Interferon γ is recognised by importin α/β: Enhanced nuclear localising and transactivation activities of an interferon γ mimetic

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Abstract Interferon (IFN) γ’s ability to localise in the nucleus and function in gene activation has been known for some time, although the role of the conventional nuclear transporting importin molecules is unclear. Here, we demonstrate for the first time the direct recognition of IFNγ and an IFNγ mimetic peptide by IMPα and the IMPα/β heterodimer, where the IFNγ mimetic shows higher affinity. Significantly, this correlates well both with in vivo ability to target green fluorescent protein to the nucleus in transfected cells as determined by quantitative confocal laser scanning microscopy, as well as GAS promoter activity of a luciferase reporter. This has important implications for IFNγ’s anti-viral action, and the potential use of the IFNγ mimetic in antiviral therapies.

Structured summary:
MINT-647853, MINT-6478550, MINT-6478516: IFN gamma (uniprotkb:P01580) directly interacts (MI:0407) with IMP beta (uniprotkb:Q6GT15) and IMP alpha (uniprotkb:P52293) by enzyme linked immunosorbent assay (MI:0411) MINT-6478503, MINT-6478490:
IFN gamma (uniprotkb:P01580) directly interacts (MI:0407) with IMP alpha (uniprotkb:P52293) by enzyme linked immunosorbent assay (MI:0411) MINT-6478601:
T-ag (uniprotkb:P00370) directly interacts (MI:0407) with IMP beta (uniprotkb:Q6GT15) and IMP alpha (uniprotkb:P52293) by enzyme linked immunosorbent assay (MI:0411) MINT-6478541:
T-ag (uniprotkb:P00370) directly interacts (MI:0407) with IMP alpha (uniprotkb:P52293) by enzyme linked immunosorbent assay (MI:0411)

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Keywords: Interferon gamma; Importin; Nuclear protein import; GAS promoter activity; Confocal laser scanning microscopy

1. Introduction

Nuclear localisation of polypeptide ligands such as interferon γ (IFNγ), growth hormone, and members of the fibroblast and epidermal growth factor families has been known for a number of years [1–5]. Importantly in this context, the nuclear function of these ligands has become clearer more recently, appearing to relate strongly to specific gene activation [6], and regulation of cell proliferation/oncogenic potential [2,4], putting into question the dogma that ligands signal solely by interaction with the extracellular domain of membrane receptors.

IFNγ is a classic example of a nuclear localising/nuclear signalling ligand [7–11], appearing to have a strongly basic nuclear localisation signal (NLS); RKRKRKRKR resembling similar sequences from viral gene products, with intact IFNγ having been shown to undergo nuclear translocation [12,13]. Significantly, the NLS of IFNγ can be replaced with the well-characterised NLS from SV40 large tumor-antigen (T-ag), without any loss of biological activity [9]. NLSs conventionally confer efficient nuclear import through the fact that they are recognised with high affinity by members of the importin (IMP) superfamily of nuclear transport molecules. The IMPS recognize NLS-containing cargoes, and ferry them into the nucleus via a series of specific interactions with components of the nuclear pore, as well as the monomeric guanine nucleotide binding protein Ran. Although there are many NLS-dependent pathways for transport into the nucleus, the best understood is that mediated by the IMPαβ heterodimer, where IMPα within the heterodimer binds the NLS, and IMPβ mediates the interactions with the nuclear pore and Ran to effect translocation into the nucleus [14]. IMP interactions with IFNγ have not been examined, although IFNγ complexed with one of the IFNγ receptor (IFNGR1) subunits is believed to be responsible for localising the STAT1γ transcription factor in the nucleus in IMPαβ-dependent fashion, dependent on the IFNγ NLS [8]. Subsequent to internalization of IFNγ [10], the IFNγ/IFNGR1/STAT1 complex is recruited to the promoters of genes activated by IFNγ, with IFNGR1 itself functioning as a transcription factor with transactivational activity [6].

