The IFNAR1 subunit of the type I IFN receptor complex contains a functional nuclear localization sequence☆

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Abstract A nuclear localization sequence (NLS) in the type II interferon (IFN) IFNγ, which is responsible for the nuclear translocation of both the ligand and the α-subunit (IFNGR1) of the receptor complex, has previously been characterized and its role in signaling examined in detail. We have now identified an NLS in the type I IFN receptor (IFNAR) common subunit IFNAR1 from humans and show that the human IFNAR1 subunit can translocate to the nucleus following human IFNβ stimulation. An NLS in human IFNAR1 is located in the extracellular domain of IFNAR1 within the sequence ^{382}RKIIEKKT (numbered for the precursor form). Nuclear import by the NLS functions in a conventional fashion requiring cytosolic import factors, is energy-dependent and inhibited by the prototypical NLS of the SV40 large T-antigen. These studies provide a mechanism for nuclear import of IFNAR1, as well as for type I IFN ligands, and a starting point for studying an alternate role for IFNAR1 in nuclear signaling within the type I IFN system.

1. Introduction

An alternate mode of signaling to the nucleus by diverse cytokines, growth factors and hormones has been known for some time that involves the direct nuclear translocation of ligands and/or their receptors [1]. These ligand/receptor systems now include, but are not limited to, those of growth hormone [2,3], members of the epidermal growth factor family [4], angiotensin [5], prolactin [6], interleukin-5 [7,8], FGFs [9], and interferon-gamma (IFNγ) [10,11]. In many of these cases, the nuclear ligand or receptor has been identified as acting as a transcription factor or transcriptional co-activator, thereby highlighting a direct role for these nuclear ligand/receptor molecules in regulating gene expression.

In the case of the IFNs, the type II IFN IFNγ has been described as a nuclear localizing ligand [12,13]. The mechanism and function of nuclear translocation of IFNγ has been studied in detail [10,11]. IFNγ has been shown to contain a nuclear localization sequence (NLS) in its C-terminus that mediates the nuclear translocation of not only the ligand but also the α-subunit of the IFNγ receptor (IFNGR-1) [14]. The IFNγ/IFNGR-1 complex appears to function as a nuclear chaperone for the STAT-1α transcription factor activated by IFNγ [10,11,14].

Within the type I IFN system, the α-subunit of the common type I IFN receptor (IFNAR1) for the large family of type I IFNs has been postulated to contain a putative NLS, ^{382}RKIIEKKT (numbering for precursor form), within its extracellular domain but this has not been experimentally tested [1]. The IFNAR1 subunit is considered the 'signaling' subunit, as it does not bind type I IFNs with detectable affinity but is absolutely required for signal transduction from the heterodimeric IFNAR complex and for type I IFN biological activity [15]. In contrast, the β-subunit, IFNAR2, appears to be the subunit that binds type I IFNs with relatively high affinity [15]. Signaling to the nucleus from the IFNAR complex occurs along multiple pathways, one of which is the well characterized JAK/STAT pathway (for reviews see [16–18]). In the JAK/STAT pathway, IFNAR1 associates with the Janus kinase (Jak) family member, Tyk2, while IFNAR2 associates with the Jak family member, Jak1, which together activate STAT transcription factors as important nuclear messengers. Besides this pathway, IFNAR1 through the Jaks participates in activating other pathways like those involving protein kinase C [19,20], insulin receptor substrate proteins and PI 3′-kinase [16,17], and p38 MAP kinase pathway [16,17], that may involve cross-talk with the STAT pathway. However, the multiple pathways activated by the type I IFNAR complex is a complicating feature since, as has been previously suggested [11,10,21], almost all cytokines, growth factors and hormones activate some or all of the same pathways, raising the question of how signaling specificity in the nucleus is ultimately maintained. The nuclear translocation of ligands and/or their receptors and their emerging roles in transcription and other chromatin events regulating gene expression has been suggested as one method of imparting a degree of specificity to nuclear signaling, since the nuclear messenger is the signal generating ligand/receptor itself [10,11,21].

