IFNγ) or IFNα (IFNα) in the absence (Prebleed) or presence of a 1:30 dilution of rabbit antibodies against the C-terminal peptide IFNγ(95-133) (Anti-C-term) corresponding to mouse IFNγ. Cells were then fixed and immunofluorescently stained for STAT1α. (B) Mouse L 929 cells were treated with 5000 units/ml of IFNγ (γ) in the presence of a 1:30 dilution of prebleed (Pre) or the anti-C-terminus antibodies to IFNγ (Ab) outlined in (A) for 20 minutes at 37°C. Total cell lysates were then analyzed by immunblotting of equal amounts of protein for STAT1α phosphorylation (upper panel) using a polyclonal antibody specific for Tyr^{701} phosphorylated STAT1α (New England Biolabs). Blots were then stripped and reprobed (lower panel) for total STAT1α protein using STAT1α antibodies.

of these digital images (Fig. 4B) showed that inhibition by the intracellular C terminus antibodies was significant, with an approximately 3-fold decrease in the amount of STAT1α accumulated in the nucleus. For comparison, quantitation of nuclear STAT1α in nearby uninjected cells in each field is also shown, along with the background levels in untreated cells (from Fig. 4A, uppermost panel).

Consistent with the IFNγ specificity of these antibodies, the microinjected C-terminal antibodies had no effect on IFNα induced nuclear translocation of STAT1α (Fig. 4C). Quantitation of these images for IFNα (Fig. 4D) show that the amount of nuclear STAT1α accumulated by IFNα in the presence of intracellular anti-C-terminus antibodies was essentially the same as that of prebleed antibodies, and similar to that in uninjected cells. Thus, the inhibition of the nuclear translocation of STAT1α by intracellular anti-C-terminus antibodies to IFNγ was specific to IFNγ.

In general, we observed that the distribution of STAT1α in IFNα treated cells was more diffuse than the more intense nuclear STAT1α signal in IFNγ treated cells. Also, as observed visually, the quantity of STAT1α accumulated (Fig. 4B,D) in the nucleus by IFNγ treatment was greater than that induced by IFNα treatment. This is possibly a reflection, in part at least, of the stoichiometry of activated STAT1α in IFNγ-treated cells within a homodimeric complex versus the heterotrimeric complex with STAT2 and p48 that forms when STAT1α is activated by IFNα.

The data from microinjection experiments show that IFNγ has an intracellular role in the events of signal transduction leading to activation and nuclear translocation of STAT1α, which are subsequent to the internalization of the ligand by receptor-mediated endocytosis. This is mediated by the C-terminal domain of IFNγ encompassing residues 95-133 that contains the NLS.

We have previously shown that human IFNγ(95-134) and mouse IFNγ(95-133) peptides (see Table 1), which also contain the NLS, induced MHC Class II expression and an antiviral state when taken up intracellularly by murine macrophages through macropinocytosis (Szente et al., 1994). Their agonist properties were lost when the NLS motif in these peptides was deleted (Szente et al., 1994). Since these biological activities require the activation of STAT1α, we determined the abilitiy of the internalized mouse peptide IFNγ(95-133) (see Table 1) and mouse peptide IFNγ(95-125) that is deleted in the NLS motif to induce nuclear translocation of STAT1α in these murine macrophages. Nuclear localization of STAT1α was monitored by immunofluorescence staining and cells were counterstained with DAPI to mark the nuclear volume. As can be seen in Fig. 5A, intact IFNγ,

