Efficiency of Importin α/β-Mediated Nuclear Localization Sequence Recognition and Nuclear Import

Differential Role of NTF2*

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Little quantitative, kinetic information is available with respect to the process of nuclear import of conventional nuclear localization sequence (NLS)-containing proteins, which initially involves recognition and docking at the nuclear pore by importin α/β. This study compares the binding and nuclear import properties of mouse (m) and yeast (y) importin (IMP) subunits with respect to the NLSs from the SV40 large tumor antigen (T-ag), and the Xenopus laevis phosphoprotein N1N2. m- and y-IMPα recognized both NLSs, with y-IMPα exhibiting higher affinity. m-IMPβ greatly enhanced the binding of m-IMPα to the T-ag and N1N2 NLSs, but y-IMPβ did not significantly affect the affinity of y-IMPα for the T-ag NLS. In contrast, y-IMPβ enhanced y-IMPα binding to the NLS of N1N2, but to a lesser extent than the enhancement of m-IMPα binding by m-IMPβ. NLS-dependent nuclear import was reconstituted in vitro using the different importin subunits together with the transport factors Ran and NTF2. Whereas T-ag NLS-mediated nuclear import did not exhibit an absolute requirement for NTF2, N1N2 NLS-mediated transport strictly required NTF2. High levels of NTF2 inhibited nuclear accumulation conferred by both NLSs. We conclude that different NLSs possess distinct nuclear import properties due to differences in recognition by importin and requirements for NTF2.

The entry of karyophilic proteins into the nucleus through the nuclear pore complex (NPC)† is effected by specific targeting signals called nuclear localization sequences (NLSs) (1, 2), and is a receptor-mediated (3, 4), energy-dependent (5, 6) process. The key factors involved are members of the NLS-binding importin/karyopherin family (7–11), and the monomeric GTPase Ran/TC4 (12, 13) and auxiliary proteins such as NTF2/ p10 (14, 15). In the first step, the NLS-containing protein is recognized by the importin heterodimer through the NLS-binding importin α subunit (3, 7, 9), and then targeted to the NPC through the affinity of the importin β subunit (8, 10, 11, 16) for NPC components (17, 18). In the second step requiring cytoplasmic RanGDP (19, 20), the complex is translocated through the NPC (21) and importin α and the NLS-containing protein released into the nucleus through the action of RanGTP (19). Alternative signal-mediated nuclear import pathways independent of the importin α/β heterodimer have recently been identified where either importin β (22, 23) or importin β-related homologs (24–26) appear to fulfil the role of both importin α and β in binding NLSs and targeting them to the NPC (24, 25, 27).

Although NLS receptors from different species share structural and functional homology, the recognition of different types of NLS by importin subunits and its importance in the nuclear import process has not been adequately investigated in quantitative terms (28, 29). In this study, we compare the NLS binding and transport properties of mouse and yeast importin subunits. We find that although mouse (m-IMPα) and yeast (y-IMPα) importin α can bind different types of NLS, y-IMPα binds with higher affinity. y-IMPβ does not significantly affect the affinity of y-IMPα for the NLS of the simian virus SV40 large tumor antigen (T-ag) but does enhance binding to the NLS of the Xenopus laevis phosphoprotein N1N2. In contrast, m-IMPα requires m-IMPβ for high affinity binding. Nuclear import of a T-ag-NLS containing fusion protein in vitro was found to be enhanced by NTF2 but did not require it absolutely, whereas import of an N1N2 NLS-containing fusion protein had an absolute requirement for NTF2. Surprisingly, high NTF2 concentrations inhibited nuclear import in all cases. The results indicate that different types of NLS have different requirements with respect to the factors mediating nuclear import, and imply a differential, regulatory role for NTF2 in nuclear transport.