There are only seven STAT transcription factors that respond to over 60 distinct ligands that use these factors for gene activation [15]; where tested, all of these different receptor systems involve receptor nuclear translocation. Understanding IFNγ nuclear import thus relates integrally to mechanisms of...
IFNγ and STAT nuclear signalling, as well as enabling the subsequent development of mimetics of particular ligands of therapeutic interest such as IFNγ. In this study the interactions of IFNγ with IMPs are examined for the first time, in addition to examining the role of IFNγ nuclear localisation in GAS-dependent gene activation. Importantly, we show that IFNγ is recognised with high affinity by IMPαβ, dependent on its NLS, and that this is central to IFNγ’s nuclear localising ability. Intriguingly, a truncated derivative, the IFNγ mimic peptide 95–132, shows higher affinity of recognition by IMPs, enhanced nuclear localising abilities, and higher gene activation activity, implying that it has all the properties of a potent anti-viral agent.

2. Materials and methods

2.1. Peptide synthesis

Synthesis of peptides using Fmoc chemistry and their purification were performed as described [16]. The peptide sequences are listed in Table 1. Full length IFNγ was from PBL interferon (Piscataway, NJ).

2.2. Cell culture and CLSM analysis

COS-7 African green monkey kidney cells were maintained in DMEM supplemented with 10% FCS in a 5% CO2 atmosphere at 37°C. COS-7 cells, 70–80% confluent were transfected with 1.5 µg of plasmid using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Subcellular localisation of GFP-IFNγ fusion proteins was visualised in living cells 24 h after transfection by CLSM (BioRad MRC600, BioRad Laboratories, Hercules, CA), using a Nikon 40x water immersion lens. The nuclear to cytoplasmic ratio (Fn/c) was determined as previously [17,18] using the Image J 1.35i public domain software (NIH), from single cell measurements for each of the nuclear (Fn) and cytoplasmic (Fc) fluorescence, subsequent to the subtraction of fluorescence due to autofluorescence/background.

2.3. ELISA-based binding assay

An established ELISA-based assay [19–21] was used to determine the binding affinity of IMP subunits to the mIFNγ protein and peptide derivatives thereof. mIFNγ and T-ag 111-135-gal fusion protein were coated onto 96-well microtiter plates at 1 µg/well, with peptides coated at 4 µmol/well. Wells were then incubated with increasing concentrations of IMPα-GST or IMPβ-GST subunits. Bound IMP-GST was detected using an anti-GST primary antibody, an alkaline phosphatase-coupled secondary antibody, and the substrate p-nitrophenyl phosphate [19]. Data were fitted as previously [19,22,23] to determine the apparent dissociation constant (Kd).

2.4. Plasmid constructs for GFP-IFNγ

Plasmid phrGFPII-C encoding GFP from Stratagene (La Jolla, CA), was used to fuse the different IFNγ and IFNγ mimic peptide sequences to the GFP C-terminus. Briefly, A plasmid with complete coding sequence for murine IFNγ was obtained from ATCC (Manassas, VA). The sequence of the complete secreted form of IFNγ was amplified by PCR and introduced downstream of the CMV promoter in the eukaryotic expression vector pShCMV (Stratagene, Jolla, CA). For the mimetic peptide, the sequence of IFNγ amino acids 95–132 was amplified by PCR and introduced similarly into plasmid pShCMV. A constitutively expressed thymidine kinase promoter driven Renilla luciferase gene (pRL-TK) was used as an internal control in all of the reporter plasmid transfections. WISH cells were seeded in 12-well plates at 50% confluency. The next day, 200 ng of GAS promoter-driven firefly luciferase was obtained from Promega (Madison, WI). A sequence containing three copies of the GAS promoter element from human IFN-1 gene, 5′-AGCCGTATTTCCGCAGAATGACGCG-3′, was inserted in the multiple cloning site of pGL3. The plasmid containing the coding sequence for IFNγ was from ATCC (Manassas, VA). The sequence of the complete secreted form of IFNγ was amplified by PCR and introduced downstream of the CMV promoter in the eukaryotic expression vector pShCMV (Stratagene, Jolla, CA). For the mimetic peptide, the sequence of IFNγ amino acids 95–132 was amplified by PCR and introduced similarly into plasmid pShCMV. A constitutively expressed thymidine kinase promoter driven Renilla luciferase gene (pRL-TK) was used as an internal control in all of the reporter plasmid transfections.

Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>mIFNγ mim</td>
<td>AKFVNNPQV QRAFHELIR VHQIIPPESS LLRRKRSSR</td>
</tr>
<tr>
<td>mIFNγ mimNLSm</td>
<td>AKFVNNPQV QRAFHELIR VHQIIPPESS LaaaasSa</td>
</tr>
<tr>
<td>mIFNγ (95-125)</td>
<td>AKFVNNPQV QRAFHELIR VHQIIPPESS LL RRKRSSR</td>
</tr>
<tr>
<td>mIFNγ (121-132)</td>
<td>PESS LRRKRSSR</td>
</tr>
<tr>
<td>mIFNγ (121-132)NLSm</td>
<td>PESS LLRRKasSR</td>
</tr>
</tbody>
</table>

*aAll peptides (single letter amino acid code) are derived from the sequence of mature form of the corresponding mouse IFNγ sequence and numbered accordingly. The NLS region is in bold, with substitutions from wild type in lower case.*

2.5. Reporter gene assays

The plasmid pGL3 promoter, which expresses the firefly luciferase was obtained from Promega (Madison, WI). A sequence containing three copies of the GAS promoter element from human IFN-1 gene, 5′-AGCCGTATTTCCGCAGAATGACGCG-3′, was inserted in the multiple cloning site of pGL3. The plasmid containing the coding sequence for IFNγ was from ATCC (Manassas, VA). The sequence of the complete secreted form of IFNγ was amplified by PCR and introduced downstream of the CMV promoter in the eukaryotic expression vector pShCMV (Stratagene, Jolla, CA). For the mimetic peptide, the sequence of IFNγ amino acids 95–132 was amplified by PCR and introduced similarly into plasmid pShCMV. A constitutively expressed thymidine kinase promoter driven Renilla luciferase gene (pRL-TK) was used as an internal control in all of the reporter plasmid transfections. WISH cells were seeded in 12-well plates at 50% confluency. The next day, 200 ng of GAS promoter-driven firefly luciferase was obtained from Promega (Madison, WI). A sequence containing three copies of the GAS promoter element from human IFN-1 gene, 5′-AGCCGTATTTCCGCAGAATGACGCG-3′, was inserted in the multiple cloning site of pGL3. The plasmid containing the coding sequence for IFNγ was from ATCC (Manassas, VA). The sequence of the complete secreted form of IFNγ was amplified by PCR and introduced downstream of the CMV promoter in the eukaryotic expression vector pShCMV (Stratagene, Jolla, CA). For the mimetic peptide, the sequence of IFNγ amino acids 95–132 was amplified by PCR and introduced similarly into plasmid pShCMV. A constitutively expressed thymidine kinase promoter driven Renilla luciferase gene (pRL-TK) was used as an internal control in all of the reporter plasmid transfections.

3. Results and discussion

3.1. Importin α/β recognizes mIFNγ with high affinity: enhanced binding of the mIFNγ mimic

Although IFNγ is known to localise in the nucleus in many diverse cellular contexts [1,6–13], its interactions with IMPs have not been reported. An established ELISA-based binding assay was used to assess the binding of IMPs to murine IFNγ encoding 126AAAAASA was used. The sequence encoding the IFNγ mimic peptide was generated by PCR using primers covering amino acids 95–132, with a reverse primer encoding 126AAAAASA used to generate the NLS mutant derivative thereof. All PCR products above were fused in frame with GFP at the C-terminus in the plasmid phrGFPH-C, and DNA sequencing performed to confirm the integrity of all constructs.

Table 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Kd (nM)</th>
<th>IMP αβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIFNγ</td>
<td>175 ± 3 (3)</td>
<td>73 ± 17 (2)</td>
</tr>
<tr>
<td>mIFNγ mimic</td>
<td>1.8 ± 0.96 (3)</td>
<td>0.49 ± 0.17 (3)</td>
</tr>
<tr>
<td>mIFNγ (95–125)</td>
<td>216 ± 99 (3)</td>
<td>167 ± 28 (4)</td>
</tr>
<tr>
<td>mIFNγ (121–132)</td>
<td>241 ± 37 (2)</td>
<td>15 ± 3 (3)</td>
</tr>
<tr>
<td>mIFNγ (121–132) NLSmut</td>
<td>583 ± 350 (2)</td>
<td>100 ± 65 (3)</td>
</tr>
<tr>
<td>T-ag 111–135β-gal</td>
<td>146 ± 78 (2)</td>
<td>3 ± 1.3 (3)</td>
</tr>
</tbody>
</table>