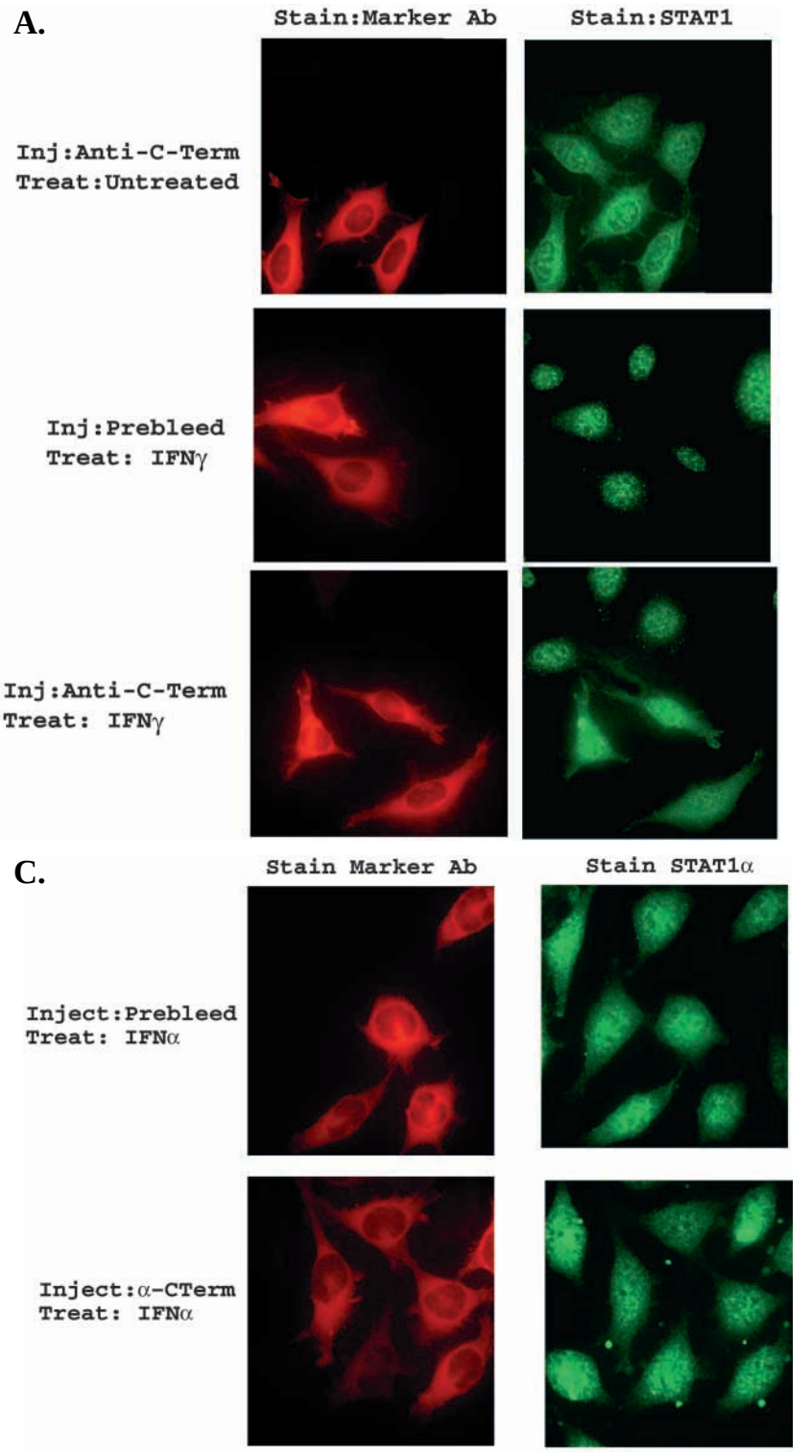
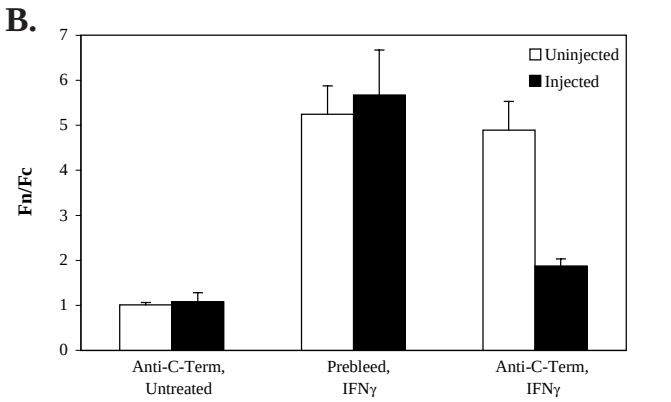
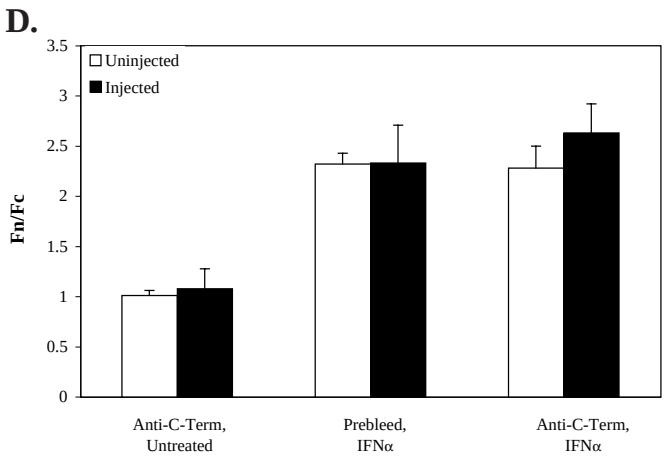


