Signal Transduction Mechanism of A Peptide Mimetic of Interferon-γ†

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ABSTRACT: The C-terminus of interferon-γ (IFNγ) contains a nuclear localization sequence (NLS) required for the activation and nuclear translocation of the transcription factor STAT1α and induction of IFNγ-activated genes. On the basis of this and other studies, we developed a peptide mimetic of IFNγ that possesses the IFNγ functions of antiviral activity and upregulation of MHC class II molecules. The mimetic also shares with IFNγ the ability to induce the activation and nuclear translocation of STAT1α and the IFNγ receptor (IFNGR)-1 subunit. The mimetic, IFNγ(95−132), is a peptide that consists of the C-terminal residues 95−132 of murine IFNγ and contains a required α-helical domain and the NLS of IFNγ. In this study, we determined the mechanism of the intracellular action of the mimetic at the level of signal transduction. We show that the mimetic mediates the nuclear transport of IFNGR-1 through its interaction with IFNGR-1 cytoplasmic region 253−287 via both the helical region and the NLS of IFNγ(95−132). Alanine substitutions of the NLS of the mimetic showed that the NLS was required for nuclear translocation and that the nuclear transport properties of the mimetic correlated with its ability to bind IFNGR-1. These data also show that the NLS of IFNγ(95−132) can interact simultaneously with IFNGR-1 and the nuclear import machinery. We found that in in vitro nuclear transport assays tyrosine-phosphorylated STAT1α failed to undergo nuclear translocation in the presence of nuclear import factors, but was transported to nucleus in the presence of IFNγ(95−132) and JAK2-phosphorylated IFNGR-1, to which STAT1α binds, as a complex of IFNγ(95−132)/IFNGR-1/STAT1α. Thus, the mimetic, which possesses IFNγ function, is directly involved as a chaperone in the nuclear transport of STAT1α and shares this mechanism of action with that previously described for IFNγ. The mimetic, like IFNγ, is able to upregulate the tumor suppressor p21WAF1/CIP1, a direct target of STAT1α, and this ability requires the NLS of the mimetic. However, unlike IFNγ, the mimetic is unable to downregulate c-myc and hence does not inhibit the cycling of cells. This suggests that IFNγ has additional functions that are not tied directly to the nuclear translocation of STAT1α.

We have shown that the polycationic sequence in the C-terminus of human and murine interferon-γ (IFNγ) is a nuclear localization sequence (NLS)[1] and is responsible for the appearance of IFNγ in the nucleus of treated cells (*1*−*3*). Several studies have shown that deletion mutants of the C-terminal polycationic NLS results in the loss of IFNγ activity (*4*−*7*). However, while the N-terminus of IFNγ has been shown to bind to the extracellular receptor domains by peptide competition and crystallographic structure determination, no extracellular receptor binding site for the NLS-containing C-terminus of IFNγ has been demonstrated (reviewed in ref *8*).

In contrast, we have been able to demonstrate and characterize a high-affinity species nonspecific binding site ($K_d \approx 10^{-8}$ M) for the IFNγ C-terminus on the intracellular cytoplasmic domain of the soluble recombinant α-subunit of the IFNγ receptor (IFNGR), IFNGR-1, of both human and murine origin (*9*). This site, also demonstrable in whole cells, was identified as a membrane proximal region of the cytoplasmic domain within amino acid residues 253−287 (*8*). Consistent with this, we were able to demonstrate that peptides from the C-terminal NLS-containing domains of human IFNγ, huIFNγ(95−134), and murine IFNγ, muIFNγ-(95−132), were sufficient to induce IFNγ-associated activities when internalized by murine macrophages (*10*). The intracellular peptide mimetics, which contain the NLS of IFN, were found to inhibit virus replication by 10^6−10^9-fold and upregulated MHC class II expression up to 10-fold in a fashion that depended strictly on the presence of the NLS in these peptide sequences (*10*). The peptide mimetics interacted exclusively with the cytoplasmic domain of IFNGR-1 within residues 253−287 and not with the extracellular region of IFNGR-1. An N-terminal α-helix in these peptides within residues 108−121 was also found to be important for function by virtue of its requirement in the mimetics for binding to IFNGR-1(253−287) (*11*). Other studies showed that the peptides behaved as IFNγ mimetics only when delivered intracellularly in cells and that their mimetic properties required expression of IFNGR-1 in such cells showing the requirement of the cytoplasmic domain of IFNGR-1 (*12*). HuIFNγ(95−134) and muIFNγ(95−132) share approximately 80% homology and interact with the membrane proximal IFNGR-1 cytoplasmic region (aa 253−

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[1] Abbreviations: IFNGR, IFNγ receptor; NLS, nuclear localization sequence; NPI-1, nucleoprotein interactor 1; APC, allophycocyanin.

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Table 1: Sequences of Peptides Used in This Study

Peptide	Sequence [a]
IFNGR-1(253-287)	cTKKNSFKRKSIMLPKSLLSVVKSATLETKPESKYS
IFNγ(95-132)	cAKFEVNNPQVQRQAFNELIRVVHQLLPESSL**RKRKRSR**
IFNγ(95-132)5Ala	cAKFEVNNPQVQRQAFNELIRVVHQLLPESSL**aaaaaSR**
IFNγ(95-132)3Ala1	cAKFEVNNPQVQRQAFNELIRVVHQLLPESSL**RKaaaSR**
IFNγ(95-132)3Ala2	cAKFEVNNPQVQRQAFNELIRVVHQLLPESSL**aaRKRSa**
IFNγ(95-132)P,P	AKFEVNNPQVQRQPAFNEPIRVVHQLLPESSL**RKRKRSR**
IFNγ(126-132)	c**RKRKRSR**

[a] Some peptides contained an extra cysteine (c, underlined) at the N-terminal end to allow for covalent coupling. All peptides are derived from the sequence of the mature form of the corresponding mouse sequences and numbered accordingly. The added cysteine, where applicable, is not included in the numbering. The NLS region is in bold. In IFNγ(95−132)P,P, the two additional prolines are underlined.

287) in a non-species-specific manner (*10*).[2] The IFNγ peptide mimetics, like intracellular IFNγ, also were able to induce the activation and nuclear translocation of STAT1α, as well as induce the endocytosis and nuclear translocation of the IFNGR-1 subunit from within the cell.

