**Novel properties of the protein kinase CK2-site-regulated nuclear-localization sequence of the interferon-induced nuclear factor IFI 16**

Lyndall J. BRIGGS*, Ricky W. JOHNSTONE†, Rachel M. ELLIOT*, Chong-Yun XIAO*, Michelle DAWSON†, Joseph A. TRAPANI‡ and David A. JANS*  

*Nuclear Signalling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra City, A.C.T. 2601, Australia, and †Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg, Victoria, Australia

Members of the interferon-induced class of nuclear factors possess a putative CcN motif, comparable with that within proteins such as the simian virus 40 large tumour antigen (T-ag), which confers phosphorylation-mediated regulation of nuclear-localization sequence (NLS)-dependent nuclear import. Here we examine the functionality of the interferon-induced factor 16 (IFI 16) CcN motif, demonstrating its ability to target a heterologous protein to the nucleus, and to be phosphorylated specifically by the CcN-motif-phosphorylating protein kinase CK2 (CK2). The IFI 16 NLS, however, has novel properties, conferring ATP-dependent nuclear import completely independent of cytosolic factors, as well as binding to nuclear components. The IFI 16 NLS is not recognized with high affinity by the NLS-binding importin heterodimer, and transport mediated by it is insensitive to non-hydrolysable GTP analogues. The IFI 16 NLS thus mediates nuclear import through a pathway completely distinct from that of conventional NLSs, such as that of T-ag, but intriguingly resembling that of the NLS of the HIV-1 transactivator protein Tat. Since the IFI 16 CK2 site enhances nuclear import through facilitating binding to nuclear components, this represents a novel mechanism by which the site regulates nuclear-protein import, and constitutes a difference between the IFI 16 and Tat NLSs that may be of importance in the immune response.

Key words: myeloid cell, nuclear transport, phosphorylation, protein kinase, transcription factor.

**INTRODUCTION**

Haematopoietic cell maturation is regulated in part by cytokines secreted by bone marrow stroma or other cells acting in a paracrine fashion [1,2]. One such group of cytokines, the interferons (IFNs; the related IFN-α/β, produced by leucocytes and fibroblasts, respectively, and IFN-γ, secreted by activated T-lymphocytes and natural killer cells) have anti-viral and immunomodulatory functions in addition to effects on cell maturation [1,2]. IFN action is mediated through transcriptional control of a variety of target genes, which include a novel family of homologous IFN-inducible nuclear factors described in both humans and mouse, whose expression appears to be associated specifically with myeloid differentiation [3–7]. Human members include IFI 16 (IFN-induced factor 16) [3], myeloid cell nuclear-differentiation antigen (MNDa) [4] and the recently described AIM-2 (absent in melanoma) [6], all of which share domain organization and overall amino acid homology with a cluster of IFN-inducible genes on mouse chromosome 1, the Gene 200 complex, of which IFI 204, IFI 202 and D3 are known to be transcribed [1,7]. A number of studies implicate a nuclear-gene-regulatory role for these proteins in inhibiting transcription through DNA binding [8–10] and/or association with nuclear factors such as the tumour-suppressing retinoblastoma or p53 [11,12] proteins, or activator protein 1 (AP-1) transcription-factor family members [13]. The IFI 16 family of proteins are distinguished by possession of at least one copy of a definitive 200-amino acid motif (see [3]). Where analysed, they have been found to localize strongly in the nucleus and/or nucleolus [1,5,14], but the sequences responsible have not been identified. Of significance in this context is a region of homology shared by IFI 16 with other members of the family (see Figure 1B) in an N-terminal domain distinct from the 200-amino acid motif referred to above [14], which contains a CcN motif, comprising phosphorylation sites for protein kinase CK2 (CK2) and the cyclin-dependent kinase (cdk) cdc2 in the vicinity of a nuclear-localization sequence (NLS). The CcN motif was characterized initially as being responsible for phosphorylation-regulated nuclear localization of the simian virus 40 large tumour antigen (T-ag) [15], and has subsequently been identified in a number of diverse proteins, including the yeast transcription factor SW15 [16], nucleoplasmin [17], phosphatase inhibitor-2 [18] and specific low-molecular-mass isoforms of microtubule-associated protein-2 (MAP2) [19]. Where tested, CK2 phosphorylation has been shown to enhance NLS-dependent nuclear import [17,20,21]; in the case of T-ag, this appears to be through phosphorylation increasing the affinity of recognition by the NLS-recognizing importin heterodimer [20], which mediates the first step of nuclear import through targeting the NLS-containing protein to the nuclear envelope [22,23].

