The Carboxyl Terminus of Interferon-γ Contains a Functional Polybasic Nuclear Localization Sequence*

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Cytokines such as interferon-gamma (IFN-γ), which utilize the well studied JAK/STAT pathway for nuclear signal transduction, are themselves translocated to the nucleus. The exact mechanism for the nuclear import of IFN-γ or the functional role of the nuclear translocation of ligand in signal transduction is unknown. We show in this study that nuclear localization of IFN-γ is driven by a simple polybasic nuclear localization sequence (NLS) in its COOH terminus, as verified by its ability to specify nuclear import of a heterologous protein allophtocyanin (APC) in standard import assays in digitonin-permeabilized cells. Similar to other nuclear import signals, we show that a peptide representing amino acids 95–132 of IFN-γ (IFN-γ(95–132)) containing the polybasic sequence 120RRKKRRSP132 was capable of specifying nuclear uptake of the autofluorescent protein, APC, in an energy-dependent fashion that required both ATP and GTP. Nuclear import was abolished when the above polybasic sequence was deleted. Moreover, deletion immediately NH2-terminal of this sequence did not affect the nuclear import. Thus, the sequence 120RRKKRRSP132 is necessary and sufficient for nuclear localization. Furthermore, nuclear import was strongly blocked by competition with the cognate peptide IFN-γ(95–132) but not the peptide IFN-γ(95–125), which is deleted in the polybasic sequence, further confirming that the NLS properties were contained in this sequence. A peptide containing the prototypical polybasic NLS sequence of the SV40 large T-antigen was also able to inhibit the nuclear import mediated by IFN-γ(95–132). This observation suggests that the NLS in IFN-γ may function through the components of the Ran/importin pathway utilized by the SV40 T-NLS. Finally, we show that intact IFN-γ, when coupled to APC, was also able to mediate its nuclear import. Again, nuclear import was blocked by the peptide IFN-γ(95–132) and the SV40 T-NLS peptide, suggesting that intact IFN-γ was also transported into the nucleus through the Ran/importin pathway. Previous studies have suggested a direct intracellular role for IFN-γ in the induction of its biological activities. Based on our data in this study, we suggest that a key intracellular site of interaction of IFN-γ is the one with the nuclear transport mechanism that occurs via the NLS in the COOH terminus of IFN-γ.

The interferons (IFNs) are cytokines that use the well studied JAK/STAT pathway for signal transduction to the nucleus. This pathway is initiated by the binding of the ligand to the extracellular domain of the appropriate receptor complex followed by the activation of select members of the JAK family of tyrosine kinases at the intracellular cytoplasmic domain of the receptor subunits. These tyrosine kinases in turn phosphorylate appropriate members of the STAT family of transcription factors present in the cytoplasm, thereby targeting these factors, through unknown mechanisms, for translocation to the nucleus to activate transcription. Transcription factors the size of STATs must be taken into the cell nucleus by an active transport process through the nuclear pore complex.

Active nuclear import of a large number of nuclear proteins occurs through the Ran/importin pathway (for review, see Ref. 2). In this pathway, the nuclear protein initially binds to a heterodimeric nuclear transport protein called importin which contains α and β subunit. Nuclear proteins interact with the α subunit, importin α, of the transporter via a specific nuclear localization sequence (NLS). These NLSs generally consist of a cluster of basic amino acids or two short clusters separated by a spacer of variable length (a bipartite NLS; 2). The canonical simple polybasic NLS is represented by the sequence of the NLS of the SV40 T-antigen, KKKRRK. The β subunit, importin β, in turn mediates the binding of the NLS-importin α complex to the GTPase Ran present at the nuclear pore complex. Nuclear import of this nuclear protein-bearing complex through the nuclear pore is initiated by Ran with the subsequent hydrolysis of GTP and ATP and hence is a strictly energy-dependent process (2).

As mentioned above, the nuclear import of STAT1 requires active transport. Indeed, it has been shown recently that the IFN-γ-activated STAT1 is transported into the nucleus via the Ran/importin pathway by binding the importin α homolog NPI-1 (3, 4). NPI-1 is known to interact with nuclear proteins via the above polycationic NLSs. However, based on current knowledge, no polybasic NLS has been identified on STAT1 (3, 4). Mutational analysis of STAT1 failed to reveal a conventional NLS on STAT1 which was responsible for mediating the binding to NPI-1 or nuclear translocation of STAT1 (4). These studies are consistent with the concept that the necessary basic NLS required for STAT1 interaction with NPI-1 is provided for by another molecule associated with STAT1. Based on the results of studies from our laboratory on the COOH-terminal domain of IFN-γ, IFN-γ(95–133), and its interaction with the α chain of the receptor, we propose that this candidate chaperone function may be served by the ligand IFN-γ (5). Crucial to this

*This work was supported by National Institutes of Health Grants CA69956 and CA38587 (to H. M. J.). This manuscript is Florida Agriculture Experiment Station Journal Series R-06627. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
hypothesis, as a first step, is demonstrating that IFN-γ contains a functional NLS. In this report, we have specifically addressed this issue.