In this report, using the IFNγ mimetic in in vitro binding assays and a standard in vitro nuclear transport assay system, we present data to suggest that the IFNγ NLS can play multiple roles in IFNγ mimetic signaling. These include binding to intracellular cytoplasmic domain of IFNGR-1 and simultaneous interaction with the nuclear import machinery to mediate the nuclear transport of IFNGR-1. Further, phosphorylated STAT1α, which binds to phosphorylated IFNGR-1, is shown to be transported to the nucleus in an in vitro system by IFNγ(95−132) and a phosphorylated IFNGR-1 subunit. Finally, the N-terminal α-helix of the IFNγ mimetic also plays an important role in IFNGR-1 binding and subsequent events. Our data on the mimetic support the conclusion that this versatility of the C-terminus of IFNγ, involving both its α-helix and NLS, is important for IFNγ mimetic-mediated STAT1α nuclear translocation and associated IFNγ-like biological activities.

MATERIAL AND METHODS

Materials. Amino acids for peptide synthesis were purchased from Bachem (Torrance, CA) and Advanced ChemTech (Louisville, KY). Cross-linked allophycocyanin (APC) activated with the bifunctional cross-linker succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was from Prozyme (San Leandro, CA). Digitonin was from Fisher Scientific. Recombinant JAK2 immobilized on agarose beads was purchased from Upstate Biotechnology (Lake Placid, NY). Agarose-conjugated monoclonal antibodies to human STAT1α were purchased from Santa Cruz Biotechnology (Camarillo, CA). Alexa Fluor 594 C_5 maleimide was purchased from Molecular Probes (Eugene, OR).

Cell Culture. WISH cells were grown in minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS) and antibiotics. A31 mouse cells were grown in Dulbecco's modified Eagle's medium (from ATCC) containing 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Before use in assays, cells were cultured for 16−24 h on glass coverslips. PC-3 cells were grown in Ham's F12 medium containing 10% FBS and antibiotics.

Peptide Synthesis. Synthesis of peptides using Fmoc chemistry and their purification were performed as described previously (*11*). All peptides contained an extra cysteine residue at the N-terminus to allow for fluorescent labeling where required. The peptide sequences are listed in Table 1.

Radiolabeling of IFNγ and Binding Experiments. Recombinant murine IFNγ (carrier-free, Peprotech, Rocky Hill, NJ) was labeled essentially as previously described (*10*), except that 2 mM tyrosine (sodium salt) was included and BSA was omitted from the stop reagents. The specific activity of the labeled protein was ~40 μCi/μg.

Solid-phase binding assays to follow interaction of ^{125}I-IFNγ to peptide mouse IFNGR-1(253−287) and ability of IFNγ and IFNγ peptides to compete with ^{125}I-IFNγ (1 nM) for binding were performed in 96-well plates as previously described (*10*). Peptide IFNGR-1(253−287) was immobilized on the plates, and competitors were added in the required concentrations to wells before the addition of ^{125}I-IFNγ. K_i values for the respective peptides were arrived at by curve fitting initial values to a simple one site competition model using GraphPad Prism software (version 4). The K_i values were determined using a K_d value of 1×10^{-8} M for the binding of IFNγ to the 253−287 region of the full length cytoplasmic domain of IFNGR-1 (see ref *9*).

Purification of STAT1α and a Soluble Version of IFNGR-1. Insect cells expressing human STAT1α using a baculovirus expression construct (bvc-10349) from Orbigen (San Diego, CA) were grown, harvested, and provided on contract by the manufacturer. Cells from 1000 mL of culture were resuspended in 10 mL of buffer A, and cell lysates were prepared by six rounds of sonication in buffer A (50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 10 μg/mL aprotinin, leupeptin, and pepstatin, and 1mM PMSF). Lysates were then clarified by centrifugation and used for immunoaffinity isolation of STAT1α. For this, lysates were adsorbed for 2 h with agarose-conjugated antibodies to human STAT1α at 4 °C (600 μl of agarose, settled volume). The agarose beads were then washed and packed into a column. The column was further washed with five bed volumes of buffer A before elution with 0.25 M glycine, pH 2.5. The eluate was collected in fractions that were immediately neutralized with 1 M Tris-HCl, pH 8.0. Fractions containing STAT1α were detected

[2] Since both mimetics are highly homologous and are indistinguishable in activity, we use the murine mimetic IFNγ(95−132) to represent the properties of the mimetic from both species.

by SDS−PAGE, and purity and identity were assessed by Coomassie Blue staining and immunoblotting. Pooled fractions were then concentrated and dialyzed against 50 mM potassium phosphate buffer, pH 7.0, containing 1 μg/mL leupeptin, aprotinin, and pepstatin.

The expression system for the soluble mouse IFNGR-1 has been previously described in detail (*13*). The secreted IFNGR-1 was recovered from medium following culture under serum-free conditions previously described (*13*), concentrated by ultrafiltration using a 50 000 MWCO membrane and dialyzed exhaustively into 50 mM potassium phosphate buffer, pH 7.0, containing 10 μg/mL leupeptin, pepstatin, and aprotinin. The protein was found to be the predominant band following SDS−PAGE and Coomassie Blue staining and verified to be IFNGR-1 by immunoblotting and used as such. Aliquots were stored at −80 °C.

Fluorescent Labeling of Proteins. The peptide IFNGR-1(253−287) was labeled with SMCC-activated APC at a molar ratio of 1:4 (APC/peptide) as previously described for IFNγ(95−132) (*1*), except that residual peptide was removed by repeated concentration through a Centricon 50 ultrafiltration unit (MWCO 50 000; Millipore) in the presence of 20 mM β-mercaptoethanol.

STAT1α was labeled with Alexa Fluor 594 C_5 maleimide according to the manufacturer's instructions. Briefly, thiol groups in STAT1α were reduced using a 10-fold molar excess of TCEP [tris-(2-carboxyethyl)phosphine (Molecular Probes, Eugene, OR) in storage buffer (50 mM potassium phosphate buffer, pH 7.0, containing 1 μg/mL leupeptin, aprotinin, and pepstatin), and the Alexa Fluor reagent was added in DMSO at a 1:4 molar ratio (STAT1α/dye). After reaction overnight at 4 °C, the solution was dialyzed against 50 mM potassium phosphate, pH 7.0, containing 20 mM β-mercaptoethanol and stored away from light.

The labeled STAT1α when tyrosine-phosphorylated with JAK2 (see below) was indistinguishable from the purified STAT1α in electrophoretic mobility shift assays (EMSA, data not shown). This is consistent with the fact that complete alkylation of free thiol groups in STAT1α with *N*-ethyl maleimide had no effect on its properties (*14*).