The present study directly examines the functionality of the IFI 16 CcN motif. We find that the IFI 16 NLS has novel properties, with the CK2 site playing a critical role in enhancing nuclear import through facilitating binding to nuclear components. The importin heterodimer does not appear to recognize the IFI 16 NLS with high affinity, and non-hydrolysable GTP analogues do not inhibit IFI 16-NLS-mediated nuclear import. The similarity of these properties of the IFI 16 NLS to those of the HIV-1 transactivator protein Tat nuclear-targeting signal [24] implies that parallel nuclear-import pathways may be of some significance in the acute phase of viral infection and the immune response thereto [3,10,14].

---

**AUTHORSHIP**

1 To whom correspondence should be addressed (e-mail David.Jans@anu.edu.au).
### MATERIALS AND METHODS

#### Chemicals and reagents
Isopropyl β-D-thiogalactoside was from Boehringer Mannheim, and the thiol-labeling reagent 5-iodacetamido-fluorescein (IAF) was from Molecular Probes. Other reagents were from the sources described previously [16,20,21,24].

#### Cell culture
Cells of the HTC rat hepatoma tissue-culture line (a derivative of Morris hepatoma 7288C) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum as described previously [16,20,21].

#### IFI 16 fusion proteins

The T-ag-CcN-β-Gal fusion protein was used as a control contained T-ag amino acids 111–135, including the CcN motif (including CK2 and cyclin-dependent kinase phosphorylation sites and NLS), fused to the N-terminus of β-Gal amino acids 9–1023 [15,26], whereas the N1N2-β-Gal fusion protein contained Xenopus laevis N1N2 amino acids 465–581, including the bipartite NLS (see Figure 1A), fused to the N-terminus of β-Gal amino acids 9–1023 [26].

β-Gal fusion proteins were expressed in E. coli, purified by affinity chromatography and labelled with IAF as described in [16,25,26]. Relatively poor levels of expression of the fusion proteins precluded certain experiments, including analysis of the nuclear-import kinetics of CK2-prephosphorylated proteins (see also [25]). In some experiments, a glutathione S-transferase (GST) fusion protein containing IFI 16 amino acids 1–159 [14], expressed and purified by affinity chromatography as described, was used. The protein was labelled using 5-(4,6-dichloro- triazinyl)aminofluorescein as described in [27]. Protein concentrations were determined using the dye-binding assay of Bradford [28], with BSA as a standard.

#### Nuclear transport
Analysis of nuclear-import kinetics at the single-cell level in micro-injected HTC cells using confocal laser-scanning microscopy (CLSM; Bio-Rad MRC-600) was performed as described previously in detail [15,16,20,21,24–26], where HTC cells were fused with poly(ethylene glycol) about 1 h prior to micro-injection to produce polykaryons. Analysis of nuclear import in vitro using mechanically perforated HTC cells in conjunction with CLSM was performed as described in [15,24,26]; NLS-dependent nuclear-protein import can be reconstituted in this system through the exogenous addition of cytosolic extract (untreated reticulocyte lysate, Promega), an ATP-regenerating system and transport substrate. Nuclear integrity is monitored routinely using a fluorescently labelled 70 kDa dextran (Sigma).

In experiments where the ATP-dependence of transport was tested, apyrase pretreatment was used to hydrolyse endogenous ATP in both cytosol (10 min at room temperature with 800 units/ml) and perforated cells (15 min at 37 °C with 0.2 units/ml) [15,24,29], and transport assays performed in the absence of the ATP-regenerating system. Where the dependence of transport on the GTP-binding protein Ran was tested, cytosolic extract was treated with 850 μM guanosine 5-[γ-thio]triphosphate (GTP[S]; non-hydrolysable GTP analogue) for 5 min at room temperature prior to use in the in vitro assay [24,25]. Image analysis of CLSM files using the Macintosh NIH Image 1.60 public-domain software and curve fitting was performed as described in [15,24–26].