Fig. 4. IFNγ regulates STAT1α nuclear translocation at an intracellular site. Mouse L 929 cells were microinjected with antibodies to the C-terminal peptide IFNγ(95-133) (Anti-C-Term) or prebleed antisera (Prebleed) as described in before being treated with 500 units/ml of mouse IFNγ, (A), for 30 minutes at 37°C, or 500 units/ml of mouse IFNα, (C). Cells were then subjected to immunofluorescence staining for the marker antibody (red, TexasRed), to indicate injected cells, and for STAT1α (green, FITC) using 2 μg/ml of a goat anti-STAT1α antibody. Quantitation of the images as described in Materials and Methods was performed for IFNγ-treated, (B), and IFNα-treated, (D), cells to obtain the average nuclear (Fn) to cytoplasmic (Fc) fluorescence ratios (Fn/Fc), in both injected and uninjected cells as indicated.





as expected, induced the nuclear localization of STAT1α. The mouse peptide IFNγ(95-133) was also able to induce the nuclear translocation of STAT1α. The STAT1α signal from IFNγ(95-133) treatment was lower than that from IFNγ, reflecting the higher specific activity of intact IFNγ (Szente et al., 1994). In contrast, peptide IFNγ(95-125) that is deleted in the NLS motif was not able to induce the nuclear translocation of STAT1α and showed a staining pattern indistinguishable from untreated cells. Quantitation of these images is presented in Fig. 5B. IFNγ(95-133) specifically induced nuclear uptake of STAT1α although it is quantitatively a weaker inducer when compared to IFNγ. Deletion of the NLS from IFNγ(95-133), as shown by IFNγ(95-125) treated cells, resulted in essentially no STAT1α nuclear translocation. These data confirm that the NLS-containing C-terminal domain of IFNγ interacts at an intracellular site to induce the nuclear translocation of STAT1α. Furthermore, the results show that this ability of the C-terminal NLS-containing domain of IFNγ to induce the nuclear localization of STAT1α is dependent on the presence of the NLS.

The above experiments demonstrate that STAT1α nuclear localization is dependent on the intracellular interactions of the C terminus of IFNγ. This intracellular interaction could be indirect, or IFNγ could exist as part of a complex with STAT1α that directly regulates nuclear trafficking of STAT1α. Accordingly, we performed immunoprecipitation experiments to determine if a complex between STAT1α and IFNγ occurred in mouse L-cells treated with mouse IFNγ. Cells were treated with ^{125}I-IFNγ at 37°C, washed, and total cell lysates were immunoprecipitated with anti-STAT1α antibodies. As a control, cells were treated with IFNγ at 4°C, where receptor-mediated endocytosis of ligand and STAT1α activation was prevented. Co-immunoprecipitation of IFNγ with STAT1α antibodies in cell lysates was monitored by autoradiography following SDS-PAGE separation. Results are presented in Fig. 6A. IFNγ co-immunoprecipitated with STAT1α only in cells following activation at 37°C, in a time-dependent fashion. Thus, in IFNγ-activated cells STAT1α is present in a complex with IFNγ. Interestingly, the complexed IFNγ appears primarily as the monomeric form. Though the exact significance of this is not known, the existence of IFNγ as a monomer complexed with STAT1α is consistent with the ability of the C-terminal peptide, which is a monomeric molecule, to behave as an agonist by forming a similar complex with STAT1α. Fig. 6C shows a quantitative analysis of the monomer bands in Fig. 6A. These data show that with increasing time of IFNγ activation, the pool of STAT1α complexed to IFNγ also increases.

Nuclear transport of STAT1α occurs through the interaction of activated STAT1α with the importin-α analog Npi-1. Thus, since IFNγ was found complexed with STAT1α we determined whether IFNγ was complexed with Npi-1. Lysates from mouse L Cells treated with ^{125}I-IFNγ as for Fig. 6, were now immunoprecipitated with antibodies to Npi-1 and IFNγ was again followed by autoradiography. The results presented in Fig. 7 show that IFNγ co-immunoprecipitated with Npi-1 in cells activated with IFNγ at 37°C. Reprobing the membranes with STAT1α antibodies also showed STAT1α to be present in the complex. The time-course for the complexation of IFNγ with STAT1α (Fig. 6) coincided with that for the Npi-1/IFNγ/STAT1α complex (Fig. 7). Thus, these data suggest that intracellular IFNγ forms a complex with the nuclear import receptor Npi-1 that is also the nuclear receptor for STAT1α. The direct complexation of IFNγ with Npi-1/STAT1α provides a mechanism for the regulation of STAT1α nuclear transport by IFNγ.