MATERIALS AND METHODS

Fusion Proteins—The amino acid sequences of the NLSs used are shown in Table I. Constructs to express fusion proteins T-ag-Gal and the NLS-defective mutant derivative T-ag-Cc-Gal containing T-ag sequences fused amino-terminal to the Escherichia coli β-galactosidase sequence (amino acids 9–1023) have been described (30, 31). The plasmid pPR-N1N2-β-Gal expressing Xenopus laevis N1N2 amino acids 465–581 fused amino-terminal to β-galactosidase has been described (32). Plasmids encoding the N1N2 NLS-mutated derivatives N1N2A-β-Gal and N1N2β-β-Gal were derived through oligonucleotide site-directed mutagenesis (U.S.E. mutagenesis kit, Amersham Pharmacia Biotech) of plasmid pPR2-N1N2-β-Gal. N1N2A-β-Gal and N1N2β-β-Gal possess Asn-Asn-Ser in place of Lys-Lys-Arg537 and Leu-Gln-Asn in place of the Ala-Lys-Lys357, respectively (see Table I).

β-galactosidase fusion proteins were expressed, purified, and labeled with 5-iodoacetamidofluorescein as previously (30, 31). m-IMPα (PTAC58), m-IMPβ (PTAC37), y-IMPα (Kap60), y-IMPβ (Kap95) subunits, and human Ran were expressed as glutathione S-transferase (GST) fusion proteins and purified as described (9, 21, 28, 34). GST-free y-IMPβ, m-IMPβ, and Ran were prepared by thrombin cleavage (21, 22, 28, 34, 35). Ran was loaded with GDP as described (36). Recombinant human NTF2 was expressed and purified using S100 HR column chro-
Fusion proteins contain the indicated amino acid sequences fused amino-terminal to E. coli β-galactosidase (amino acid 9—1023) (see “Materials and Methods” and Refs. 30—32). The single letter amino acid code is used with small letters indicating variation from the wild type sequences (33).

**RESULTS**

**y-IMPα Has Higher NLS Binding Affinity Than m-IMPα**—We have used an ELISA-based assay to determine the binding affinities of mouse importin subunits for different NLSs (28, 34, 40), with the results revealing relatively low NLS-binding affinity for m-IMPα compared with that for the m-IMPαβ heterodimer (28, 34). We set out to compare the NLS binding affinity of m-IMPα, y-IMPα, m-IMPαβ, and y-IMPαβ for proteins containing either the T-ag NLS, or the bipartite NLS of the X. laevis nuclear factor N1N2 (see Table I). y-IMPα bound these fusion proteins with significantly (p < 0.05) higher affinity (about 2-fold) than m-IMPα (Figs. 1 and 2; see Table II), the latter only attaining high affinity in the presence of m-IMPαβ. The specificity of binding in all cases was indicated by the fact that binding to NLS mutant derivatives was severely reduced (Table II). The T-ag NLS mutant showed negligible binding affinity for IMPαβ from either mouse or yeast, whereas the two N1N2-NLS mutant derivatives showed different importin binding affinity (Fig. 2). N1N2A-β-Gal, mutated in the proximal arm of the bipartite NLS, showed apparent dissociation constants (Kd values) of 48 and 35 nM for y-IMPαβ and m-IMPαβ, respectively, whereas N1N2A-β-Gal, mutated in the distal arm, exhibited negligible binding. Clearly, mouse and yeast IMPαβ are able to bind both types of NLS specifically, with y-IMPα binding with higher affinity than m-IMPα (Figs. 1 and 2; Table II).
Table II
Binding parameters of NLS-containing β-galactosidase fusion proteins for yeast and mouse importins as measured using an ELISA-based assay

Sequence details are presented in Table I. Data represent the mean ± S.E. (number in parentheses) for the apparent dissociation constant ($K_d$) determined as outlined under "Materials and Methods" (28, 34).