#### Phosphorylation
HeLa cells (5 × 10⁶) were incubated in lysis buffer (0.5% Brij 96/5 mM EDTA/10 mM Tris/HCl, pH 8/140 mM NaCl/2 mM NaF/2 mM Na₃PO₄/2 mM Na₂VO₃) for 15 mins on ice, after

---

A. **Protein**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Bipartite NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMN5</td>
<td>KEKENVYVRSPRSPSFKS55</td>
</tr>
<tr>
<td>NIM2</td>
<td>KEKENVYVRSPRSPSFKS54</td>
</tr>
<tr>
<td>Nucleoplasmin</td>
<td>KPAATAVKAKGQAKKK174</td>
</tr>
</tbody>
</table>

C. **IFI 16 Fusion proteins**

<table>
<thead>
<tr>
<th>IFI16-(127–145)</th>
<th>β-Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFI16-(127–145: Ala132β-Gal)</td>
<td>QAKKKSKRKEKAPGKEKSVSROW145</td>
</tr>
<tr>
<td>IFI16-(127–145: Asp133β-Gal)</td>
<td>QAKKKSKRKEKAPGKEKSVSROW145</td>
</tr>
<tr>
<td>IFI16-(127–138: β-Gal)</td>
<td>QAKKKSKRKEKAPGKEKSVSROW145</td>
</tr>
<tr>
<td>IFI16-(127–138: Ala132β-Gal)</td>
<td>QAKKKSKRKEKAPGKEKSVSROW145</td>
</tr>
</tbody>
</table>

Figure 1 Comparison of the relevant amino acid sequences of selected proteins with bipartite NLSs (see [32]) (A), of the CcN motifs [19,22] of IFI family members (B) and of the IFI 16 sequences within the β-Gal fusion proteins examined in this study (C).

Basic residues are shown in bold and the CK2 sites are underlined, with phosphorylation-site serines highlighted by double underlining. Unlike the other proteins shown in (B), MNDa (myeloid cell nuclear-differentiation antigen), which may have a role distinct from those of the other IFI proteins (see [24]), lacks a consensus CK2 site in the vicinity of its NLS. There are putative CK2 sites S197E and T198E three and six residues distal to the sequences shown for AIM-2 (absent in melanoma) and IFI202, respectively, whereas nucleoplasmin similarly has a five-amino acid spacer to a dual CK2 site S177S178EED. IFI202 is the only IFI family member for AIM-2 (absent in melanoma) and IFI202, respectively, whereas nucleoplasmin similarly has a five-amino acid spacer to a dual CK2 site S177S178EED.
which cell debris was removed by centrifugation. Soluble recombinant β-Gal/IFI 16-β-Gal fusion proteins (20 μg) were mixed with approx. 50 μg of HeLa cell lysate and incubated at 4 °C for 2 h, after which immunoprecipitations were performed by adding 1 μg of monoclonal antibody recognizing β-Gal (Promega) and 20 μl of Protein A–Sepharose beads and incubating at 4 °C for 1 h. The beads were washed five times in ice-cold lysis buffer and once in ice-cold kinase buffer (25 mM Hepes, pH 8/5 mM MnCl₂/5 mM MgCl₂), resuspended in 20 μl of kinase buffer containing 2 μl of kinase buffer containing 2 μl of γ-[³²P]ATP and incubated for 20 min at room temperature. The beads were then washed three times in ice-cold lysis buffer and proteins separated by SDS/PAGE (10% gel). Phosphorylated proteins were visualized by autoradiography and signal intensities quantified using a PhosphorImager [20,25].

For phosphorylation experiments in vitro, 20 μg of β-Gal/IFI 16-β-Gal fusion proteins were mixed with 0.1 μg of CK2, expressed in E. coli as a GST fusion protein (GST-CK2α) and purified as described in [30], in 100 μl of lysis buffer for 2 h at 4 °C. Immunoprecipitations and kinase assays in vitro were then performed as described above.

Importin binding

Binding to IFI 16 fusion proteins by mouse importin subunits [31], expressed as GST fusion proteins in E. coli, was quantified using dot-blot or ELISA-based assays [20,24–26]. A parallel β-Gal ELISA was performed to enable correction for differences in coating efficiency with respect to the latter [20,24–26].