Phosphorylation of STAT1α and Alexa Fluor-Labeled STAT1α with JAK2. The STAT1α molecules were tyrosine-phosphorylated with recombinant JAK2 immobilized on agarose beads essentially as described by the manufacturer's instructions. Tyrosine phosphorylation was verified by SDS−PAGE and immunoblotting with an antibody specific for Tyr701-phosphorylated STAT1α (Cell Signaling Technology, Beverly, MA). Further, tyrosine-phosphorylated labeled and unlabeled STAT1α was found to be fully functional in electrophoretic mobility shift assays (data not shown). In general, we found that in the presence of JAK2−agarose, STAT1α was retained on the beads at the end of the phosphorylation reaction, most likely through binding to JAK2. However, incubation of aliquots of the STAT1α/JAK2 bearing beads in the presence of oligonucleotides in EMSA procedures released STAT1α from the beads. Similarly, STAT1α was released from JAK2 beads in nuclear import reactions described below.

Nuclear Import Assays. For IFNGR-1 peptide IFNGR-1(253−287), the basic nuclear import assay was essentially as we have previously described for IFNγ (*1*). For the substrate•IFNGR-1(253−287), APC (1 μM) was used in the presence or absence of peptide IFNγ(95−132) (1 μM). To test for energy dependence, ATP depletion was carried out as previously described (*1*), and GTP was simultaneously omitted from the reaction mixture.

For assays addressing the nuclear translocation of STAT1α, standard reticulocyte lysate nuclear import mixtures (*1*) were incubated with 1 μM of soluble IFNGR-1, Alexa Fluor-labeled tyrosine-phosphorylated STAT1α (as the agarose complex; see above), and 10 μL of JAK2−agarose for 20 min at 30 °C in the absence or presence of 1 μM IFNγ-(95−132) peptide. The reaction mixtures were centrifuged, and supernatants were used on digitonin-permeabilized cells to assess nuclear import.

Immunoblotting. PC-3 cells were lysed and extracted at 4 °C into 50 mM Tris-HCl, pH 7.5, containing 0.25 M NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 20 mM β-glyceroyl phosphate, 2 mM sodium orthovanadate, 2 mM DTT, 10% glycerol, and 10 μg/mL each leupeptin, pepstatin, and aprotinin. Equal amounts of protein from extracts were subjected to SDS−PAGE, and proteins were transferred to nitrocellulose membranes. Membranes were analyzed by immunoblotting using antibodies to p21WAF1/CIP1 and c-myc and then stripped and reprobed for actin as a protein loading control.

Nuclear extracts used for immunoblotting of STAT1α were prepared from PC-3 cells essentially as previously described (*15*). Extracts were analyzed as described above.

Cell Cycle Analysis. Staining of PC-3 cells for cell cycle analysis by flow cytometry (FACS) and flow cytometry data analysis were performed as previously described (*16*). PC-3 cells (2×10^6 total) were synchronized by starvation for 72 h in growth medium that contained only 0.5% FBS. Cells were then incubated with the appropriate IFN or peptide in complete growth medium (10% FBS) for a further 24 h, after which cells were fixed and stained for analysis by FACS.

RESULTS

To determine more precisely the role of the NLS of the mimetic IFNγ(95−132) in IFNGR-1 binding, we synthesized peptide analogues of muIFNγ(95−132) in which groups of arginines and lysines of the NLS were replaced by alanines. The replacements were as follows: aaaaaSR (5Ala), RKaaaSR (3Ala1), and aaRKRSa (3Ala2). The sequences of muIFNγ-(95−132) and the alanine-substituted peptides are shown in Table 1. These peptides were first tested for binding to muIFNGR-1(253−287) in competition solid-phase binding assays using ^{125}I-labeled muIFNγ as the ligand. The results of the binding assays using these peptides are shown in Figure 1. Binding to muIFNGR-1(253−287) by ^{125}I-IFNγ itself was specifically inhibited by "cold" IFNγ in a dose-dependent fashion (Figure 1A). The competition of alanine-substituted peptides for the binding of ^{125}I-IFNγ compared to that of muIFNγ(95−132) is shown in Figure 1B. The peptides inhibited binding in the decreasing order IFNγ(95−132) > IFNγ(95−132)3Ala1 > IFNγ(95−132)3Ala2 ≈ IFNγ(95−132)5Ala. K_i estimates for the respective peptides by curve fitting data to a one-site competition model (GraphPad Prism 4 software) are 3.87×10^{-6} M for IFNγ-(95−132), 6.057×10^{-6} M for IFNγ(95−132)3Ala1, 456.5 $\times 10^{-6}$ M for IFNγ(95−132)3Ala2, and 684.2 $\times 10^{-6}$ M for IFNγ(95−132)5Ala. Thus, the IFNγ NLS sequence

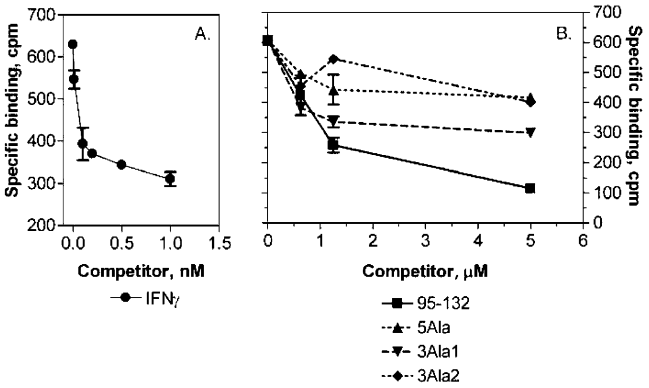

FIGURE 1: Competition of IFNγ, IFNγ(95−132), and alanine-substituted derivatives of peptide IFNγ(95−132) (see Table 1) for binding of ^{125}I-IFNγ to IFNGR-1(253−287). Binding assays were performed as described in Materials and Methods. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled IFNγ and has been subtracted. All samples were run in triplicate, values represent mean specific binding ± SD, and data are representative of five different experiments. In panel A, binding of ^{125}I-IFNγ (1 nM) to IFNGR-1(253−287) was competed with the indicated concentrations of unlabeled IFNγ. In panel B, binding of ^{125}I-IFNγ to IFNGR-1(253−287) was competed by peptides at the indicated concentrations. The peptides were as follows (see Table 1 for sequences): 95−132 = IFNγ(95−132); 5Ala, = IFNγ-(95−132)5Ala; 3Ala1 = IFNγ(95−132)3Ala1; 3Ala2 = IFNγ(95−132)3Ala2.