To further gain insight into the role of the C-terminal NLS of IFNγ in this complexation and regulation, we used the human deletion mutant IFNγ(1-123) that lacks the NLS in similar studies and compared the results with wild-type IFNγ. ^{125}I-labeled human IFNγ and human IFNγ(1-123) were used to treat human WISH cells and lysates were immunoprecipitated with Npi-1 antibodies. As seen in Fig. 8A (top panel), wild-type IFNγ was again recovered as a complex with Npi-1, similar to that seen in mouse L cells although with slightly different kinetics. However, the NLS-mutant IFNγ(1-123) was not found to be complexed with Npi-1. These data show that the NLS is required for complexation with Npi-1. When these complexes were examined for the present of STAT1α (Fig. 8A, lower panel), STAT1α was found to co-immunoprecipitate in significant amounts only in cells treated with wild-type IFNγ. Thus, the interaction of the NLS of IFNγ with the importin Npi-1 is required for the formation of a stable complex between STAT1α and its nuclear transporter Npi-1. Npi-1 immunoprecipitates from cells treated with IFNγ(1-123) showed a low and transient signal for STAT1α, suggesting some complexation of STAT1α with Npi-1 could occur in a NLS-independent manner but this complex is weak and short-lived.

To compare the significance of these results with respect to the specific phosphorylation status and nuclear accumulation