RESULTS

Mutations within the IFI 16 CcN motif

In identical fashion to other members of the IFI gene family (see Figure 1), IFI 16 possesses a CcN motif (within amino acids 127–145) comparable with that of T-ag [15], with the NLS resembling bipartite NLSs from a variety of proteins including nucleoplasmin [32]. To assess functionality, an array of IFI 16-β-Gal fusion proteins was generated as described in Materials and methods section (see Figure 1C). Derivatives were made either lacking the distal arm (amino acids 139–145) of the putative bipartite NLS, or containing mutations within the CK2 site where Ser132 was substituted by either Ala or Asp.

Cytosolic extracts and purified CK2α were initially used to phosphorylate the IFI 16 fusion proteins in vitro, results being compared with those for β-Gal alone (Figure 2). The wild-type IFI16-(127–145)-β-Gal and IFI16-(127–138)-β-Gal fusion proteins were phosphorylated by both cytosol and CK2α, in contrast to β-Gal (Figure 2, top and middle panel). Quantitative results (Figure 2, bottom panel) confirmed these observations for both cytosolic extract and CK2α. The specificity of CK2 phosphorylation for the IFI 16 proteins was indicated by the negligible phosphorylation of Ala132- or Asp132-substituted mutant derivatives (Figure 2, middle and bottom panels). It was concluded that the CK2 site was functional, and able to be phosphorylated in cell extracts.

Nuclear-import kinetics of IFI 16-β-Gal fusion proteins

The IFI 16-β-Gal fusion proteins were assessed for their nuclear-import properties at the single-cell level both in vitro and in vivo in the presence of exogenous cytosol and an ATP-regenerating system (Figure 3 and Table 1) using established experimental systems that have been used to examine signal-dependent nuclear import of a number of nuclear-localizing proteins [15,16,19,24–26,33,34]. All of the IFI16-(127–145)-β-Gal (Lanes 2) and fusion proteins IFI16-(127–138)-β-Gal (lanes 3), IFI16-(127–138; Ala132)-β-Gal (lanes 4), IFI16-(127–145)-β-Gal (lanes 5) and IFI16-(127–145; Asp132)-β-Gal (lanes 6) were mixed with either HeLa cell lysate (top-left panel) or purified recombinant CK2 (top-right panel), immunoprecipitated and in vitro kinase assays performed as described in the Materials and methods section. As a control, HeLa cell lysate containing no β-Gal or IFI 16-β-Gal fusion proteins was included (lanes 1). Molecular-mass standards in kDa are shown on the left, with the positions of the β-Gal/IFI 16-β-Gal fusion proteins indicated on the right. Quantification of the extent of phosphorylation (bottom panel) was performed using a PhosphorImager, with intensities calculated by subtracting the background values for immunoprecipitated β-Gal.
Figure 3  Nuclear uptake of IFI 16-β-Gal fusion protein derivatives in vivo and in vitro
(Top panels) Nuclear-transport kinetics of IFI 16 fusion-protein derivatives as measured using quantitative CLSM in micro-injected HTC cells (top-left panel) and mechanically perforated HTC cells (top-right panel) in the presence of an ATP-regenerating system and exogenous cytosol. Measurements, performed as described in the Materials and methods section [15,16,19–21,24–26], represent the mean of at least two separate experiments, where each point represents the mean of six to ten separate measurements (S.E.M. < 10.8% of the mean) for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence respectively, with autofluorescence subtracted. Data were fitted for the function \( \text{Fn/c}(t) = \text{Fn/c}_{\text{max}} (1 - e^{-kt}) \), where \( t \) is time in min, \( \text{Fn/c}_{\text{max}} \) is the maximal level of nuclear accumulation and \( k \) is the first-order rate constant [16,24–26]; collated data are presented in Table 1. (Bottom panel) Visualization of nuclear uptake of IFI 16 fusion-protein derivatives in vitro, performed as described for the top panels, after 30–35 min incubation at room temperature. Confocal images such as these were used to generate the data shown in the top panels.