^{126}RKRKRSR^{132} is required for binding to the IFNGR-1 cytoplasmic domain described by IFNGR-1(253−287), though some amino acids within this sequence appear to be more important than others.

We next determined the effects of these alanine substitutions on the NLS activity associated with IFNγ(95−132). The peptides were used in nuclear import assays as functional competitors to IFNγ(95−132), where IFNγ(95−132) coupled to the fluorescent protein APC ($M_r \approx 105$ kDa) was used as the import substrate in in vitro nuclear import assays (Figure 2A). The pattern of inhibition was, in decreasing order, IFNγ-(95−132) > IFNγ(95−132)3Ala1 > IFNγ(95−132)3Ala2 ≈ IFNγ(95−132)5Ala. This was similar to the pattern of peptide competition for binding to IFNGR-1(253−287).

We have previously shown that a helical domain upstream of the NLS was also required for binding to IFNGR-1(253−287) (*11*). A double proline substitution [see Table 1, peptide IFNγ(95−132)P,P] in the region 108−121 was shown to destroy helical structure and consequently the mimetic/agonist activities of IFNγ(95−132) by disrupting the binding of IFNγ(95−132) to IFNGR-1(253−287) (*11*). When used as a competitor in the above nuclear transport assays, IFNγ-(95−132)P,P was just as effective as IFNγ(95−132) in blocking nuclear localization of IFNγ(95−132)−APC (Figure 2A, bottom row). Likewise, peptide IFNγ(126−132) that contains only the NLS sequence and is devoid of the region upstream of the NLS was also an effective competitor. However, peptide IFNγ(95−125), which contains the complete upstream region but is devoid of the NLS, was unable to compete with the nuclear transport of IFNγ(95−132)−APC. Thus, the NLS sequence is necessary for nuclear import of IFNγ(95−132).

The pattern of competition of the alanine-substituted peptide analogues was verified by coupling the individual peptides to APC and using them directly as nuclear import substrates in similar assays (Figure 2B). Consistent with the functional competition results, the peptide nuclear translocation activity trend was, in decreasing order, IFNγ (95−132) > IFNγ(95−132)3Ala1 > IFNγ(95−132)3Ala2 ≈ IFNγ(95−132)5Ala. Thus, the basic residues in the NLS of IFNγ are required for binding to IFNGR-1(253−287) as well for NLS activity. Notable in Figure 2A,B is the strong staining within the nucleolus with IFNγ(95−132) but not with IFNγ(95−132)3Ala1. We have previously observed strong nucleolar staining of IFNγ(95−132) (*1*). The functional significance of this for IFNγ is not known. Notwithstanding, the data suggest that the amino acids of the NLS that are involved in binding of IFNγ to IFNGR-1(95−132) are also involved in its nuclear translocation.

Our earlier studies using intact IFNγ led us to propose a model in which the IFNγ C-terminus, identified by the mimetic IFNγ(95−132), was suggested to direct the nuclear transport of a IFNγ/IFNGR-1/STAT1α complex by simultaneously interacting with the cytoplasmic domain of IFNGR-1 and the nuclear import machinery (*17*). This implies that IFNγ(95−132), as well as intact IFNγ, should be able to mediate the nuclear transport of IFNGR-1(253−287). This is consistent with the fact that, in intact cells, both intracellular mimetic IFNγ(95−132) and intracellularly expressed IFNγ can induce the nuclear translocation of IFNGR-1 (*18*, *19*). We thus coupled IFNGR-1(253−287) to APC and followed its nuclear translocation in the presence and absence of IFNγ(95−132) or IFNγ. The results are shown in Figure 3. IFNGR-1(253−287)−APC alone failed to undergo nuclear transport. However, addition of IFNγ(95−132) to the reaction resulted in nuclear transport of IFNGR-1(253−287)−APC. Nuclear import was also mediated by intact IFNγ. Both processes were energy-dependent since in the absence of ATP/GTP, the presence of IFNγ(95−132) or IFNγ resulted in the accumulation of IFNGR-1(253−287)−APC only at the nuclear pores producing the characteristic "rimming" of the nucleus. Excess unlabeled IFNGR-1(253−287) was able to inhibit the nuclear translocation of IFNGR-1(253−287)−APC compared to positive control with residual presence in the nucleolus. These data demonstrate that IFNγ NLS can simultaneously interact with IFNGR-1 cytoplasmic domain and the nuclear import machinery.

Because nuclear cotransport of IFNGR-1 occurs through binding of IFNγ(95−132) to IFNGR-1(253−287), we also tested the specific contribution of the helical region in IFNγ-(95−132), by using peptide IFNγ(95−132)P,P in this assay, which contains proline substitutions at positions 108 and 113 as described above. As can be seen in Figure 4, under conditions where IFNγ(95−132) mediated nuclear transport of IFNGR-1(253−287)−APC, IFNγ(95−132)P,P was unable to mediate the nuclear transport. Likewise, peptide IFNγ(126−132), which contains the NLS sequence but is devoid of the region upstream of the NLS, was unable to mediate nuclear transport of IFNGR-1(253−287)−APC, even though as we showed in Figure 2A it had NLS function. Thus, the NLS as well as the helical region upstream of the NLS are both required for nuclear transport of IFNGR-1(253−287). These data, along with those of Figure 2, suggest that the helical region of IFNγ(95−132) provides a binding determinant, while the NLS provides both a binding determinant and the NLS for nuclear transport of IFNGR-1(253−287). Thus, the IFNγ mimetic IFNγ(95−132) contains two binding sites for the cytoplasmic domain of