| Fusion proteins | y-IMPα (A) | y-IMPα/β (B) | y-IMPαβ (C) | A/B | m-IMPα (D) | m-IMPα/β (E) | m-IMPβ (F) | D/E |
|-----------------|------------|---------------|-------------|-----|------------|---------------|------------|-----|
| T-ag-CeN-β-Gal  | $2.0 \pm 0.12 (5)$ | $2.1 \pm 0.3 (4)$ | $2.2 \pm 0.3 (2)$ | 0.9 | $5.5 \pm 0.4 (2)$ | $2.7 \pm 0.1 (2)$ | LB (2) | 2.1 |
| T-ag-Ce-β-Gal   | LB (1)     | LB (1)        | LB (2)      | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) |
| N1N2-β-Gal      | $12.8 \pm 4.3 (3)$ | $5.7 \pm 1.0 (3)$ | $2.2 \pm 0.3 (2)$ | 0.7 | $22.3 \pm 2.2 (3)$ | $2.5 \pm 0.5 (3)$ | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) |
| N1N2A-β-Gal     | LB (1)     | LB (1)        | LB (1)      | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) |
| N1N2B-β-Gal     | $35.2 \pm 3.3 (1)^d$ | $47.8 \pm 5.5 (1)^d$ | $29.4 \pm 2.3 (2)$ | LB (1) | LB (2) | LB (2) | LB (2) | LB (2) | LB (2) |

$^a$ Significant differences (Student’s t-test) ($p < 0.05$) were observed between $K_d$ values for y-IMPα compared to m-IMPα (columns A and D, $p = 0.0009$), and m-IMPα in the absence or presence of m-IMPβ (columns D and E, $p = 0.0181$).

$^b$ LB, low binding; $K_d$ value not able to be determined.

$^c$ Significant differences were observed between $K_d$ values for y-IMPα in the absence or presence of y-IMPβ (columns A and B, $p = 0.0218$), for y-IMPα compared with m-IMPα (columns A and D, $p = 0.0984$), and for m-IMPα in the absence or presence of m-IMPβ (columns D and E, $p = 0.0297$).

$^d$ S.E. from curve fit.
Interestingly, the affinity of γ-IMPα after precomplexation to γ-IMPβ for the T-ag NLS was not significantly different from that in its absence (Fig. 1 and Table II), but was enhanced 2-fold ($p < 0.02$) in the case of the N1N2 NLS (Fig. 2; Table II). This was in contrast to the significantly larger effect of m-IMPβ on NLS recognition by m-IMPα ($p < 0.03$), where the presence of m-IMPβ increased the binding affinity 2- and 4-fold for the T-ag and N1N2 NLSs respectively (Table II). Clearly, γ-IMP α is a higher affinity NLS receptor than m-IMPα, which requires m-IMPβ to achieve high affinity. That γ-IMPβ increased the binding affinity of m-IMPα for the bipartite N1N2 NLS but not for the T-ag NLS implies that different NLSs have different requirements to achieve high affinity recognition by importin.

**Nuclear Import Conferred by the T-ag NLS Does not Require NTF2**—The ability of m-IMPα/β and γ-IMPα/β to mediate nuclear import of a T-ag NLS-containing transport substrate independent of importin subunits (Fig. 3, A–C and Table III). This was in contrast to the combination of m-IMPα/β where maximal nuclear accumulation relative to that in the cytoplasm ($F_{n,max}$) was about 5-fold, half-maximal levels ($t_{1/2}$) being achieved within 5.3 min. In the presence of NTF2, the nuclear import rate was enhanced significantly ($t_{1/2} = 2.6$ min; $p < 0.05$), whereas $F_{n,max}$ remained unaffected ($F_{n,max}$ of 5.0; see Table III).

In the absence of exogenously added NTF2, γ-IMPα/β mediated nuclear import to levels ($F_{n,max}$ of 5.8; $t_{1/2} = 4.9$ min) only slightly higher than m-IMPα/β, while in the presence of NTF2, both the import rate and the level of accumulation ($F_{n,max}$ of 9.4; $t_{1/2} = 1.8$ min) were increased further ($p < 0.05$; see Table III). The specificity of transport in all cases was demonstrated by the fact that the T-ag-Ccβ-Gal protein, containing a nonfunctional NLS, did not accumulate to any significant extent (Fig. 3, A and C, and Table III). The results thus demonstrate that both m-IMPα/β and γ-IMPα/β can mediate nuclear import of an T-ag NLS-containing transport substrate independent of importin.
**Differential Role of NTF2 in Nuclear Import**

Results represent the mean ± S.E. from data (see Figs. 3C and 4C) fitted to the function \( Fnlc(t) = Fnlc_{\text{max}}*(1 - e^{-kt}) \), where \( Fnlc_{\text{max}} \) is the maximal level of nuclear accumulation, \( k \) is the rate constant, and \( t \) is time in minutes. NTF2 was used at the optimal concentration for nuclear accumulation (see Fig. 5).