β-Gal derivatives accumulated to levels markedly higher than those of β-Gal alone, wild-type IFI16-(127–145)-β-Gal protein being imported to the highest extent with half-maximal transport at about 1.5 and 9 min in vivo and in vitro, respectively (Figure 3, top panels; Table 1). Results for IFI16-(127–145)-β-Gal were comparable with those for the fusion protein IFI16-(1–159)-GST (Table 1, and results not shown), the latter's faster accumulation rate in vitro probably attributable to its smaller size (≈ 90 kDa) compared with the β-Gal fusion proteins (≈ 476 kDa).

IFI16-(127–138)-β-Gal showed nuclear exclusion (\( \text{Fn/c}_{\text{max}} < 0.9 \); where \( \text{Fn/c}_{\text{max}} \) is the maximal level of nuclear accumulation) both in vivo and in vitro (Figure 3 and Table 1), comparable with that of β-Gal itself (\( \text{Fn/c}_{\text{max}} < 0.7 \) [19], indicating that amino acids 139–145 of the NLS are required for nuclear entry/accumulation, and implying the bipartite nature of the IFI 16 NLS. Transport of IFI16-(127–145)-β-Gal appeared to be enhanced by the CK2 site in the presence of ATP and cytosol, since replacement of Ser\(^{137} \) by the non-phosphorylatable residues Ala or Asp reduced nuclear accumulation (Figure 3 and Table 1). Analysis of CK2-prephosphorylated proteins was not possible due to technical problems (see the Materials and methods section). It was concluded that IFI 16 amino acids 127–145 are sufficient to target a large heterologous protein to the nucleus, with full activity requiring both the intact bipartite NLS and a functional CK2 site.

Dependence of IFI 16 nuclear import on ATP but not cytosolic factors
Conventional NLS-mediated nuclear-protein import in vitro is dependent on energy in the form of ATP and exogenous cytosol (see [29,34–37]), the latter supplying the NLS-recognizing
Table 1  Nuclear-import kinetics of IFI 16-β-Gal fusion proteins compared with those of a T-ag fusion protein and control molecules

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>In vivo</th>
<th>In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fn/cmax</td>
<td>f1/2 (min)</td>
</tr>
<tr>
<td>IFI16-(127–145)-β-Gal</td>
<td>1.2 ± 0.3</td>
<td>1.5 ± 1.0 (6)</td>
</tr>
<tr>
<td>ATP</td>
<td>0.8 ± 0.1</td>
<td>ND (3)</td>
</tr>
<tr>
<td>Cytosol</td>
<td>3.3 ± 0.4</td>
<td>12.4 ± 2.9 (2)</td>
</tr>
<tr>
<td>IFI16-(127–145: Asp132)-β-Gal</td>
<td>0.6 ± 0.1</td>
<td>ND (2)</td>
</tr>
<tr>
<td>ATP</td>
<td>0.6 ± 0.1</td>
<td>ND (1)</td>
</tr>
<tr>
<td>Cytosol</td>
<td>3.1 ± 0.5</td>
<td>29.7 ± 3.0 (3)</td>
</tr>
<tr>
<td>IFI16-(127–138)-β-Gal</td>
<td>0.5 ± 0.1</td>
<td>ND (4)</td>
</tr>
<tr>
<td>IFI16-(1–159)-GST</td>
<td>1.2 ± 0.1</td>
<td>2.4 ± 0.5 (3)</td>
</tr>
<tr>
<td>β-Gal</td>
<td>0.4 ± 0.0</td>
<td>ND (2)</td>
</tr>
<tr>
<td>T-ag-CcN-β-Gal</td>
<td>5.9 ± 0.3</td>
<td>4.9 ± 0.8 (2)</td>
</tr>
<tr>
<td>ATP</td>
<td>0.8 ± 0.1</td>
<td>ND (2)</td>
</tr>
<tr>
<td>Cytosol + GTP[S]</td>
<td>2.2 ± 0.4</td>
<td>ND (1)</td>
</tr>
<tr>
<td>70 kDa Dextran</td>
<td>0.2 ± 0.0</td>
<td>ND (3)</td>
</tr>
</tbody>
</table>

Figure 4  Nuclear uptake of IFI 16-β-Gal fusion-protein derivatives in vitro is independent of cytosolic factors

