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Kinetic Properties of Nuclear Transport Conferred by the Retinoblastoma (Rb) NLS

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Abstract The retinoblastoma (RB) tumor suppressor is a nuclear phosphoprotein central to control of cellular proliferation. We have previously shown that human RB possesses an evolutionarily conserved bipartite nuclear localization sequence (NLS) (KRSAEGSNPPKPLKKLR^{877}) resembling that of nucleoplasmin. Here we analyze the kinetic properties of the RB NLS in detail with respect to recognition by cellular nuclear import factors, the importins (IMPs), and nuclear transport properties, comparing results to those for the NLSs from SV40 large tumor antigen (T-ag) and the *Xenopus laevis* phosphoprotein N1N2. Binding affinities of different IMPα subunits for the Rb NLS, in the absence or presence of IMPβ subunits were determined, and NLS-dependent nuclear import reconstituted in vitro for the first time using purified IMPα/β subunits together with recombinant human RanGDP and nuclear transport factor 2 (NTF2). RB NLS-mediated transport had a strict requirement for all components, with high NTF2 concentrations inhibiting transport. As in the case of transport mediated by the T-ag- and N1N2-NLSs, nuclear import of an RB-NLS containing β-Gal fusion protein was reduced or abolished when anti-IMPα or β antibody was added to cytosolic extract, respectively, confirming that RB NLS-mediated nuclear import occurs through action of IMPα/β. We conclude that although mediated by IMPα/β, and similar in most respects to transport mediated by the similarly bipartite N1N2 NLS, nuclear import conferred by the RB NLS has distinct properties, in part due to the affinity of its interaction with IMPα. J. Cell. Biochem. 95: 782–793, 2005.

Key words: tumor suppressor; bipartite nuclear localization signal; importins; nuclear protein import; in vitro reconstituted system

Facilitated transport into the nucleus through the nuclear envelope (NE)-localized nuclear pore complex (NPC) requires targeting signals that confer interaction with the cellular nuclear transport machinery. The key factors involved are members of the nuclear localization sequence (NLS)-binding importin (IMP)/karyopherin family [Gorlich et al., 1994; Imamoto et al., 1995], the monomeric guanine nucleotide binding protein Ran/TC4 [Moore and Blobel, 1993), and nuclear transport factor 2 (NTF2) [Paschal and Gerace, 1995]. In the first step, the NLS-containing protein is recognized by the IMPα/β heterodimer through the NLS-binding IMPα subunit and targeted to the NPC by the IMPβ subunit [Gorlich et al., 1994]. The complex subsequently translocates through the NPC via transient IMPβ-mediated interactions with nucleoporins, the FG-repeat-containing components of the NPC. Finally, IMPα and the NLS-containing protein are released from IMPβ into the nucleoplasm through binding of RanGTP to IMPβ [Gorlich et al., 1996b], which requires high concentrations of RanGTP in the

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nucleus. Although NTF2 has been shown to function as the nuclear import receptor for Ran [Ribbeck et al., 1998], we and others have demonstrated that, depending on the nuclear import substrate, NTF2 can have inhibitory effects on nuclear import [Tachibana et al., 1996; Hu and Jans, 1999; Lam et al., 1999].

The retinoblastoma (RB) tumor suppressor gene, expressed in various cell types, is mutated in many human cancers [Goodrich, 2003]. As a key regulator of cell-cycle progression and differentiation, RB exerts its function in the nucleus at the transcriptional level by modulating the activities of various transcription factors. Human RB contains an evolutionarily conserved bipartite NLS (amino acids 860–877: KRSAEGSNPPKPLKKLR^{877}) [Zacksenhaus et al., 1993; Efthymiadis et al., 1997]. Bipartite NLSs comprise two clusters of basic amino acids separated by a 10–12 amino acid spacer [Robbins et al., 1991; Fontes et al., 2003], and thereby differ from monopartite NLSs, which consist of a single basic amino acid cluster resembling the NLS (PKKKRKV^{132}) of the simian virus SV40 large tumor antigen (T-ag) [Kalderon et al., 1984].

This study investigates the kinetic properties of the RB NLS, comparing it to other IMPα/β recognized NLSs, examining nuclear import in vitro at the single cell level using a defined system for the first time. We assess the dependence of nuclear import on IMPs in vitro using specific antibodies, and use direct binding assays to determine IMP binding properties for RB-NLS compared to the NLSs from T-ag and the *Xenopus laevis* histone assembly factor N1N2. Although there are similarities with the N1N2 bipartite NLS, we find differences between the IMP binding properties of the RB-NLS compared to the other NLSs, and accompanying differences in nuclear import properties. The results are consistent with our previous work indicating that NLS-IMP interaction is a key determinant in nuclear protein import.

MATERIALS AND METHODS

Peptides

NLS-containing peptides were synthesized using the Merrified solid-phase procedure [Pearson and Choi, 1993]. The NLS peptides were as follows: pepRB includes human Rb amino acids 860–877 (KRSAEGSNPPKPLKKLR^{877}), with pepRBM identical in sequence except that it contains the TT$^{880/861}$ substitutions inactivating the proximal arm of the bipartite NLS; pep101Lys, includes T-ag amino acids 111–132 including the NLS (PKKKRKV^{132}), with pep101Thr identical in sequence except that it contains the NLS inactivating T^{128} substitution [see Akhlynina et al., 1997]; and pepN1 includes *Xenopus laevis* amino acids 465–488 including the bipartite NLS (RKKRKTEEESPLKDKAKKSK^{554}), with pepN1A identical in sequence except that it contains the NNS$^{466-468}$ substitutions inactivating the proximal arm of the bipartite NLS.