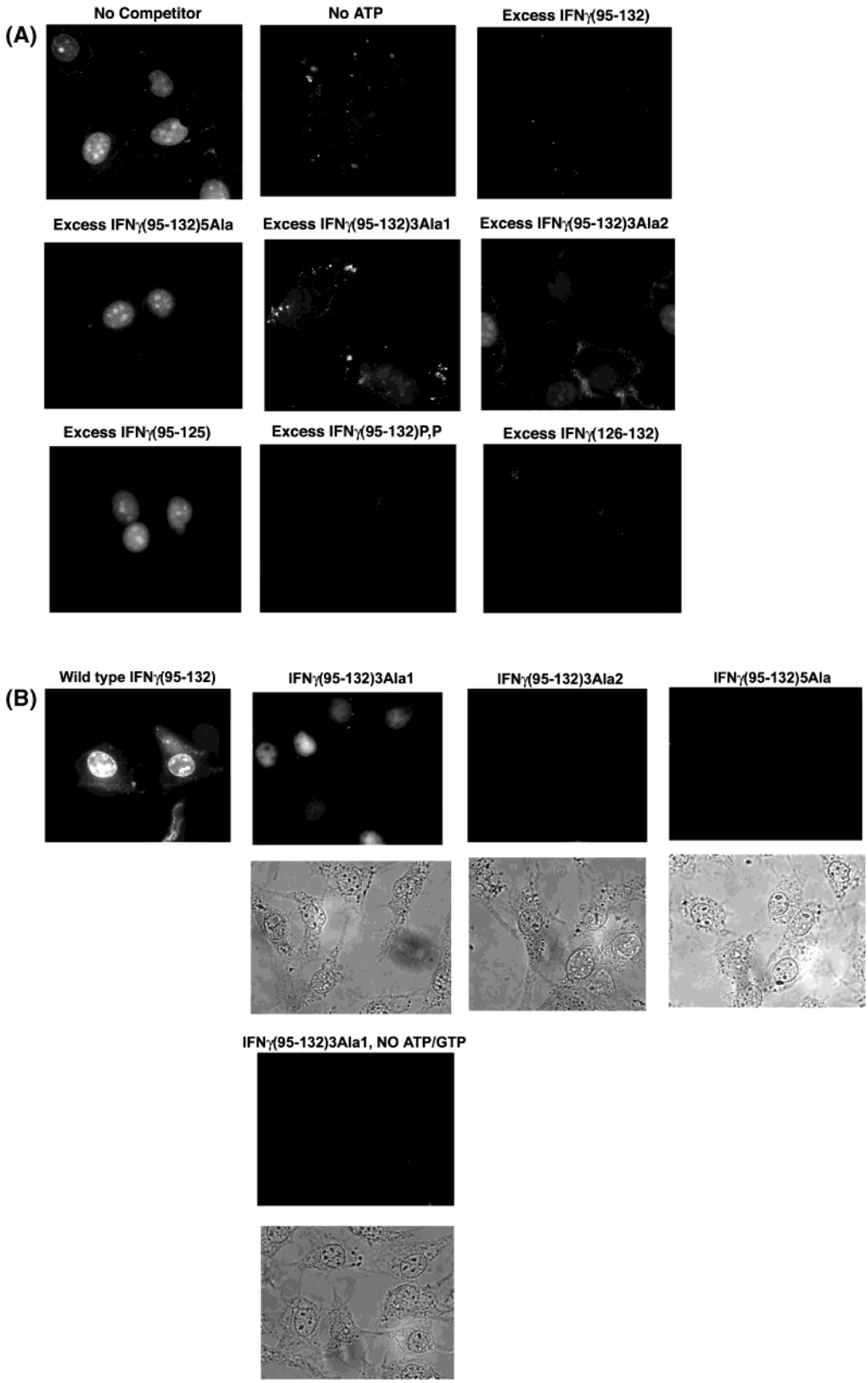

FIGURE 2: Alanine substitutions within the NLS of IFNγ inhibit nuclear translocation activity. Nuclear translocation was assessed in standard digitonin permeabilization-based nuclear import assays as described in Materials and Methods. In panel A, a 10-fold excess of the individual alanine-substituted peptides were used as competitors to the nuclear translocation of substrate IFNγ(95−132)−APC. No ATP/GTP refers to reactions run in the absence of ATP or GTP as described in Materials and Methods. In panel B, the alanine-substituted derivatives of IFNγ(95−132), as well as IFNγ(95−132), were individually coupled to APC and compared directly for their ability to function as substrates in nuclear import assays.

IFNGR-1, one of which also possesses NLS functions.

We previously also showed that the complex of IFNγ-(95−132) and the cytoplasmic domain of IFNGR-1 can act as a chaperone system for STAT1α nuclear translocation when the phosphorylated IFNGR-1 binds STAT1α (*17*, *19*). To determine whether the mimetic IFNγ(95−132) could

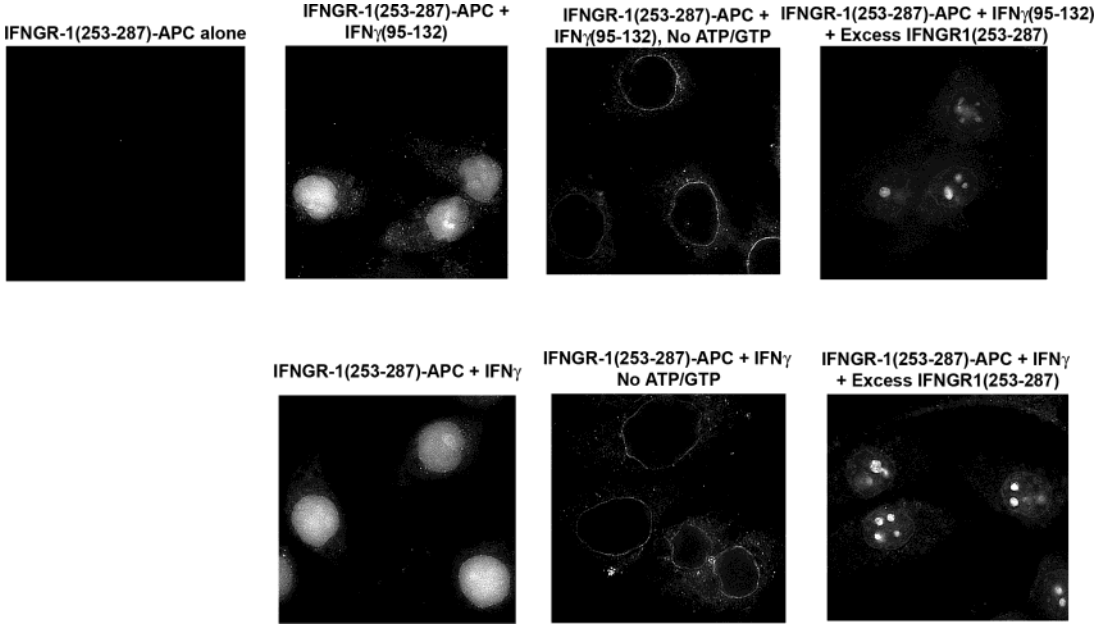

FIGURE 3: IFNγ(95−132) and IFNγ are able to translocate IFNGR-1(253−287) into the nucleus. IFNGR-1 coupled to APC was used as substrate in nuclear import assays in the presence or absence of an equimolar amount [with respect to IFNGR-1(253−287)−APC] of IFNγ(95−132) or IFNγ, as indicated. Reactions labeled with "Excess IFNGR-1(253−287)" contained a 30-fold excess of unlabeled IFNGR-1(253−287) peptide.