We show for the first time in this study that within the type I IFN system, the IFNAR1 subunit of the IFNAR complex is translocated to the nucleus following ligand stimulation. Further, we show that a NLS lies in the extracellular domain region of IFNAR1 containing the sequence ^{382}RKIIEKKT that was previously postulated. IFNAR1, thus, behaves similarly to other receptors like c-ErbB-3, FGFR-1, Notch and angiontensin II type I receptor that have been shown to be actively transported to the nucleus via NLSs in their sequence, and is, thus, at least one component within the type I IFN

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Table 1
Sequences of peptides used in this study

Peptide	Sequence[a]
IFNAR1(374–390)	cWENTSNAERKIIEKKTD
SV40 NLS	cGGGPKKKRKVED
IFNγ(95–125)	cAKFEVNNPQVQRQAFNELIRVVHQLLPESSL

[a]A cysteine (underlined) was introduced as the first amino acid residue of each peptide sequence to allow for coupling to APC, where required. The IFNAR1 sequence is from the precursor form of human IFNAR1 and that of IFNγ from the mature form of murine IFNγ, and residues in peptides are numbered after the first cysteine.

system that could possibly participate in direct nuclear signaling through nuclear translocation.

2. Materials and methods

2.1. Cell culture

The LpZRαβ$_L$.10 clone expressing the IFNAR1 subunit and the long form of the IFNAR2 subunit were a kind gift of Dr. O.R. Colamonici (University of Illinois, Chicago, IL), and have previously been described [22]. WISH cells were grown in EMEM containing 10% fetal bovine serum.

2.2. Peptide synthesis

Peptides used in this study (see Table 1) were synthesized on a PerSeptive Biosystems 9050 automated peptide synthesizer using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry as outlined previously [23].

2.3. Immunofluorescent staining

LpZRαβ$_L$.10 cells, or parental L 929 cells, were grown on microscope slides and treated with 3000 U/ml of recombinant human or murine IFNβ (Biosource International, Camarillo, CA) for 1 h, before fixing and staining with antibodies to IFNAR1 (Santa Cruz Biotechnology, Santa Cruz, CA; #sc-845 recognizing both human and mouse IFNAR1). Fixation and staining procedures were as described previously [24].

2.4. Preparation of import substrate (Allophycocyanin conjugation)

IFNAR1(374–390) peptide was coupled to SMCC-activated allophycocyanin (APC; Prozyme, San Leandro, CA) using a molar ratio of 4:1 peptide:APC as previously described [25,14] except that reduced peptide was separated on Sephadex G-10 before addition to the activated APC.

2.5. Nuclear import assays

Nuclear import assays in digitonin-permeabilized WISH cells were performed as previously described [25] using IFNAR1(374–390)-APC as a substrate. Energy-dependence with respect to ATP and GTP requirements was performed as before [25]. Competition experiments with unlabeled peptides were also performed as before [25], except that a 30-fold excess of unlabeled peptide was used.

3. Results and discussion

To first determine whether the human IFNAR1 subunit could translocate to the nucleus, we tested the subcellular localization of human IFNAR1 in mouse L cells overexpressing both human IFNAR1 and the long form IFNAR2 subunit of the IFNAR receptor (clone LpZRαβ$_L$.10 [22]). These cells are fully responsive to both human IFNα and IFNβ and have been characterized previously [22]. Treatment of these cells with human IFNβ for 1 h followed by immunofluorescent staining for the IFNAR1 subunit showed a clear nuclear localization of the human IFNAR1 subunit (Fig. 1A and B). Treatment with human IFNγ did not induce nuclear localization of human IFNAR1, showing the specificity of the response (Fig. 1C). LpZRαβ$_L$.10 cells, or parental L 929 cells, treated with murine IFNβ (which does not activate the human IFNAR complex) showed a weaker positive signal for nuclear murine IFNAR1 (data not shown), as expected given the relatively