### Table III

| Substrate | Addition | \( Fnlc_{\text{max}} \) | \( t_{1/2} \) | \( n \) | Addition | \( Fnlc_{\text{max}} \) | \( t_{1/2} \) | \( n \) |
|-----------|----------|-----------------|----------|------|----------|-----------------|----------|------|
| T-ag-CeN-β-Gal | y-IMPα/β + Ran + NTF2 | 9.4 ± 0.8 | 1.8 ± 0.5 | 2 | m-IMPαβ + Ran + NTF2 | 5.0 ± 0.9 | 2.6 ± 1.1 | 3 |
| y-IMPα + Ran | 5.8 ± 0.1 | 4.9 ± 0.2 | 2 | m-IMPαβ + Ran | 4.8 ± 1.2 | 6.3 ± 1.0 | 3 |
| y-IMPα + NTF2 | 1.7 ± 0.9 | 2.6 ± 1.8 | 2 | m-IMPαβ | 1.0 ± 0.04<sup>a</sup> | 2.1 ± 0.4 | 1 |
| Ran + NTF2 | 0.9 ± 0.2 | 1.1 ± 0.1 | 2 | m-IMPαβ | 0.8 ± 0.06 | LA<sup>b</sup> | 5 |
| T-ag-CeN-β-Gal | y-IMPα/β + Ran + NTF2 | 0.6 ± 0.1<sup>b</sup> | 7.1 ± 0.1<sup>b</sup> | 1 | m-IMPαβ + Ran + NTF2 | 1.4 ± 0.1<sup>b</sup> | 55 ± 0.3<sup>b</sup> | 1 |
| N1N2-β-Gal<sup>c</sup> | y-IMPα/β + Ran + NTF2 | 4.2 ± 0.3 | 4.1 ± 0.5 | 2 | m-IMPαβ + Ran + NTF2 | 3.3 ± 0.5 | 7.8 ± 0.8 | 2 |
| y-IMPα + Ran | 1.5 ± 0.3 | 6.2 ± 1.4 | 2 | m-IMPαβ + Ran | 1.2 ± 0.1 | 2.5 ± 0.1 | 2 |
| Ran + NTF2 | 1.4 ± 0.1 | 3.7 ± 0.6 | 2 | m-IMPαβ + NTF2 | 1.6 ± 0.2<sup>c</sup> | 7.2 ± 0.03<sup>c</sup> | 1 |
| N1N2A-β-Gal | y-IMPα/β + Ran + NTF2 | 1.0 ± 0.1 | 7.3 ± 2.1 | 2 | m-IMPαβ + Ran + NTF2 | 0.9 ± 0.2 | 4.8 ± 1.4 | 2 |
| y-IMPα + Ran | 1.0 ± 0.3 | 9.0 ± 3.2 | 2 | m-IMPαβ + Ran | 1.0 ± 0.1 | 32 ± 2.7 | 2 |
| none | 1.1 ± 0.1 | 4.8 ± 1.1 | 2 | m-IMPαβ + Ran + NTF2 | 2.6 ± 0.04 | 8.4 ± 5.7 | 2 |
| N1N2B-β-Gal | y-IMPα/β + Ran + NTF2 | 2.8 ± 0.1 | 13.9 ± 1.6 | 2 | m-IMPαβ + Ran + NTF2 | 2.6 ± 0.04 | 8.4 ± 5.7 | 2 |
| y-IMPα + Ran | 1.6 ± 0.3 | 7.3 ± 2.1 | 2 | m-IMPαβ + Ran | 1.5 ± 0.1 | 6.4 ± 0.3 | 2 |
| none | 1.2 ± 0.2<sup>b</sup> | 10 ± 0.02<sup>b</sup> | 1 | m-IMPαβ + Ran + NTF2 | 2.6 ± 0.04 | 8.4 ± 5.7 | 2 |

<sup>a</sup> Significant differences (\( p < 0.05 \)) were observed with or without NTF2 for y-IMPα/β + Ran between \( Fnlc_{\text{max}} \) (\( p = 0.0251 \)) and \( t_{1/2} \) values (\( p = 0.0490 \)), and for m-IMPα/β + Ran between \( t_{1/2} \) (\( p = 0.0474 \)) values, as well as for \( Fnlc_{\text{max}} \) for transport in the presence of Ran and NTF2 mediated by y-IMPα/β and m-IMPα/β (\( p = 0.0344 \)).