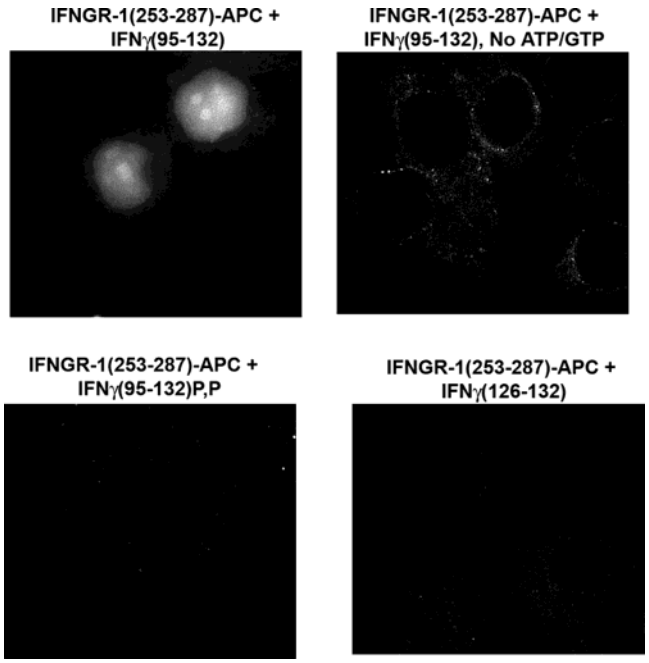

FIGURE 4: Nuclear cotransport of IFNGR-1 by IFNγ(95−132) requires integrity of the 108−121 helical region. IFNγ(95−132)P,P, where prolines are substituted at positions 108 and 113 (see Table 1), and IFNγ(126−132), which contains the NLS but is devoid of the region upstream of the NLS including the α-helix, were assessed for their ability to chaperone IFNGR-1(253−287)−APC into the nucleus. Nuclear import assays were run as described in Figure 3 using IFNGR-1(253−287)−APC as substrate in the presence of the indicated peptides.

chaperone the nuclear transport of tyrosine-phosphorylated STAT1α, we used a soluble form of the IFNGR-1 protein and fluorescently labeled phosphorylated STAT1α. In vivo, STAT1α binds to the tyrosine-phosphorylated IFNGR-1 cytoplasmic domain after JAK2 phosphorylation of IFNGR-1 and is itself phosphorylated (*20*). Thus, we treated IFNGR-1 with JAK2 in the presence of STAT1α and added the nuclear import mixture to determine STAT1α nuclear translocation activity in the absence and presence of IFNγ(95−132). IFNGR-1 and STAT1α itself were successfully tyrosine-phosphorylated in vitro by the recombinant JAK2 as determined by Western blotting, and the phosphorylated STAT1α bound to DNA as determined by electromobility shift assays (data not shown). The results of the nuclear import assays are shown in Figure 5. In the absence of IFNγ-(95−132), STAT1α associated with IFNGR-1 did not translocate to the nucleus (upper panel). Addition of IFNγ-(95−132) resulted in the nuclear translocation of STAT1α (middle panels). Thus, tyrosine phosphorylation by JAK2 alone was not sufficient to induce nuclear transport of STAT1α. Nuclear transport of phosphorylated STAT1α in the presence of IFNγ and IFNGR-1 was energy-dependent since in the absence of ATP and GTP transport of STAT1α was not observed (Figure 5, lower panel).

We have previously shown that IFNγ(95−132), like IFNγ, induced an antiviral state in a non-virus-specific manner and upregulated both MHC class I and class II molecules (*10*). We have also shown that IFNs inhibit cell cycling in various cell lines (*16*, *21*−*23*). We have previously determined that IFNs inhibited cell cycling via a combination of the induction of the tumor suppressor gene p21WAF/CIP1 and the down-regulation of the c-myc protein (*23*). The p21WAF1/CIP1 gene is a direct target of STAT1α (*24*). To determine the relationship between functional effects of the mimetic and its nuclear translocation as well as that of STAT1α, we compared IFNγ(95−132) with IFNγ for regulation of the cell cycle. We and others have previously shown that attachment of a lipid group, palmitic acid, to the N-terminus of IFNγ(95−132), lipo-IFNγ(95−132), allowed the peptide to cross the cell membrane and exert its effects (*18*, *25*). Thus, peptides used in cell cycle experiments were the N-terminal lipid-derivatized forms of those listed in Table 1.

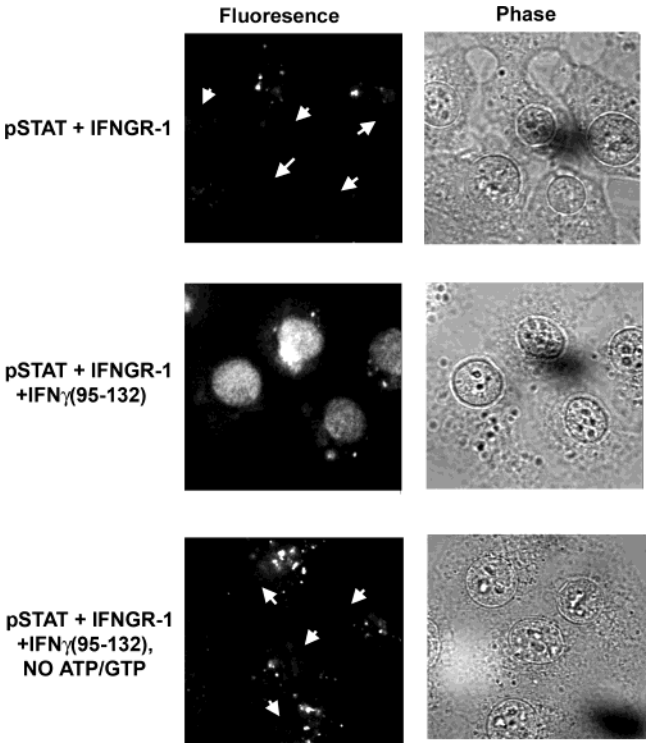

FIGURE 5: Nuclear transport of STAT1α can be directly mediated by a combination of IFNGR-1 and IFNγ. Standard nuclear import reaction mixtures were incubated with Sepharose-immobilized JAK2, tyrosine-phosphorylated STAT1α labeled with Alexa Fluor 594 (Alexa-pSTAT), and murine IFNGR-1 in the presence or absence of IFNγ(95−132) as indicated, before centrifugation to remove JAK2. Supernatants were then added to digitonin-permeabilized WISH cells to follow nuclear import of STAT1α. Arrows in the fluorescence fields indicate the position of the nuclei.