Nuclear-transport kinetics were determined as described in Figure 3 for mechanically perforated HTC cells in the absence or presence of ATP or exogenous cytosol, or in the presence of cytosol/ATP/GTP[S] (GTP[S]), as indicated. Measurements represent a single typical experiment, from a series of at least two separate experiments, where each point represents the mean of five to eight separate measurements for each of Fn and Fc respectively, with autofluorescence subtracted. Collated data are presented in Table 1.
accumulation conferred by the IFI 16 CcN motif uniquely resembled the properties conferred by the novel nuclear-targeting signal of HIV-1 Tat [24], and was in stark contrast to the conventional NLS-containing proteins (see above) analysed using this and other experimental systems (see above and [19,24–26,34–37]). Further, the non-hydrolysable GTP analogue GTP[βS] had no significant inhibitory effect on nuclear import (Figure 4, left-hand panel), implying that transport, in contrast to that mediated by the T-ag NLS, was Ran-independent (Table 1).

Dependence of nuclear accumulation on ATP and cytosolic factors was examined for the CK2-site IFI 16-(127–145) variants (Figure 4, right-hand panel; Table 1), indicating results similar to those for wild-type IFI 16-(127–145); nuclear import was severely reduced in the absence of ATP, exhibiting nuclear exclusion (Fn/c < 1.0). Strikingly, in the presence of ATP, nuclear import was enhanced in the absence compared with in the presence of cytosol, the Ala and Asp variants of IFi16-(127–145)-β-Gal showing 70 and 55% increased maximal accumulation in the absence of cytosol, compared with in its presence (Table 1; compare Figure 4, right-hand panel with Figure 3, top-right panel). These results suggested that a functional CK2 site is required to overcome the inhibition of transport by exogenous cytosol, which could be mediated either through competition for the nuclear-import machinery, or a cytoplasmic retention mechanism (see the Discussion). In the presence of ATP but the absence of cytosol, IFi16-(127–145)-β-Gal and IFi16-(127–145): Asp132β-Gal achieved maximal accumulation in the nucleus at rates significantly faster (P < 0.0001 and P < 0.02, respectively) than that of IFi16-(127–145): Ala132β-Gal, implying that the CK2 site, in addition to the maximal level of nuclear accumulation, may modulate the nuclear-import rate of IFI 16. That the Asp132 derivative showed faster nuclear-import kinetics than the Ala132 derivative implied that the negatively charged residue approximates phosphoserine in functional terms, at least to some extent [15,21,25].

The IFI 16 CcN motif confers intranuclear binding

The detergent CHAPS can be used to perforate the nuclear envelope to enable molecules to diffuse freely between cytoplasm and nucleoplasm, so that in its presence, nuclear accumulation can only occur through binding to nuclear components [24,33,34]. Under these conditions, T-ag-CcN-β-Gal did not accumulate, instead equilibrating between the nuclear and cytoplasmic compartments (see Figure 5), in a fashion similar to other conventional NLS-containing proteins that we have examined [24,25,34]. Both IFi16-(127–145)-β-Gal and IFi16-(127–138)-β-Gal exhibited nuclear accumulation in the presence of CHAPS (Figure 5). Interestingly and intriguingly, despite the lack of a barrier to diffusion in the presence of CHAPS, the absence of ATP resulted in a lack of nuclear accumulation in all cases (Figure 5, top-right and bottom panels). Thus these results for the IFI 16 NLS resemble those for the novel Tat nuclear-targeting signal (GRKKRQRRRAPR) [24], which has a strict requirement for ATP hydrolysis for binding to nuclear components. The presence of cytosol reduced the level of accumulation of IFi16-(127–145)-β-Gal only slightly (Figure 5, top-left panel); this level of accumulation (Fn/c 2.2) is comparable with that conferred by Tat in vivo [24], indicative of its significance. IFi16-(127–145)-β-Gal accumulated more efficiently than IFi16-(127–138)-β-Gal, but the difference between the two was not significant (Figure 5, top-left panel), suggesting that IFI 16 amino acids 127–138 were sufficient to confer binding to nuclear components. By comparison, the CK2-site-mutated IFI 16 proteins showed reduced nuclear accumulation, implying that the CK2 site is required for binding in the nucleus, with IFi16-(127–145): Asp132β-Gal most severely affected. This role of the CK2 site in mediating nuclear binding was supported by measurement of nuclear accumulation of IFi16-(127–138): Ala132β-Gal in the presence of CHAPS. In the presence of ATP and the absence of cytosol, only equilibration between nucleus and cytoplasm was observed (Fn/c ≈ 1.0), which contrasted strongly with the strong nuclear accumulation on the part of IFi16-(127–138):β-Gal (Fn/c ≈ 2.5; Figure 5, top panels). The CK2-site Ser132 was thus concluded to be essential for intranuclear binding.