<sup>b</sup> S.E. is from curve fit.

<sup>c</sup> LA, low accumulation; \( t_{1/2} \) unable to be determined.

<sup>d</sup> Significant differences were observed with or without NTF2 for y-IMPα/β + Ran between \( Fnlc_{\text{max}} \) values (\( p = 0.0183 \)), and for m-IMPα/β + Ran between \( Fnlc_{\text{max}} \) (\( p = 0.0470 \)) and \( t_{1/2} \) (\( p = 0.0310 \)) values.

Nuclear Import Conferred by the N1N2 NLS Requires NTF2—Nuclear import kinetic measurements were also performed using the bipartite NLS-containing substrate N1N2-β-Gal (Fig. 4 and Table III). As for T-ag-CeN-β-Gal, nuclear accumulation was negligible in the absence of RanGDP or m-IMPαβ. In the presence of m-IMPαβ and RanGDP but in the absence of NTF2, a lack of nuclear accumulation was also evident, although nuclear accumulation was clearly observed in the presence of NTF2, with an \( Fnlc_{\text{max}} \) of 3.3 and \( t_{1/2} \) of 7.8 min.

Similar results were observed for y-IMPα/β in the presence of Ran-GDP, no nuclear accumulation being observed in the absence of NTF2 (Fig. 4; Table III); in its presence y-IMPα/β yielded an \( Fnlc_{\text{max}} \) of 4.2 (\( t_{1/2} = 4.1 \) min). Of the two N1N2-NLS mutant derivatives, N1N2A-β-Gal was excluded from the nucleus, whereas N1N2B-β-Gal showed reduced nuclear accumulation in the presence of RanGDP and NTF2 (\( Fnlc_{\text{max}} \) of 2.8, \( t_{1/2} = 13.9 \) min and \( Fnlc_{\text{max}} \) of 2.6, \( t_{1/2} = 8.4 \) min for y-IMPα/β and m-IMPαβ, respectively) compared with wild type N1N2-β-gal. Clearly, although both arms of the bipartite N1N2 NLS are necessary for optimal nuclear import, mutation of the proximal arm has a much greater effect on nuclear import (Fig. 4) through its more severe effect on importin binding (Fig. 2).

### Inhibition of Nuclear Import by High Levels of NTF2—Previous studies have shown that NTF2 is necessary for nuclear import in several vertebrate systems, being able to bind directly to RanGDP, importin β and proteins of the NPC (15, 21, 41–44). Because the T-ag and N1N2 NLSs appeared to have different requirements for NTF2 for nuclear import, we performed transport measurements in the presence of increasing concentrations of NTF2 (Fig. 5). Although NTF2 concentrations up to 0.15–0.3 μM enhanced nuclear import of the T-ag-NLS-containing fusion protein in the case of both m-IMPαβ and y-IMPαβ, higher concentrations inhibited transport strongly (Fig. 5A). Optimal nuclear import efficiency appeared to be obtained with an NTF2 concentration of about 0.08 μM.

Similar results were also observed for N1N2-NLS-mediated nuclear import (Fig. 5B), whereby m-IMPαβ or y-IMPαβ-mediated import was enhanced in the presence of NTF2 concentrations up to 0.3 and 0.6 μM, respectively, with the optimal concentration of NTF2 for N1N2-NLS-mediated nuclear accumulation being about 0.15 μM. Nuclear accumulation was inhibited essentially completely by NTF2 concentrations above 0.6 μM. NTF2 thus appears to play a regulatory role in nuclear import, its concentration determining whether it has enhancing or inhibitory effects.