It has been well documented that human and murine IFN-γ are themselves translocated to the nucleus (6–8). The sequence of murine IFN-γ contains a putative polybasic NLS within the above mentioned COOH-terminal domain. It has been known for some time that this conserved sequence in murine and human IFN-γ is absolutely required for the biological activities of the corresponding IFN-γ (9–16). We have also shown that both human and murine IFN-γ interact with the cytoplasmic domain of the α chain of the corresponding IFN-γ receptor complexes via this COOH-terminal domain containing this putative NLS sequence (17–19). STAT1 binds to the cytoplasmic domain of the α chain of the receptor at a downstream site. The interaction of the COOH terminus of IFN-γ with the α chain is dependent on the presence of the putative NLS sequence and in turn increases the affinity of the receptor-activated tyrosine kinase JAK2 to an immediately proximal site on the α chain of the receptor (18). JAK2, along with JAK1, is involved in the receptor recruitment and phosphorylation of STAT1, events that precede the subsequent nuclear translocation of STAT1. Thus, this COOH-terminal domain of IFN-γ has many characteristics that it make a good candidate to be involved in the chaperoning of STAT1α to the nucleus, probably as part of a ligand-receptor-STAT1α complex.

Given the properties of this sequence, we tested in this study whether the above polybasic sequence in the COOH-terminal domain of IFN-γ can function as an NLS and facilitate the nuclear translocation of a heterologous protein. We demonstrate that this sequence is, indeed, an NLS and can function as a natural import signal for the biological activities of the corresponding IFN-γ.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse 3T3 cells (Balb/c, clone A31; from ATCC) were grown in ATCC-modified Dulbecco’s modified Eagle’s medium containing 10% bovine serum. WISH cells were grown in Eagle’s minimal essential medium containing 10% fetal bovine serum. Cells were plated onto coverslips for 24 h before use.

**Peptide Synthesis**—Peptides used in this study (see Table I) were synthesized on a PerSeptive Biosystems 9050 automated peptide synthesizer using Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry as detailed previously (10).

**Preparation of Import Substrates (APC Conjugates)—**Allophycocyanin (APC) activated with the bifunctional cross-linker succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid was purchased from Prozyme (San Leandro, CA) and used according to the manufacturer’s suggestions. Briefly, peptides or human IFN-γ (Biosource International, Camarillo, CA) reduced with dithiothreitol (50 mM) was coupled at a 1:1 or 1:2 molar ratio (APC:peptide) in 5 mM MES, pH 6.0, containing 5 mM EDTA. After the initial separation of uncoupled peptides by gel filtration through an Econo 10DG column (Bio-Rad) in 20 mM HEPES, pH 7.3, any residual peptide was removed by repeated concentration in the same buffer through a Centricon 50 ultrafiltration unit (MWCO 50,000; Amicon, Inc., Beverly, MA) and the conjugate stored at 4 °C. Coupling efficiency (two or three peptides/APC) and peptide removal were established by SDS-polyacrylamide gel electrophoresis.

**Nuclear Transport Assays**—Transport assays with mouse A31 cells were based on methods described previously (for review, see Ref. 20). (Cells; both A31 and WISH cells) grown on coverslips were incubated at 4 °C with transport buffer: 20 mM HEPES, pH 7.3; 110 mM potassium acetate; 5 mM sodium acetate; 2 mM magnesium acetate; 1 mM EGTA; 2 mM dithiothreitol; 10 µg/ml each leupeptin, pepstatin, and aprotinin; and 2 mM dithiothreitol. Cells were then permeabilized with digitonin (at 40 µg/ml) in transport buffer for 5 min at 4 °C. After washing with transport buffer, cells were incubated with the transport reaction mixtures for 30 min at 30 °C. Complete reaction mixtures (60–1 µl final volume) contained 20 mM HEPES, pH 7.3; 110 mM potassium acetate; 5 mM sodium acetate; 2 mM magnesium acetate; 1 mM EGTA; 2 mM dithiothreitol; 10 µg/ml each leupeptin, pepstatin, and aprotinin; 0.5 mM GTP; 2.5 mM ATP; 5 mM phosphocreatine (Calbiochem); 50 units/ml creatine phosphokinase (Calbiochem); approximately 200 mM appropriate import substrate; and 20 µl of rabbit reticulocyte lysate (untreated; Promega, Madison, WI). Coverslips were washed in transport buffer containing 1% bovine serum albumin, mounted on slides, and observed under a fluorescence microscope (cooled-CCD deconvolution microscopy).