IFNγ inhibited movement of PC-3 prostate cancer cells from G1 to S phase (Figure 6A). Associated with this, IFNγ upregulated p21WAF/CIP1 at 24 h and downregulated c-myc (Figure 6B). By contrast, lipo-IFNγ(95−132) failed to inhibit cell cycling of PC-3 cells (Figure 6A). Lipo-IFNγ(95−132) did upregulate p21WAF/CIP1, similar to IFNγ (Figure 6B), in a NLS-dependent fashion (Figure 6B, compare lanes labeled 95−132 to 95−125). However, unlike IFNγ, lipo-IFNγ(95−132) failed to downregulate c-myc (Figure 6B). As previously demonstrated (*16*), lipo-IFNγ(95−132), like IFNγ, induced nuclear translocation of STAT1α (Figure 6C). This suggests that the NLS activity associated with the mimetic is required for p21WAF1/CIP1 induction, implying that IFNγ nuclear translocation is associated with induction of p21WAF1/CIP1. This is consistent with the fact that STAT1α has been shown to be a direct regulator of the p21WAF1/CIP1 gene (*24*), and the data here that show that the mimetic directly effects STAT1α nuclear transport. But with respect to cell cycle inhibition, the inability of IFNγ-(95−132) to downregulate c-myc, despite its ability at the same time to induce p21WAF/CIP1, results in its failure to inhibit the cell cycle of PC-3 cells. Therefore, although the IFNγ mimetic, IFNγ(95−132), possesses significant functions associated with IFNγ, it lacks structural features associated with intact IFNγ that convey all of the signaling events required for regulation of the cell cycle. Such events may not be directly linked to the nuclear translocation of STAT1α.

DISCUSSION

In this report, we have examined the structure−function relationships of key elements of a peptide mimetic of IFNγ, namely, IFNγ(95−132), which acts only when delivered intracellularly, and describe a mechanism by which these structural elements function in signal transduction by the mimetic. Two structural elements of the mimetic required for this intracellular function, which also are important in intact IFNγ, are a NLS in residues 126−132 and an α-helical structure upstream of this NLS within residues 108−121 (*11*). Our data suggest a mechanism in which the mimetic chaperones the nuclear transport of STAT1α via an IFNγ/IFNGR-1/STAT1α complex in which the above two structural elements play a crucial role.

The interaction of the mimetic with IFNGR-1 controls the association of JAK2 with IFNGR-1 and the subsequent phosphorylation of STAT1 by JAK2 in a manner similar to that found for intact IFNγ (reviewed in ref *26*). Further, the intracellular mimetic IFNγ(95−132) is able to also induce the nuclear translocation of IFNGR-1, as well as STAT1α, again similarly to IFNγ (*18*, *19*). Here, we found that the interaction of the mimetic IFNγ(95−132) with IFNGR-1(253−287) was sufficient to induce the nuclear translocation of IFNGR-1(253−287) in in vitro nuclear import assays. This required both of the above-described structural elements of the mimetic, α-helix and the NLS. With respect to the NLS, alanine substitutions within the NLS disrupted nuclear translocation of the mimetic, binding of the mimetic to IFNGR-1(253−287), and cotransport of IFNGR-1(253−287) to the nucleus. These data show that the mimetic IFNγ(95−132) can simultaneously interact with the nuclear import machinery and IFNGR-1 through its NLS. Disrupting the α-helix structure required for binding to IFNGR-1 also disrupted the ability of IFNγ(95−132) to mediate the transport of IFNGR-1. This is consistent with its role in binding to IFNGR-1 and further supports the conclusion that direct interaction of IFNGR-1 and IFNγ(95−132) mediates the nuclear translocation of IFNGR-1.

We found that tyrosine phosphorylation of STAT1α on Tyr^{701} by JAK2 alone was not sufficient to support nuclear transport of STAT1α. Likewise, treatment of the combination of STAT1α and IFNGR-1 with JAK2 was also not sufficient to support the nuclear transport of STAT1α. However, addition of mimetic IFNγ(95−132) was found to be required for the nuclear transport of STAT1α, supporting a role for the IFNγ NLS in the nuclear translocation of STAT1α and providing a mechanism for the intracellular action of the mimetic. The intracellular mimetic can effect the activation of JAK2 in intact cells, which controls the subsequent events that result in phosphorylation of STAT1α (*26*). Thus, the mimetic functions by activating the phosphorylation of STAT1α and chaperoning activated STAT1α, the key nuclear transcription factor of IFNγ responses, to the nucleus.

In cell cycle experiments, we found that the NLS activity of mimetic IFNγ(95−132), which is required for nuclear translocation of STAT1α, also contributes to the induction of p21WAF1/CIP1, which is a direct target of STAT1α. This is consistent with the fact that the mimetic can directly mediate nuclear transport of STAT1α. However, the mimetic is unable to inhibit the cell cycle due to its failure to downregulate c-myc, which suggests that it is unable to