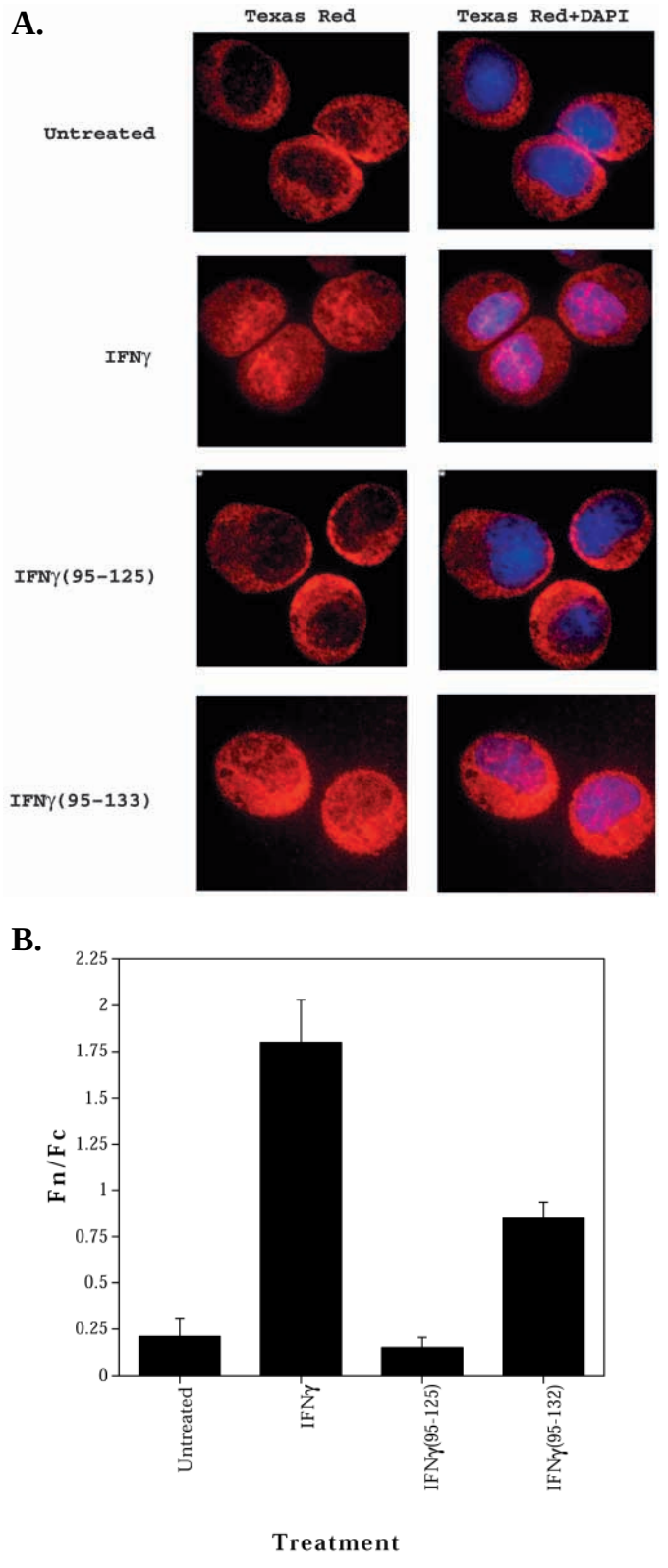


Fig. 5. The NLS motif in IFNγ is required for intracellular regulation of STAT1α nuclear translocation. (A) Mouse P388D$_1$ macrophages were treated with IFNγ (10,000 units/ml), mouse peptide IFNγ(95-133) (25 μM), or mouse peptide IFNγ(95-125) (25 μM) or left untreated for 20 minutes at 37°C following which cells were cytocentrifuged onto microscope slides, fixed and subjected to immunofluorescence staining for STAT1α localization (left panels, TexasRed). Cellular nuclei were counterstained with DAPI. Overlapping images of DAPI and STAT1α staining are presented (right panels, TexasRed+DAPI). (B) Quantitation of images in A showing average nuclear to cytoplasmic fluorescence (Fn/Fc) ratios as described in Materials and Methods.

of STAT1α, we examined cells treated with IFNγ and IFNγ(1-123) by immunoprecipitating STAT1α from separated cytoplasmic and nuclear extracts. Phosphorylation of STAT1α was followed with antibodies specific for Tyr^{701}-phosphorylated STAT1α. As shown in Fig. 8B, wild-type IFNγ induced strong tyrosine phosphorylation of STAT1α coupled with its nuclear translocation. Treatment of cells with IFNγ(1-123) resulted in markedly reduced STAT1α tyrosine phosphorylation, and almost no nuclear translocation of STAT1α Thus, taken in conjunction with the data from Fig. 8A we conclude that the NLS of IFNγ is required for the ability of STAT1α to form a a stable complex with the nuclear importin Npi-1 and its consequent nuclear translocation. These data strongly suggest that the NLS of IFNγ directly regulates the Npi-1-mediated entry of STAT1α into the nucleus.

In the latter experiments, a small amount of phosphorylated STAT1α was detectable in the nucleus of cells treated with IFNγ(1-123)(Fig. 8B) that coincided with the transient Npi-1:STAT1α complex seen in Fig. 8A in similarly treated cells. The kinetics of this transport differed from that of wild-type IFNγ. This suggests that small amounts of STAT1α can translocate to the nucleus through other mechanisms. This may be related to the fact that a second weaker NLS in human IFNγ exists upstream of the one studied here (J. L. Larkin et al., unpublished; see also Bader and Witzerbin, 1994). It remains to be determined if this NLS can function similarly to that described here.

DISCUSSION

We have shown in this report that internalized IFNγ interacts at an intracellular site to regulate the nuclear translocation of STAT1α. This intracellular function of IFNγ is specifically mediated by a C-terminal domain of IFNγ encompassed by residues 95-133, which also contains a NLS that is required for its ability to function intracellularly. Intracellular IFNγ can be recovered as part of a complex with STAT1α in IFNγ activated cells. This complex also contains the nuclear importin-α analog Npi-1, which has previously been show to mediate the nuclear import of STAT1α Further studies showed that the NLS of IFNγ is required for the ability of STAT1α and IFNγ to form this trimeric complex with Npi-1. The tyrosine phosphorylation of STAT1α, the formation of the complex IFNγ/Npi-1/STAT1α complex and the subsequent nuclear translocation of STAT1α were all found to be dependent on the presence of the IFNγ NLS. Previous mutational studies on STAT1α failed to identify an NLS motif responsible for the nuclear import of STAT1α (Sekimoto et al., 1997) via Npi-1. Our data strongly support the conclusion that the required NLS for the nuclear localization of STAT1α is provided by IFNγ, and IFNγ acts as a chaperone for the nuclear delivery of STAT1α in the form of a_IFNγ:STAT1α:Npi-1 complex.