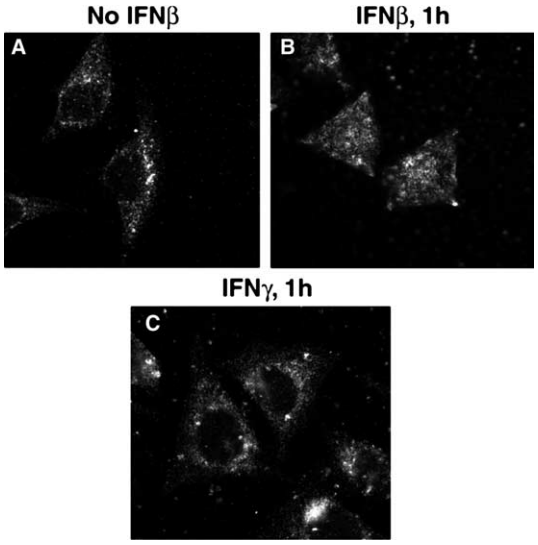

Fig. 1. Nuclear translocation of the human IFNAR1 subunit following IFNβ stimulation of cells. LpZRαβ$_L$.10 cells grown on slides were left untreated (A), or treated with 3000 U/ml of recombinant human IFNβ (B) or recombinant human IFNγ (C) at 37 °C for 1 h. Cells were then fixed and immunofluorescently stained with antibodies to IFNAR1.

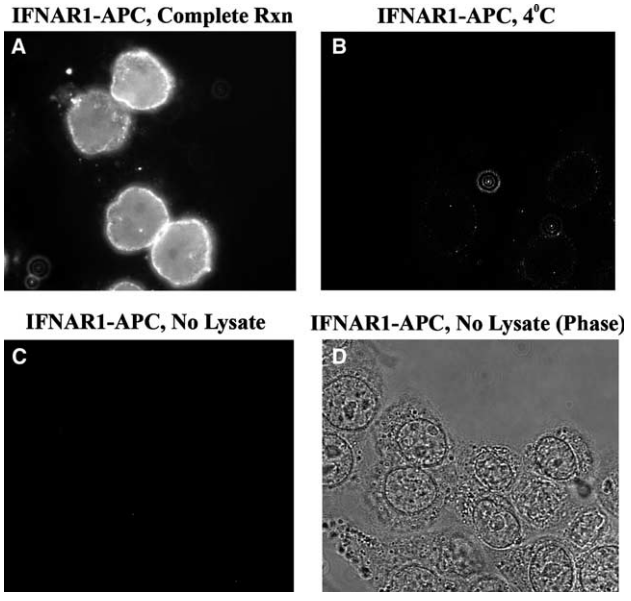

Fig. 2. The peptide IFNAR1(374–390) mediates the nuclear import of the heterologous fluorescent protein APC (IFNAR1-APC). Digitonin-permeabilized WISH cells were incubated for the assay duration (30 min) with the complete import reaction mixture at 30 °C (A) or 4 °C (B). In (C) cells were incubated with an import reaction mixture in which the reticulocyte lysate was replaced by 4% BSA (w/v). (D) The phase contrast image of the field of cells in (C).

lower expression of the endogenous murine IFNAR1 subunit in these cells.

To specifically examine the putative NLS in IFNAR1 for nuclear localization properties, we used a standard fluorescence-based nuclear import assay using digitonin-permeabilized cells that are routinely used to study NLS function [26–28], such as with the IFNγ NLS [14,25,29] or that of the prototypical SV40 large T-antigen NLS (SV40 NLS) [26–28]. For use as substrate in the assays, a peptide containing the sequence RKIIEKKT (see Table 1) was coupled to the autofluorescent protein APC. As can be seen in Fig. 2A, IFNAR1(374–390)-APC was translocated to the nucleus in the complete nuclear import assay. The nuclear accumulation was inhibited when the assay was run at 4 °C (Fig. 2B) and was also dependent on the presence of reticulocyte-derived cytosolic factors (Fig. 2C). These data show that the IFNAR1(374–390) is an NLS that is capable of mediating the nuclear import of a heterologous protein (APC), which is typical of many conventional NLSs.