*aResults (mean ± S.E.M, with n in parentheses) were derived using an ELISA - based binding assay as described in Section 2, and exemplified in Fig. 1B and C.*

Although IFNγ is known to localise in the nucleus in many diverse cellular contexts [1,6–13], its interactions with IMPs have not been reported. An established ELISA-based binding assay was used to assess the binding of IMPs to murine IFNγ.
Fig. 1. High affinity recognition of IFNγ mimetic peptide compared to IFNγ and the truncated 95–125 derivative by IMP α and the α/β heterodimer. (A) Schematic representation of the IFNγ showing the mimetic peptide region along with other truncations and NLS mutations used in this study. (B) Microtiter plates were coated with mIFNγ (A, left) or a control β-gal fusion protein containing the T-ag 111–135 including the NLS or the indicated mIFNγ peptides and then incubated with increasing amounts of IMPα or α/β complex (see Section 2). Binding curves were fitted for the function \( B = B_{\text{max}} (1 - e^{-Kd/C_0}) \), where the \( K_d \)s represent the concentration of IMPα or α/β complex yielding half-maximal binding. The regression for the curve fits in all cases were >0.98. The results are from a single typical experiment with pooled data shown in Table 1.
and synthetic peptides (see Table 1 for sequences), and results compared to those for the control molecule T-ag 111–135-β-gal which contains the well-characterised IMPα/β recognised NLS of T-ag. Protein/peptides were coated onto microtiter plates and incubated with increasing amounts of IMPα-GST or preformed IMPα/β-GST heterodimer, and binding quantitated subsequent to sequential incubations with antibodies specific to GST and an alkaline-phosphatase-labeled secondary antibody [19,21].

Both mIFNγ and the mIFNγ mimetic peptide were found to be recognised by IMPα/β with high affinity (apparent dissociation constants, $K_d$s, of 72 and 0.5 nM, respectively), implying that IMPα/β is likely to be the mediator of nuclear import of IFNγ and/or protein complexes containing it. The affinity of binding of IMPα for both mIFNγ and the mIFNγ mimetic peptide was about 2–3 times lower than that for the IMPα/β heterodimer. This is consistent with results for other conventional NLS-containing cargoes of IMPα/β such as T-ag [19–21], where IMPα recognizes NLS-containing proteins with much lower affinity in the absence of IMPβ. The mIFNγ 95–125, 121–132 and 121–132 NLSmut peptides showed no significant binding by IMPα, while the binding affinity of the IFNγ mimetic peptide for IMPα/β was >300 times higher than that for mIFNγ 95–125 (Table 2), clearly indicating that the NLS (126RKRKRSR) is critical for IMP binding. The affinity of mIFNγ 121–132 peptide for IMPα/β ($K_d$ of 15 nM) was 30-fold lower than that of the mIFNγ mimetic peptide, but 5–11-fold higher than that of mIFNγ 95–125, 121–132 NLSmut or mIFNγ itself (see Table 2). Overall, the results indicate that IFNγ mimetic peptide shows much higher IMPα/β binding affinity than full length IFNγ, and are consistent with previous findings [7,8,16] that IFNγ contains a functional NLS (see also below). The fact that IFNγ is recognised by IMPα/β in apparently conventional fashion, in contrast to reports of binding of the transcription factor STAT1 to a non-conventional site on IMPα [24], would appear to be consistent with the idea that nuclear translocation of STAT1 is likely to be mediated by IMPα/β interaction with IFNγ, rather than STAT1, with IFNGR1 as the adaptor between the two (see [1,6]). That full length mIFNγ did not bind IMPs as well as the truncated mimetic peptide is probably attributable to conformational properties that hinder access to the NLS in the full protein [7,16].