Earlier studies on the binding of STAT1α to Npi-1 have

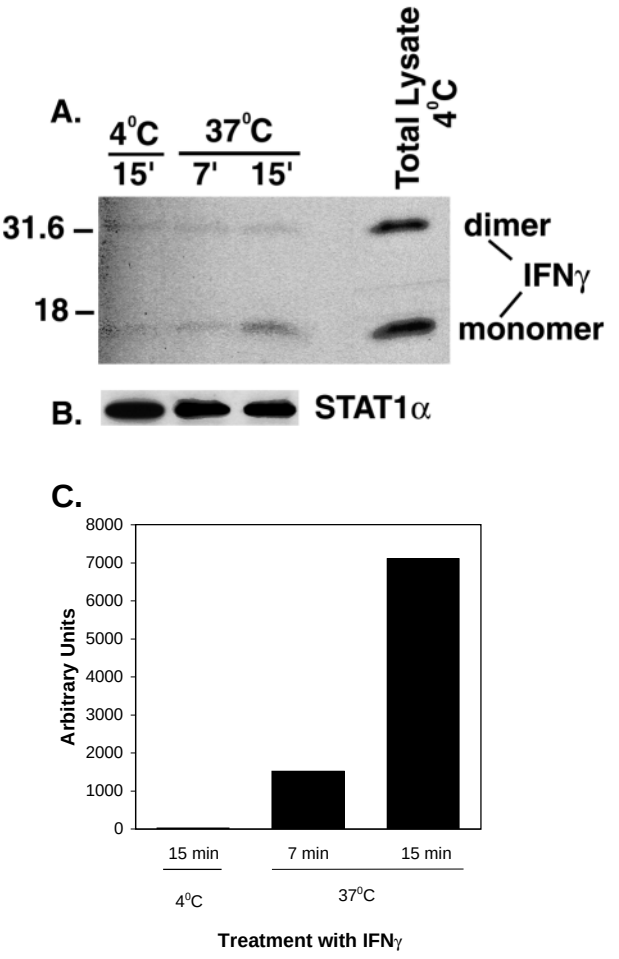


Fig. 6. IFNγ co-imunoprecipitates with STAT1α from cytoplasmic extracts in IFNγ-treated cells. Mouse L cells were incubated with 0.33 μg/ml of ^{125}I-labeled mouse IFNγ at 4°C for 1 hour before cells were moved to 37°C for 7 minutes (37°C, 7′) or 15 minutes (37°C, 15′). Control cells were maintained at 4°C (4°C, 15 minutes). (A) Cytoplasmic lysates were immunoprecipitated with anti-STAT1α antibodies, as described in Materials and Methods. Following SDS-PAGE separation of immune complexes and transfer to nitrocellulose membrane, ^{125}I-IFNγ was detected by autoradiography. To follow total ^{125}I-IFNγ a sample of the total (not immunoprecipitated) extract from control cells (Total lysate 4°C) was also run. (B) The membrane from A was immunoblotted with goat anti-STAT1α antibodies. (C) Quantitiation of data from A. Band intensities for monomeric IFNγ in A were quantitated by densitometric analysis and plotted.

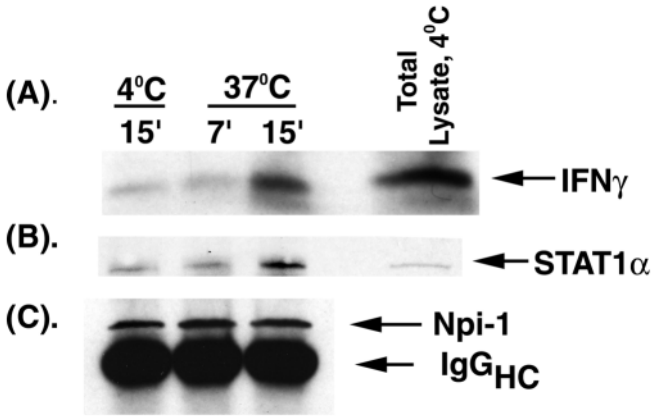


Fig. 7. IFNγ is complexed with the importin analog Npi-1 and STAT1α in IFNγ activated cells. Mouse L cells were treated with ^{125}I-IFNγ as for Fig. 6 and cytoplasmic lysates were immunoprecipitated with antibodies to Npi-1. Following transfer of proteins to nitrocellulose IFNγ was detected by autoradiography (A). The membrane was then probed by immunoblotting sequentially with antibodies to STAT1α (B), and Npi-1 (C).

suggested that STAT1α binds to the C terminus of Npi-1 (residues 456-538) at a site that cannot be competed for by the basic SV-40 T-NLS (Sekimoto et al., 1997), suggesting that STAT1α binds outside of the 'conventional' NLS binding site. However, an alternate interaction site for basic NLSs has been identified with the C-terminal residues 501-510 (Moroianu et al., 1996), which falls within the STAT1α binding region described on Npi-1 (Sekimoto et al., 1997). Interaction of the IFNγ NLS at this site on Npi-1 would provide a mode for binding of an IFNγ/STAT1α complex to Npi-1 in an IFNγ NLS-dependent fashion. Further, amino acid differences within NLSs are known to alter both the specificity and affinity for importin binding sites (for review see Jans et al., 2000). Thus, while the SV-40 T-NLSs would qualitatively bind both sites on Npi-1, its affinity for the C-terminal STAT1α site on Npi-1 may be too low to compete with the high specific binding of the IFNγ NLS within the IFNγ/STAT1α complex. This would explain why in the presence of STAT1α already bound to Npi-1, the SV-40 T-NLS cannot displace STAT1α, as determined in the earlier studies (Sekimoto et al., 1997). Different binding sites for IFNγ NLS and SV-40 T-NLS are also consistent with the ability of SV-40 T-NLS to 'compete' in functional assays (Subramaniam et al., 1999), since in functional import assays utilization of Npi-1 by excess SV-40 T-NLS would prevent import of IFNγ. Further studies into the binding of IFNγ to Npi-1 should help provide insight into these phenomenona.