For ATP depletion experiments reticulocyte lysates were first treated with a mixture of hexokinase (~300 units/ml), glucose (8 mM), and glucagon (0.2 units/ml) at 30 °C for 15 min before the addition of the rest of the components. For GTP dependence, GTP was omitted from the reaction mixture, and the reticulocyte lysate was incubated at room temperature with the analog GTP·S (Calbiochem) at 0.5 mM before the addition of other components.

For peptide competition experiments, peptides were added at 600-fold molar excess, calculated with respect to APC, in the presence of all other components except the import substrate. After incubation for 5 min at room temperature, the APC substrate was then added and the mixture incubated with the cells.

**RESULTS**

The competence of the putative nuclear localization sequence within the COOH-terminal domain of IFN-γ to function in nuclear import was evaluated by testing its ability to mediate the nuclear import of a heterologous protein. This was performed using the standard in vitro nuclear transport assay (20) in digitonin-permeabilized mouse A31 cells.

In this study, we used as substrate a peptide corresponding to amino acids 95–132 of murine IFN-γ (IFN-γ(95–132); see Table I) coupled to the heterologous autofluorescent protein APC to provide a substrate for the assay. As shown in Fig. 1, essentially a 100% of the A31 cells accumulated the IFN-γ(95–132)-APC substrate in the nucleus and nucleoli (compare Fig. 1A and B). This accumulation was inhibited at 4 °C (Fig. 1C) and was dependent on the addition of reticulocyte-derived cytosolic factors (Fig. 1D). Addition of a simple mixture of IFN-γ(95–132) with carrier protein without covalent coupling did not lead to any nuclear staining (data not shown). These data show that the IFN-γ(95–132) was capable of mediating the nuclear import of a heterologous protein when coupled to it.

The transport of karyophilic proteins across the nuclear membrane via the nuclear pore complex is an active, energy-dependent event (2). It depends strictly on the availability of both ATP and GTP. This energy-independent binding to the nuclear pore complex leads to a typical “rimming” pattern at the nuclear periphery (21). As shown in Fig. 2, the nuclear import of IFN-γ(95–132)-APC was strictly energy-dependent.
Cytosolic extracts depleted of ATP did not support the nuclear import of IFN-\(\gamma\)\((95–132)\)-APC into the nucleus of A31 cells (compare Fig. 2, A and B). In the absence of ATP the IFN-\(\gamma\)\((95–132)\)-APC substrate accumulates as a rim at the nuclear envelope. In a similar fashion, the absence of exogenous GTP coupled with the addition of the nonhydrolyzable analog GTP\(_{\text{gS}}\) also resulted in failure of transport into the nucleus (Fig. 2C) and resulted in the accumulation of the substrate at the nuclear periphery. Thus, these data further demonstrate that the domain represented by IFN-\(\gamma\)\((95–132)\) contains an NLS that functions in a strictly energy-dependent fashion characteristic of nuclear import signals (2).

To establish further the specificity of this NLS-bearing domain, we performed competition experiments using peptides to block the transport of the IFN-\(\gamma\)\((95–132)\)-APC substrate. The peptides used were the following (see Table I): a peptide containing the NLS of the SV40 T-antigen (SV40 T-NLS); the IFN-\(\gamma\)\((95–132)\) peptide; and the peptide IFN-\(\gamma\)\((95–125)\), which is derived from IFN-\(\gamma\)\((95–132)\) by deletion of the polybasic putative NLS, 126RKRKRSR132. The SV40 T-NLS is one of the best characterized NLSs that utilizes the well studied Ran/importin pathway for nuclear import (2). It contains an NLS that is a simple polybasic cluster similar to that in IFN-\(\gamma\)\((95–132)\).