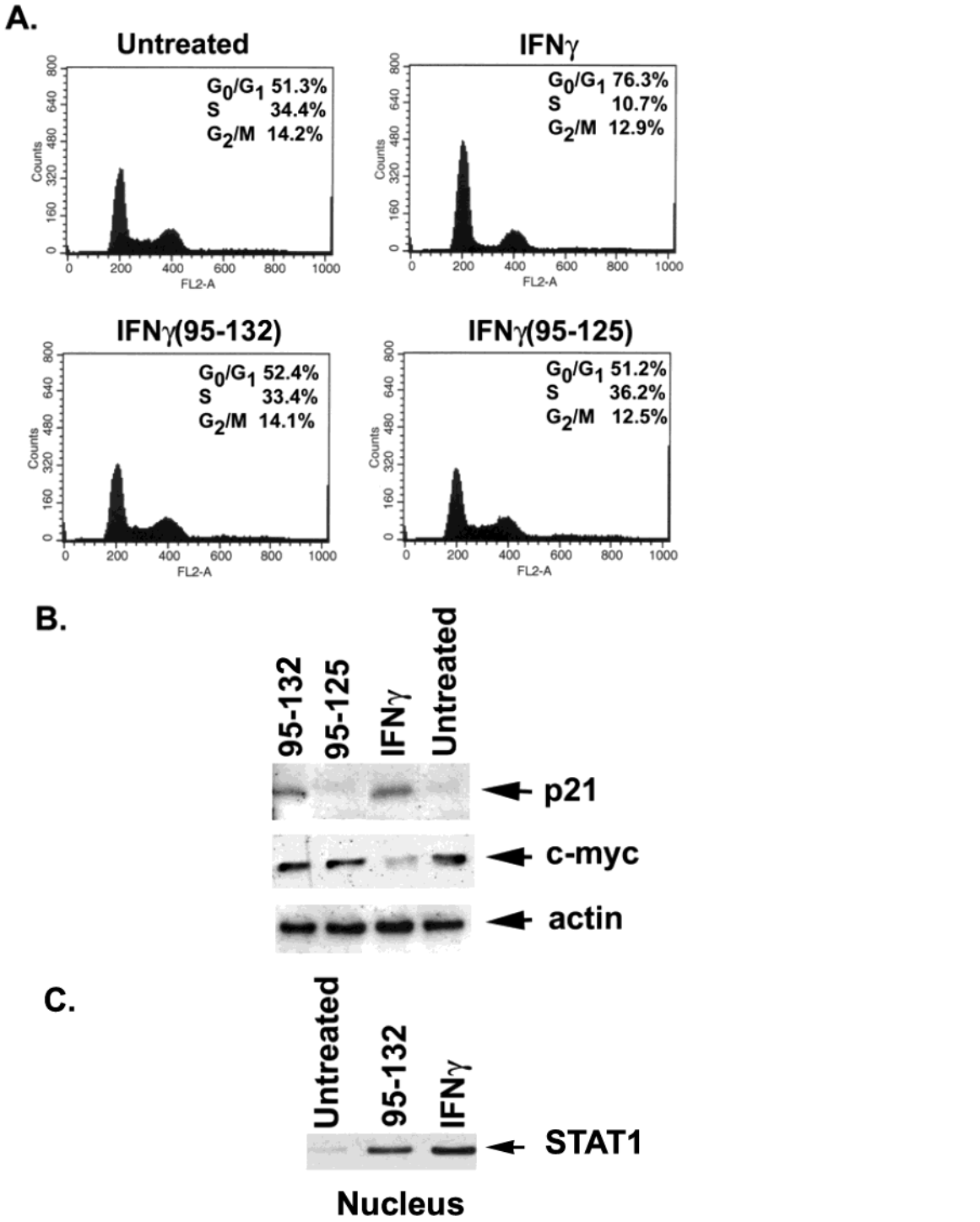

FIGURE 6: Effect of mimetic IFNγ(95−132) on cell cycle progression and its relationship to expression profiles of p21WAF1/CIP1 and c-myc. In panel A, human prostate cancer PC-3 cells were incubated in starvation media for 72 h to synchronize cells and then incubated in complete growth medium to initiate cell cycling in the presence or absence of IFNγ (5000 U/ml) or the indicated peptides (10 μM) for a further 24 h. Cell cycle progression was then determined using propidium iodide staining and FACS analysis as outlined in Materials and Methods. In panel B, PC-3 cells were treated as described for panel A, and immunoblot analysis was performed on whole cell lysates after IFNγ or peptide treatment using antibodies specific for p21WAF1/CIP1 and c-myc. Actin levels were followed on blots by stripping and reprobing with anti-actin antibodies as a protein loading control: 95−132 = lipo-IFNγ(95−132); 95−125 = lipo-IFNγ(95−125). In panel C, PC-3 cells were treated for 1 h with IFNγ (5000 U/ml) or IFNγ(95−132) (10 μM). Cells were lysed, and nuclei were isolated. Nuclear extracts were then analyzed by immunoblotting for STAT1α using antibodies specific for STAT1α: 95−132 = lipo- IFNγ(95−132).

activate all pathways that are activated by IFNγ. These pathways may not be directly related to nuclear translocation of STAT1α.

Nuclear translocation of STAT1α has been shown to be carried out by the importin-α analogue NPI-1 in a Ran-dependent manner (*27*), but studies did not clearly indicate the presence of an intrinsic NLS in STAT1α. In contrast, several recent studies have examined the nuclear translocation of STAT1α using overexpression of STAT1α fusion proteins (*28*−*31*). Through the use of mutations, it has been concluded that STAT1α contains a novel intrinsic NLS that differs from the classical NLSs such as those of the SV40 T

antigen or IFNγ (*28*−*31*). None of these studies tested STAT1α nuclear translocation in vitro via the digitonin permeabilization-based nuclear import assay used here for studying nuclear import; hence it is not possible to directly compare our negative results with STAT1α with those of the overexpression studies with respect to an intrinsic NLS. In this regard, there are no reports of an intrinsic NLS, either conventional or unconventional, in any other STAT that has been identified using the digitonin-based in vitro nuclear import assays that we have used here. This assay has been used routinely to identify NLSs in other proteins (*32*). However, we do not rule out other routes for getting STAT1α into the nucleus. Nonetheless, our data establish one pathway for nuclear translocation of STAT1α that does not need to invoke the presence of an intrinsic unconventional NLS on STAT1α.

The mechanism of action of the mimetic has important consequences for the mechanism of signal transduction of intact IFNγ. We have previously shown that the IFNγ NLS plays an active role in the internalization of IFNγ into cells (*19*). More importantly, our previous data suggest that IFNγ, following internalization, traverses the membrane of the endocytic vesicle to interact via its C-terminus (which contains the NLS) with the cytoplasmic domain of IFNGR-1 at the site identified by peptide IFNGR-1(253−287). Our data in this report suggest that this binding is ultimately responsible for the cotransport of IFNGR-1 and STAT1α to the nucleus via formation of the IFNγ/IFNGR-1/STAT1α transport complex (*19*) and is consistent with the fact that the mimetic IFNγ(95−132) by itself can induce the nuclear translocation of both IFNGR-1 and STAT1α from within the cell (*18*). Thus, the NLS of IFNγ plays a central role in the internalization of IFNγ, in the formation of the IFNγ/IFNGR-1/STAT1α transport complex, and its subsequent migration via the Ran/importin nuclear import pathway into the nucleus, providing a mechanism for the IFNγ-assisted "piggy-back" nuclear transport of STAT1α. This sequence of events thus describes a unique pathway for the nuclear translocation of STAT1α that follows the IFNγ stimulation of the IFNGR complex on the cell surface. Future studies will examine the specifics of the interactions of intact IFNγ/IFNGR-1 with NPI-1 (importin-α5) and the subsequent steps in their nuclear translocation in the context of the nuclear translocation of STAT1α.

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