While our data demonstrate that the interaction of IFNγ with Npi-1 regulates the trafficking of STAT1α to the nucleus, it remains to be determined what the exact contribution of intracellular IFNγ is to signaling events that lead to the formation of this complex with STAT1α. Studies from our laboratory and that of others provide strong support for one mechanism by which intracellular IFNγ may regulate early receptor events leading to the activation of the JAK/STAT pathway (Johnson et al., 1998a,b), and the subsequent nuclear translocation of STAT1α. As has been mentioned before, the mouse IFNγ(95-133) and human IFNγ(95-134) peptides, which contain the NLS, are agonists of IFNγ when delivered intracellularly (Johnson et al., 1998a,b). Since it is well known that the ability of IFNγ to induce MHC Class II and an antiviral state is dependent on a functional JAK/STAT pathway (Darnell, 1998), the agonist peptides must be able to activate the JAK/STAT pathway intracellularly to manifest their biological effects. We have shown in this study that murine IFNγ(95-133) can induce the nuclear localization of STAT1α when internalized by murine macrophages. The ability of this peptide to induce STAT1α nuclear localization intracellularly is dependent on the presence of the NLS motif. The NLS motif is also required for the agonist properties of the peptide (Szente

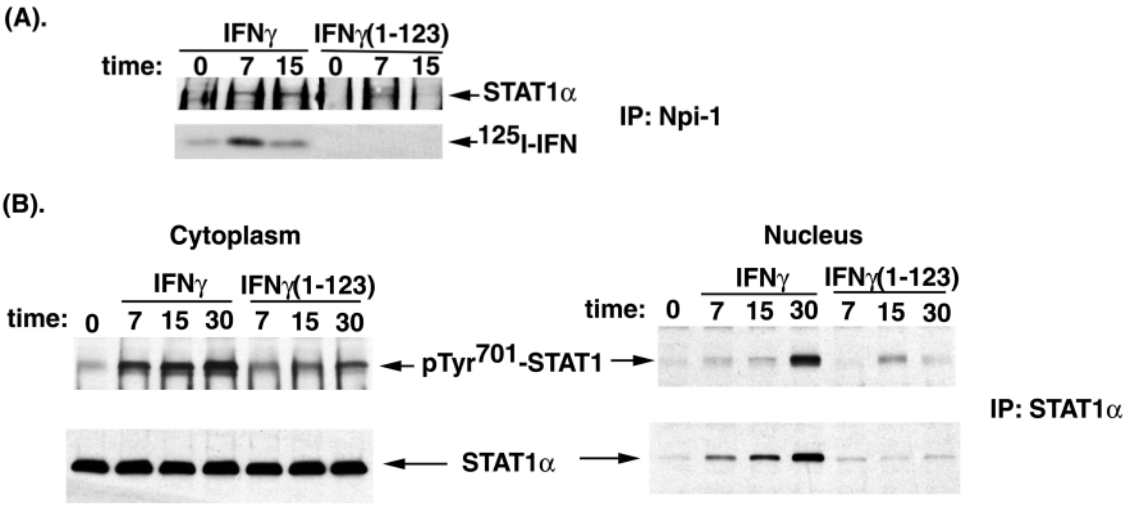


Fig. 8. (A) The formation of an Npi-1/STAT1α complex requires the IFNγ NLS. Human WISH cells were treated with 0.33 μg/ml each ^{125}I-IFNγ or ^{125}I-IFNγ(1-123), as indicated, for 7 minutes (7′) or 15 minutes (15′) at 37°C. Control cells were incubated with the appropriate ligands at 4°C (0). Cells were lysed and immunoprecipitated with antibodies to Npi-1 as for Fig. 7. IFNs were followed by autoradiography, while STAT1α was detected by immunoblotting with anti-STAT1α antibodies. (B) Tyrosine phosphorylation and nuclear translocation status of STAT1α in WISH cells treated with IFNγ and IFNγ(1-123). Cells were treated with unlabeled IFNs as in A, except that control cells were left untreated. Nuclear and cytoplasmic extracts were separated and immunoprecipitated with antibodies to STAT1α. Phosphorylation of STAT1α was followed immunoblotting with antibodies specific for Tyr^{701}-phosphorylated STAT1α, and STAT1α was detected with anti-STAT1α antibodies.

et al., 1994). Thus, the intracellular interaction of the NLS motif of IFNγ is required for STAT1α nuclear uptake.