Several important conclusions can be drawn from these results. First, the ability of IFN-\(\gamma\)\((95–132)\) to inhibit transport of IFN-\(\gamma\)\((95–132)\)-APC clearly demonstrates that the nuclear import of the substrate is specifically driven by the attached peptide IFN-\(\gamma\)\((95–132)\), further establishing the specific ability of IFN-\(\gamma\)\((95–132)\) to act as an NLS. Second, the ability of the SV40 T-NLS to inhibit transport of the substrate suggests that the nuclear import of IFN-\(\gamma\)\((95–132)\)-APC occurs through the Ran/importin pathway that is utilized by the prototypic polybasic SV40 T-NLS. Third, the fact that IFN-\(\gamma\)\((95–125)\) that is deleted in the polybasic putative NLS is unable to block the import of IFN-\(\gamma\)\((95–132)\)-APC strongly suggests that the NLS...
The presence of the putative NLS \(126^RKRKR_132^SR\) is necessary and sufficient for nuclear import directed by IFN-\(\gamma(95–132)\). Assays were performed on mouse A31 cells with the corresponding peptide-APC conjugates as substrates using the following peptides: IFN-\(\gamma(95–132)\) (panel A); peptide IFN-\(\gamma(95–125)\) (panel B), which is deleted in the NLS; or peptide IFN-\(\gamma(121–132)\) (panel C), which is derived from IFN-\(\gamma(95–132)\) by deletion immediately NH$_2$-terminal to the NLS (for sequences, see Table I). Panel D shows the phase-contrast image corresponding to the field shown in panel B.

is contained in the deleted sequence \(126^RKRKR_132^SR\). This sequence is very similar to that of the classical polybasic NLS contained in the SV40 T-NLS. Thus, it is consistent that the SV40 T-NLS peptide can block the import of IFN-\(\gamma(95–132)\)-APC. We conclude that IFN-\(\gamma(95–132)\) contains a classical polybasic NLS that clearly functions in nuclear import in a fashion similar to that of the prototypical SV40 T-NLS. The IFN-\(\gamma\) NLS probably utilizes components of the Ran/importin pathway common to the nuclear import pathway of the SV40 T-NLS.

To confirm further that the polybasic sequence \(126^RKRKR_132^SR\) is responsible for the nuclear localization properties of IFN-\(\gamma(95–132)\) we tested directly the ability of the IFN-\(\gamma(95–125)\)-APC conjugate to function as a substrate in the nuclear import assays compared with IFN-\(\gamma(95–132)\)-APC. As shown in Fig. 4, in contrast to IFN-\(\gamma(95–132)\)-APC (Fig. 4A), IFN-\(\gamma(95–125)\)-APC (Fig. 4B and its phase-contrast image in 4D), which is deleted in the polybasic NLS region, was not able to function as a nuclear import substrate. We also tested the ability of a peptide derived from IFN-\(\gamma(95–132)\) where the sequence immediately NH$_2$-terminal to the NLS has been deleted, namely IFN-\(\gamma(121–132)\) (see Table I). IFN-\(\gamma(121–132)\)-APC retained its ability to function as a substrate for nuclear import and appeared as effective as IFN-\(\gamma(95–132)\)-APC in nuclear import (Fig. 4C). These data clearly show that this polybasic sequence is necessary and sufficient for the nuclear targeting properties of the COOH-terminal domain IFN-\(\gamma(95–132)\).

Finally, we demonstrated in these assays that intact IFN-\(\gamma\) itself has the ability to mediate nuclear import of a heterologous protein. As can be seen in Fig. 5, IFN-\(\gamma\)-APC was also transported into the nucleus (Fig. 5A) in a fashion that was strictly dependent on the presence of cytosolic factors (Fig. 5B). A lower signal was observed in these assays compared with the transport of peptide-APC conjugates because of the lower coupling efficiency to APC. Nuclear import of IFN-\(\gamma\)-APC was strongly inhibited by competition with the IFN-\(\gamma(95–132)\) peptide and the SV40 T-NLS peptide. These data show that the nuclear import of IFN-\(\gamma\) itself is mediated in a fashion similar to IFN-\(\gamma(95–132)\) and the SV40 T-NLS and probably occurs via the classical Ran/importin pathway for nuclear import.