Recognition of the IFI 16 NLS by the importin heterodimer

We used dot-blot and ELISA-based binding assays, employed previously to characterize importin binding on the part of a...
the concentration of importin 58/97 and Gal fusion proteins, as well as importin 58 to determine the binding affinity of the IFI 16 NLS for the mouse number of different NLS-containing proteins [20,24–26,33,34], the raw data were B Materials and methods section [20,24–26]. Curves were fitted for the function plates and hybridized with increasing amounts of importin 58/97-GST as described in the alkaline phosphatase-conjugated second antibody and Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate [20]. (Bottom panel) Fusion proteins were coated on to microtitre-based (bottom panel) approaches

![Graph](image)

Figure 6 Recognition of IFI 16-β-Gal fusion-protein derivatives by the mouse importin 58/97 heterodimer using dot-blot (top panel) and ELISA-based (bottom panel) approaches

(Top panel) IFI 16 fusion proteins (16 pmol), together with conventional NLS-containing T-ag and N1N2-β-Gal fusion proteins and β-Gal itself as controls, were blotted on to nitrocellulose, and then incubated with 150 nM importin 58/97-GST, prior to staining with anti-GST antibody, alkaline phosphatase-conjugated second antibody and Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate [20]. (Bottom panel) Fusion proteins were coated on to microtitre plates and hybridized with increasing amounts of importin 58/97-GST as described in the Methods and methods section [20,24–26]. Curves were fitted for the function $B(x) = B_{max}(1 - e^{-kx})$, where $B$ is the extent of binding in mOD/min per pmol of protein coated, $x$ is the concentration of importin 58/97 and $k$ is the concentration at which binding is half-maximal. The $K_v$ values are the concentrations required for half-maximal binding [28,37]. The S.E.M. for the raw data were < 10.1% of the mean.

number of different NLS-containing proteins [20,24–26,33,34], to determine the binding affinity of the IFI 16 NLS for the mouse importin 58/97 heterodimer using the IFI 16 fusion-protein derivatives (Figure 6). Results were contrasted with those for the conventional NLS-containing T-ag-CcN-β-Gal and N1N2-β-Gal fusion proteins, as well as β-Gal itself (Figure 6). Both T-ag-

Figure 6 Recognition of IFI 16-β-Gal fusion-protein derivatives by the mouse importin 58/97 heterodimer using dot-blot (top panel) and ELISA-based (bottom panel) approaches

Ccn-β-Gal and the bipartite NLS-containing N1N2-β-Gal were recognized strongly in the dot-blot assay, whereas the IFI16-(127–138)-β-Gal proteins showed poor binding, similar to that of β-Gal (Figure 6, top panel); the IFI16-(127–145)-β-Gal derivatives exhibited slightly better binding.

IFI16-(127–145)-β-Gal was also tested using the ELISA assay, exhibiting much lower binding (70%, reduced) compared with that for T-ag-CcN-β-Gal (Figure 6, bottom panel). The apparent dissociation constant ($K_v$) for IFI16-(127–145)-β-Gal was 46.2 ± 4.5 nM, ≈ 15-fold higher than that for T-ag-CcN-β-Gal (3.0 ± 0.3 nM, see Figure 6, bottom panel). Clearly, the bipartite NLS of IFI 16 was able to be recognized by the importin heterodimer, but with greatly reduced efficiency compared with the NLSs in T-ag or N1N2 ($K_v$ ≈ 5 nM [26,34]).