The C-terminal domain of mouse and human IFNγ represented by these IFNγ peptides (IFNγ(95-133) and IFNγ(95-134), Table 1), and containing the NLS, have also been shown to interact with the intracellular cytoplasmic domain of the α-chain of the IFNγ receptor with high-affinity (Johnson et al., 1998a,b; Green et al., 1998). The requirement for this interaction is demonstrated by the fact that IFNGRα –/– cells do not respond to the intracellular agonist peptides or to intracellular IFNγ itself (Will et al., 1996; Thiam et al., 1998). Further, we have shown that this interaction of the C terminus of IFNγ with the cytoplasmic domain of IFNGRα induces the binding of JAK2 to IFNGRα at an immediately downstream site (Johnson et al., 1998a,b). In cells treated with the agonist peptides, JAK2 can be immunoprecipitated as a complex with the α-chain of the receptor following peptide uptake (Szente et al., 1995). This is similar to the observation by others that a stable complex formed between JAK2 and the α-chain only following IFNγ stimulation (Kotenko et al., 1995). Thus, the transfer of JAK2 from the IFNGRβ to IFNGRα appears to be mediated by the intracellular interaction of C terminus of IFNγ with IFNGRα. The α-chain also contains the phosphotyrosine binding site for STAT1α recruitment to the receptor complex and its subsequent phosphorylation by JAK2 (reviewed by Bach et al., 1997; Pestka et al., 1998). Thus, the C terminus of IFNγ modulates the recruitment of JAK2 to IFNGRα, which is involved in the direct activation and recruitment of STAT1α to IFNGRα. This requirement of intracellular IFNγ to interact with the cytoplasmic domain of IFNGRα to regulate receptor activation provides an explanation for why either IFNγ or the agonist peptide IFNγ(95-133) with intact NLSs cannot induce the nuclear translocation of STAT1α in cells devoid of the IFNGRα cytoplasmic domain. In effect, the interaction of the NLS within the C terminus of IFNγ with the nuclear import machinery is a necessary, but not a sufficient, condition for enabling the nuclear translocation of STAT1α. We conclude that the C terminus requires an additional interaction, namely the one at the cytoplasmic domain of IFNGRα to be fully competent for activation and nuclear translocation of STAT1α. This conclusion is consistent with the microinjection studies presented here, showing that intracellular antibodies to the C-terminal domain of IFNγ block the activation and nuclear translocation of STAT1α, since these antibodies also neutralize the ability of the C terminus to bind the cytoplasmic domain of IFNGRα.

As mentioned above STAT1α appears to be deficient in a NLS that can mediate its nuclear import (Sekimoto et al., 1997). Hence we propose that the C-terminal domain of IFNγ, subsequent to its role in recruitment of STAT1α to IFNGRα and priming of STAT1α at IFNGRα for nuclear translocation, via its NLS motif may directly interact with the Ran/importin pathway to mediate the nuclear delivery of STAT1α via a IFNγ/IFNGRα/STAT1α complex. This proposal is consistent with our recent findings that following extracellular binding of IFNγ to the receptor complex IFNGRα is selectively translocated to the nucleus. This nuclear translocation of IFNGRα occurs with the same kinetics as that of STAT1α, and IFNGRα appears to co-localize with STAT1α during this time. IFNGRα and STAT1α can also be recovered as a complex during these processes following IFNγ treatment. We have shown in this study that IFNγ similarly can also be recovered as a complex with STAT1α from the cytoplasm of IFNγ treated cells. These data, thus, argue for the presence of a IFNγ/IFNGRα/STAT1α complex in STAT1α nuclear import.

One major advantage of such ligand-assisted nuclear chaperoning of STAT transcription factors is the high-degree of specificity that is inherent in sequestering a pool of STAT within the ligand-receptor complex that activated it. This

would imply that ligand and/or receptor are/is involved in the specificity of STATs at the level of transciption. Further, this would explain why different ligands with different biological functions on a given cell, activate the same STATs in these cells.

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