Active nuclear import across the nuclear pore complex (NPC) is energy-dependent and depends on the availability of ATP and GTP. In the absence of ATP or GTP, binding to the NPC occurs without transport into the nucleus. As can be seen in Fig. 3, the nuclear import of IFNAR1(374–390)-APC was also strictly energy-dependent. Depletion of ATP from cytosolic extracts blocked nuclear import of IFNAR1(374–390)-APC (compare Fig. 3A and B), and IFNAR1(374–390)-APC characteristically 'rimmed' the nuclei as it accumulated at the nuclear pores without further translocation. Similarly, omission of GTP and inclusion of the non-hydrolyzable analog GTPγS blocked nuclear import (Fig. 3D) with substrate 'rimming' the nuclei. These data are typical of conventional NLSs that function in active nuclear import and show that the IFNAR1(374–390) shares these properties.

To further examine the specificity of IFNAR1(374–390) NLS properties, we performed competition experiments with unlabeled peptides in the above assays. The peptides used (see Table 1 for sequences) were IFNAR1(374–390) itself, a peptide including the SV40 NLS, and as a negative control, peptide IFNγ(95–125) derived from the C-terminus of IFNγ that has no NLS activity [25]. The SV40 NLS is one of the best characterized conventional polybasic NLSs and has been shown to utilize the Ran/importin pathway for nuclear import. As shown in Fig. 4, nuclear import of IFNAR1(374–390)-APC was inhibited by an excess of the unlabeled IFNAR1(374–390) peptide, showing that import was specifically driven by the IFNAR1 peptide in IFNAR1(382–389)-APC (Fig. 4A and B). The nuclear import of IFNAR1-APC was also inhibited by an excess of a peptide containing the prototypical polybasic SV40 NLS (Fig. 4C), but was not inhibited by the non-NLS IFNγ(95–125) control peptide (Fig. 4D). This suggests that

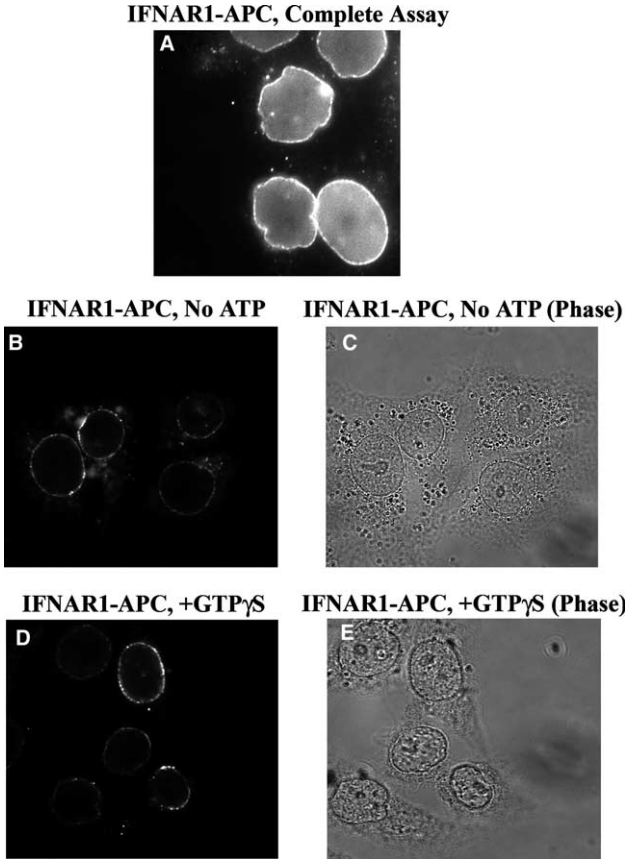

Fig. 3. Nuclear import directed by IFNAR1(374–390) is strictly energy-dependent. Permeabilized cells for assay were incubated, as in Fig. 1A, either with the complete import mixture (A), or with an import mixture that had been depleted of ATP as described under Section 2 (B), or an import mixture in which exogenous GTP was omitted, and the non-hydrolyzable analog, GTPγS, was included at a final concentration of 0.5 mM (D). (C) and (E) are phase contrast images of the field of cells in (B) and (D), respectively. IFNAR1-APC=IFNAR1(374–390)-APC.