DISCUSSION

This study represents the first examination of the sequences responsible for nuclear localization of IFI nuclear factor family members. We demonstrate that IFI 16 amino acids 127–145, the CcN motif, are sufficient to target the 476 kDa protein β-Gal to the nucleus, our quantitative analysis indicating a number of novel properties. Although ostensibly resembling the conventional bipartite NLSs of nucleoplasmin and N1N2 in terms of sequence (see Figure 1), the IFI 16 NLS does not appear to be recognized with high affinity by the importin heterodimer in contrast to these proteins [26,38,39], and also does not require exogenous cytosol to mediate nuclear import in vitro. Consistent with this, a non-hydrolysable GTP analogue does not inhibit import, implying Ran-independence. Further, the NLS is able to mediate binding to nuclear components, and has a strict ATP-dependence for nuclear import in the presence and absence of an intact nuclear envelope. These properties, summarized in Table 2, clearly differ from those of conventional NLSs [15,19–26,33,34,36–39], implying that the IFI 16 NLS mediates a novel nuclear-import pathway. Intriguingly, however, the distinctive characteristics of IFI 16-mediated nuclear import concur with those of the HIV-1 Tat nuclear-targeting signal [24], although there are differences primarily in the regulation of IFI 16 NLS-mediated nuclear accumulation by the CK2 site, which appears to be required both for maximal accumulation in the presence of an intact nuclear envelope, and for binding within the nucleus (see Table 2). Whereas the Tat nuclear-targeting signal appears to be regulated by a novel cytoplasmic retention mechanism controlled by ATP hydrolysis [24], which may also hold true for the IFI 16 NLS, the latter shows reduced nuclear import in the presence of exogenous cytosol (Figure 4), which appears to be abolished in the presence of a functional CK2 site.

One hypothesis to explain this observation is that CK2 phosphorylation is important for release from association with a cytoplasmic retention factor; alternatively, IFI 16 requires an intact CK2 site to compete effectively with factors present in cytosol for components of the nuclear import machinery. That the latter are unlikely to be importins is indicated by the relatively poor binding of the latter to the wild-type IFI 16 NLS (Figure 6, and results not shown).

This study shows for the first time that IFI 16 nuclear accumulation is enhanced by CK2. While the CK2 site is important for entry into intact nuclei in the presence of cytosol (see above), it also appears to play a role in binding within the nucleus. Hence, although the IFI 16 CK2 site enhances nuclear import, it would appear to do so through a novel mechanism distinct from that of the T-ag CcN motif where the CK2 site directly modulates the affinity of recognition by importin (see
Table 2 Summary of the nuclear-import properties conferred by the IFI 16 NLS compared with conventional NLSs and the HIV-1 Tat NLS

| Protein                | NLS Conferring Pathway | Cytoplasmic nuclear-import receptor interaction | Dependence on ATP | Intranuclear binding | ATP requirement for intranuclear binding
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional NLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-ag [20,26]</td>
<td>GRKKRRQRRR</td>
<td>PKKKRKV</td>
<td>Yes*</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Retinoblastoma [34]</td>
<td></td>
<td>KKLR</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-conventional NLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFI 16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1 Tat [24]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- The ATP requirement for conventional NLS-mediated nuclear import is not consistent (see [23,24–37]).
- ATP hydrolysis appears to be required for release from cytoplasmic retention [24], with CK2 phosphorylation also being involved in the case of IFI 16 (see Discussion).
- * The ATP requirement for conventional NLS-mediated nuclear import is contentious (see [23,24,29,34–37]).

That the nuclear-import characteristics of the IFI 16 CcN motif concur with those of the HIV-1 Tat nuclear-targeting signal raises interesting questions with respect to parallel pathways for the nuclear targeting of proteins involved in viral infection/mounting the immune response, and in particular how this may be differentially regulated. In short, the IFI 16 NLS-conferred nuclear-import pathway appears to be essentially comparable with that of a viral gene product expressed during infection in response to which IFI 16 expression is triggered. The CK2 site and its regulation of both nuclear entry and intranuclear binding of IFI 16 may well constitute a physiologically important difference in this context. The extent to which the observations here with respect to IFI 16 nuclear localization are central to T-cell-dependent immune responses [1,3,10,14] is the focus of future work in this laboratory.

REFERENCES


© 2001 Biochemical Society


16-17 Paine, P. L. (1999) Importin b recognizes parathyroid hormone-related protein (PTHrP) with high affinity and mediates nuclear import of the human retinoblastoma protein bipartite nuclear localization sequence with high affinity and mediates nuclear import independent of importin b, J. Biol. Chem., 274, 22610–22617


Received 19 June 2000/5 September 2000; accepted 16 September 2000