**DISCUSSION**

In this report, we have demonstrated that the nuclear translocation of IFN-\(\gamma\) occurs via a classical polybasic NLS sequence, \(126^RKRKR_132^SR\), in the COOH terminus of the molecule. This sequence functions in the nuclear import of IFN-\(\gamma\) via an active energy-dependent process. Competition studies suggest that the NLS most likely utilizes components of the Ran/importin pathway that is common to the nuclear import mechanism of the prototype of this class of NLSs, namely the SV40 T-NLS. We have shown previously that this sequence is a critical requirement for the biological activity of the IFN-\(\gamma\) molecule (9, 10). Several similar studies using human IFN-\(\gamma\) have shown that a highly homologous polybasic region within the COOH terminus is absolutely required for biological activity (11–16). Thus, in conjunction with these studies our data show that the nuclear localization of IFN-\(\gamma\) via this NLS serves a crucial role in the biological activity of IFN-\(\gamma\).

Previous studies have suggested a biological role for intracellular IFN-\(\gamma\) and IFN-\(\alpha\). These include the observations that (i) human IFN-\(\gamma\) delivered by a liposomal vector was able to activate murine macrophages (22); (ii) secretion-defective human IFN-\(\gamma\) expressed in murine fibroblasts induced an antiviral state in these cells (23); (iii) microinjected IFN-\(\gamma\) can induce If expression on murine macrophages (24); and (iv) intracellularly expressed IFN-\(\alpha\) is also active and can activate the DNA binding activity of the ISGF3 transcription complex containing STAT1$\alpha$ and STAT2$\alpha$ (25). Thus, the IFN-\(\gamma\) most likely interacts with intracellular element(s) to induce a biological response. Based on our data in this study we suggest that a key intracellular interaction is the one with the nuclear transport mechanism which occurs via the NLS in the COOH terminus of IFN-\(\gamma\).

Our studies in this report have clearly identified for the first time the NLS in IFN-\(\gamma\). Although it appears critical for biolog-
Nuclear Localization Signal of IFN-γ

407

ical activity of IFN-γ, the exact contribution of this NLS to biological function is less clear. Based on previous studies from our laboratory on the interaction of this domain of IFN-γ with the receptor and its effect on signaling components, we have speculated that a role for the NLS could be as a chaperone for the nuclear localization of activated STAT1α (5). Recent studies have shown that STAT1α is transported to the nucleus via the Ran/importin pathway (3, 4). STAT1α binds to the importin α homolog NPI-1 that mediates the nuclear import of substrates, like the SV40 T-NLS, in a conventional NLS-dependent fashion (4). However, mutation analysis of STAT1α has failed to reveal any sequence responsible for nuclear import of STAT1α (4). These studies are consistent with a role for a STAT1α-associated molecule that binds importin α and provides the required NLS, i.e. the interaction of STAT1α with the Ran/importin system could be indirect. We suggest that the NLS required for nuclear import of STAT1α may be provided by the ligand IFN-γ, which interacts intracellularly at the level of the nuclear transport mechanism. Others have noted the presence of putative NLSs in other ligands and their receptors (26, 27) and have suggested a broad role for the nuclear targeting of ligands and/or their receptors in signaling to the nucleus (26). In fact, recent studies have demonstrated that interleukin-1 and interleukin-5 possess functional NLSs (28–30). We have found that when a comparison is made between such cytokines and their signaling pathways, a remarkable similarity that this model of ligand/receptor-assisted nuclear translocation of ligands and/or their receptors in signaling to the nucleus may extend to other cytokine/receptor systems.

In this regard, as outlined in the Introduction, we have already demonstrated that the COOH-terminal domain represented by IFN-γ(95–132) interacts specifically with the cytoplasmic domain of the α chain of the IFN-γ receptor (9, 17–19). This interaction is also dependent on the presence of the NLS (9, 17). The α chain of the IFN-γ receptor is the subunit that binds activated STAT1α. We have recently shown using immunofluorescence and immunoprecipitation techniques that after IFN-γ treatment of intact WISH cells, the cytoplasmic domain of the α chain of the IFN-γ receptor is also translocated rapidly to the nucleus. This is specific to the α chain of the receptor because the β chain of the receptor complex, which does not bind STAT1α, did not undergo endocytosis or nuclear translocation. Furthermore, the pattern of receptor and STAT1α distribution throughout this process and the kinetics of receptor α chain and STAT1α translocation to the nucleus are very similar, consistent with the proposition that these events may be coupled. Because a putative NLS sequence cannot be identified in the receptor α chain, a likely scenario is that translocation of the receptor complex bearing STAT1α after ligand-dependent activation events is ultimately directed by the NLS in the ligand via the interaction of the NLS-containing domain in the ligand with the cytoplasmic domain of the receptor α chain. This scenario can be tested and is being examined in our ongoing studies.

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J. Larkin III, M. R. Paddy, H. M. Johnson, and P. S. Subramaniam, manuscript in preparation.