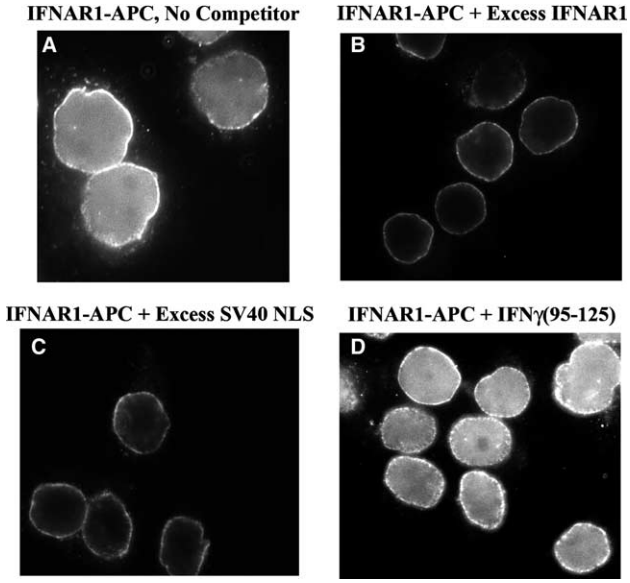

Fig. 4. Nuclear import by IFNAR1(374–390) is peptide-specific and is inhibited by the SV40 NLS. Import reaction mixtures containing the substrate IFNAR1(374–390)-APC (IFNAR1-APC; 220 nM) were incubated in the absence of any competitor peptides (A) or in the presence of the following competitor peptides: the cognate peptide IFNAR1(374–390) (B), the SV40 T-NLS peptide (C), or a non-NLS control peptide IFNγ(95–125) (D) (for sequences, see Table 1). Competitor peptides were incubated at a 30-fold molar excess, with respect to the substrate IFNAR1(374–390)-APC, in the reaction mixture at room temperature for 5 min just before the addition of the substrate and assay on mouse A31 cells.

the IFNAR1 NLS uses components of the classical Ran/importin pathway that are utilized by the SV40 NLS peptide.

The above study identifies for the first time an NLS domain within a component of the type I IFN system, namely the receptor subunit IFNAR1. Previous studies have already identified an NLS in the type II IFN IFNγ and have examined in detail its role in signaling [11,10]. The NLS of IFNAR1 lies within the extracellular domain of the human IFNAR1. This region appears to function as a conventional NLS in a manner similar to that of the SV40 NLS. In this regard, the IFNAR1 NLS contains basic residues interspersed with hydrophobic residues. This is broadly similar to the well characterized NLS of the c-myc protein, AAKRVKLD [30], where hydrophobic residues were found to enhance nuclear localization [31]. The c-myc NLS also utilizes the Ran/importin pathway similar to the SV40 NLS [32]. The specific contributions of individual residues in the IFNAR1 NLS remain to be determined. This report also documents the fact that the IFNAR1 subunit is capable of translocating to the nucleus following ligand (IFNβ) stimulation. These studies provide a framework for further detailed study of an alternate mode of signaling to the nucleus within the type I IFN system.

4. Note added in proof

A recent report [33] has shown that the second chain of the type I IFN receptor complex, IFNAR2, is subject to regulated intramembrane proteolysis and the intracellular cytoplasmic domain (ICD) of IFNAR2 is translocated to the nucleus. Although the NLS for this translocation was not identified, the nuclear ICD can function as a repressor of transcription from the Interferon Stimulated Response Element, ISRE, in gene promoters.

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