### DISCUSSION

This study represents the first quantitative comparison of two conventional NLS binding receptors and two different NLSs, and their ability to mediate nuclear protein import in a system using purified components. Although in vitro systems for nuclear transport have existed since 1990 (4), surprisingly little kinetic analysis has been performed. True understanding of the subtleties of signal-dependent nuclear import requires rigorous quantitation and this study can be seen as a first step in this direction. One of our important findings is that the importin α subunits from mouse and yeast appear to be different in their requirement for importin β to achieve high affinity NLS binding. This is highlighted by the results for the T-ag NLS, where y-IMPα and y-IMPαβ have almost identical binding affinities, in stark contrast to m-IMPα, which requires m-IMPβ to obtain high affinity binding. In the case of the N1N2...
FIG. 4. Nuclear import of N1N2-β-Gal and its mutant derivatives reconstituted in vitro mediated by m-IMPαβ and y-IMPαβ in the absence or presence of NTF2. A, visualization (after 30 min at room temperature) of nuclear accumulation of N1N2-β-Gal and its mutant derivatives (N1N2A-β-Gal and N1N2B-β-Gal) mediated by m-IMPαβ (left panels) or y-IMPαβ (right panels) in the presence of RanGDP and NTF2. B, images after 30 min of nuclear accumulation of N1N2-β-Gal mediated by m-IMPαβ (left panels) and y-IMPαβ (right panels) in the presence of RanGDP in the absence (top panels) and presence (bottom panels) of NTF2 (0.15 μM). C, nuclear import kinetics of N1N2-β-Gal (top panels) or mutant derivatives N1N2A-β-Gal (middle panels) and N1N2B-β-Gal (bottom panels) mediated by m-IMPαβ (left panels) or y-IMPαβ (right panels) in the presence of RanGDP in the absence or presence of NTF2. Results shown are from a single typical experiment where each data point represents at least five separate measurements for each of Fn, Fr, and background fluorescence. Pooled data are presented in Table III.
FIG. 5. Relationship between NTF2 concentration and nuclear import efficiency. Maximal nuclear accumulation (Fn/c max) and import rate (t1/2, the time in min at which nuclear accumulation is half-maximal) is plotted against the NTF2 concentration for nuclear import of T-ag-CcN-β-Gal (A) or N1N2-β-Gal (B) mediated by m-IMPA/β (left panels) or y-IMPa/β (right panels) in the presence of Ran. Results are for the means ± S.E. from a series of three separate experiments.
NLS, y-IMPβ does enhance y-IMPα binding, but not to such a great extent as the enhancement effected on m-IMPα binding by m-IMPβ. y-IMPα thus appears to resemble importin α from the plant Arabidopsis thaliana to some extent, the latter being able to bind a variety of NLS types in the absence of importin β (32). There would thus appear to be a gradation through evolution in the properties of importin α, the vertebrate form being much more strongly dependent on the additional presence of importin β to achieve high affinity NLS binding.

As shown here, both y-IMPαβ and m-IMPαβ can mediate the nuclear import of proteins carrying the T-ag or N1N2 NLSs in combination with human Ran and NTF2, but the role of NTF2 in nuclear import of T-ag is different from that of N1N2. Nuclear import mediated by the T-ag NLS does not require NTF2 absolutely, whereas nuclear accumulation in the case of the N1N2 NLS is completely dependent on NTF2. Varying the concentration of NTF2 changes nuclear import dramatically, high levels of NTF2 inhibiting nuclear accumulation mediated by both the T-ag and N1N2 NLSs. This is consistent with the observations of Tachibana et al. (45), who found that cytoplasmically injected NTF2 strongly inhibited nuclear import mediated by the T-ag NLS, as well as nuclear export mediated by the nuclear export sequence of the cAMP-dependent protein kinase inhibitor PKI, in a dose-dependent manner. The basis of these observations of Tachibana et al. (45) is that the NLS is capable of binding a variety of NLS types in the absence of importin α, and Michael Rexach for providing the NTF2, Ran, and y-IMPβ expression constructs, respectively, and to Lyndall Briggs for skilled technical assistance.

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