![Fig. 2. IFNγ or IFNγ mimetic can target GFP to the nucleus dependent on the NLS sequence 126–132. (A) CLSM images are shown for the indicated GFP-IFNγ constructs 21 h post-transfection of Cos-7 cells compared to GFP alone (right). (B) Results (mean ± S.E.M., $n > 43$) of image analysis, performed using the Image J software, for the nuclear to cytoplasmic ratio ($F_{nc}$ representing measurements of nuclear ($F_n$) and cytoplasmic ($F_c$) fluorescence). $p$-Values indicate significant differences between $F_{nc}$ values.](image-url)
3.2. IFNγ mimetic confers nuclear accumulation of GFP dependent on NLS

To correlate the results for IMP binding with in vivo nuclear localisation ability, COS7 cells were transfected to express various IFNγ-GFP-fusion protein constructs and the extent of nuclear accumulation assessed by quantitative CLSM. As evident from Fig. 2, both IFNγ and the IFNγ mimetic could target GFP efficiently to the nucleus, consistent with the idea that IFNγ contains a functional NLS. Interestingly, the IFNγ mimetic peptide conferred a significantly higher (p = 0.0037) level of nuclear accumulation upon GFP than full length IFNγ (Fig. 2A left column; F/DF values of 12.5 and 6.5, respectively – Fig. 2B). Mutations within the NLS had a significant (p < 0.0001) effect, abrogating nuclear accumulation of either GFP fusion protein (Fig. 2A, second column), to levels observed for GFP alone (Fig. 2A far right; F/DF value of c 1.2, Fig. 2B). The clear implication was that the IFNγ NLS is functional for nuclear targeting in intact cells.

3.3. The IFNγ mimetic has higher transactivation activity than the non-secreted IFNγ at the GAS promoter

To test the ability of the various IFNγ-derivatives to activate the IFNγ activated sequence (GAS) promoter, plasmids expressing non-secreted IFNγ or IFNγ mimetic peptide were transfected into WISH cells together with a reporter plasmid expressing firefly luciferase from a GAS promoter (Fig. 3), and a constitutively expressed Renilla luciferase plasmid as an internal control to standardise relative luciferase units. When high amounts of the plasmid were used for transfection, non-secreted IFNγ and IFNγ mimetic caused 3- and 5-fold increases in GAS promoter activity respectively, compared to the empty vector. Similarly, at low amounts of the transfected plasmids, 2- and 3.5-fold increases in GAS promoter activity were observed with non-secreted IFNγ and IFNγ mimetic, respectively. Clearly, IFNγ mimetic peptide was more active at the GAS promoter than non-secreted IFNγ, which is completely consistent with its increased ability to be recognised by Impα/β (Fig. 1) and its superior in vivo activity in targeting GFP to the nucleus (Fig. 2). We have shown previously that non-secreted IFNγ can recapitulate essentially all of the properties of secreted IFNγ in terms of intracellular signalling via the cytoplasmic domain of IFNγ receptor subunit, IFNGR1 [8,25]. Thus, consistent with the higher binding of IFNγ mimetic peptide to IMPs, the IFNγ mimetic is more efficient in activating transcription at the GAS promoter.

4. Conclusion

The results here document for the first time that IFNγ is recognised by the Impα/β heterodimer in conventional fashion, dependent on its NLS (amino acids 126–132), and that this is central to the role of IFNγ in the nucleus in GAS promoter activation. Intriguingly, the truncated mIFNγ mimetic peptide binds to Impα/β with higher affinity, and, as a result, has enhanced nuclear localisation activity, as well as higher GAS promoter activation activity, emphasizing the importance of the nuclear signalling role of IFNγ, thereby resembling that of other polypeptide ligands such as the fibroblast and epidermal growth factors [1–5]. Importantly, in the sequence of events that lead from ligand/receptor interaction culminating in expression of specific genes, it would appear that the efficiency of nuclear import of ligand/receptor may be a critical factor in target gene activation. Since the IFNγ mimetic peptide shows stronger binding to IMPα/β than IFNγ itself, as well as a greater ability to enhance transactivation of IFNγ promoters, it would appear to have all the desirable features to enhance anti-viral responses [26], underlining its potential as an anti-viral therapeutic.

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References