Fusion Proteins

The amino acid sequences of the NLSs present in the different β-Gal fusion proteins used were identical to those present within the NLS peptides (see above). RB- [Efthymiadis et al., 1997), N1N2- [Hu and Jans, 1999), and T-ag [Hubner et al., 1997] fusion proteins (RB-Bip-β-Gal, RB-BipMut-β-Gal, N1N2-β-Gal, N1N2A-β-Gal, T-ag-CcN-β-Gal, and T-ag-Cc-β-Gal) have all been described previously. β-Gal fusion proteins were expressed, purified, and labeled with 5-iodoacetamido fluorescein (IAF) as previously [Jans et al., 1991; Rihs et al., 1991]. m-IMPα (PTAC58), m-IMPβ (PTAC97), y-IMPα (Kap60), y-IMPβ (Kap95) subunits, and human Ran were expressed as glutathione-*S*-transferase (GST) fusion proteins and purified as described [Imamoto et al., 1995; Efthymiadis et al., 1997; Hu and Jans, 1999]. GST-free y-IMPβ, m-IMPβ and Ran were prepared by thrombin cleavage [Hu and Jans, 1999]. Ran was loaded with GDP as described [Chi et al., 1996].

ELISA-Based Binding Assay

An established ELISA-based assay [Efthymiadis et al., 1997; Hubner et al., 1997; Xiao et al., 1997; Efthymiadis et al., 1998; Hu and Jans, 1999] was used to determine the binding affinity of IMP subunits (±GST moieties) to NLS-containing proteins and peptides. The latter were coated onto 96-well microtiter plates, incubated with increasing concentrations of IMP subunits and bound IMP-GST detected using an anti-GST primary antibody, an alkaline phosphatase-coupled secondary antibody, and the substrate *p*-nitrophenyl phosphate [Hubner et al., 1997]. In experiments where IMPα/β combinations were tested, IMPα-GST and thrombin cleaved GST-free IMPβs were used [Hu and Jans, 1999].

In Vitro Nuclear Transport

Nuclear import kinetics were measured at the single cell level using mechanically perforated HTC rat hepatoma cells in conjunction with CLSM as previously [Jans et al., 1991; Xiao et al., 1997; Hu and Jans, 1999]. NLS-dependent nuclear protein import can be reconstituted in this system through the exogenous addition of cytosolic extract (untreated reticulocyte lysate-Promega Cat. No. L415A), an ATP regenerating system (0.125 mg/ml creatine kinase, 30 mM creatine-phosphate, and 2 mM ATP), GTP (2 mM) and transport substrate (0.2 mg/ml IAF-labeled fusion protein or a 70 kDa FITC-labeled dextran-Sigma Chem. Co.). Where indicated, transport assays were also performed in the presence of 40 μg/ml anti-IMP antibodies: anti-*Arabidopsis thaliana* IMPα [At-IMPα; Hicks et al., 1996; Hubner et al., 1999], anti-IMPα1 (Santa Cruz.), and anti-IMPβ [Chi et al., 1995]. Antibody was preincubated with cytosolic extract for 20 min at room temperature prior to addition to the in vitro assay. Where cytosol was replaced with purified subunits, reticulocyte lysate was substituted by 30 mg/ml BSA, 2 mM GTP, and 4 μM RanGDP, 1 μM IMPβ, 1 μM IMPα, NTF2 (0.04–6 μM) where indicated. Image analysis/curve-fitting were performed as previously [Hubner et al., 1997; Xiao et al., 1997; Hu and Jans, 1999]; the level of accumulation at the NE, relative to medium fluorescence, was measured using NIH Image 1.60 in the line plot mode [Piller et al., 1998].

RESULTS

y-IMPα/β Has Higher Affinity for the RB NLS Than m-IMPα/β

Initial experiments examined the binding of mouse (m) and yeast (y) IMP subunits to peptides and fusion proteins containing the RB NLS, and a RB mutant derivative thereof (mutated in the proximal arm of the bipartite NLS) (see Materials and Methods). y-IMPα bound the RB NLS-containing fusion protein (RB-Bip-β-Gal) with significantly ($P < 0.01$) higher affinity (about seven-fold) than m-IMPα (Fig. 1; Table I), the latter only attaining high affinity in the presence of m-IMPβ ($P < 0.02$). The specificity of binding in all cases was indicated by the fact that binding to the RB NLS-mutant derivative (RB-BipMut-β-Gal) was negligible (Table I). Clearly, m and y-IMPα/β bind the RB NLS specifically, with y-IMPα/β binding with significantly higher affinity than m-IMPα/β ($P < 0.01$) (Figs. 1 and 2; Table I).

In contrast to the result for the RB β-gal fusion proteins, the binding affinities of y-IMPα and y-IMPα/β for the RB NLS-containing peptide (pepRB) or RB-Bip-β-Gal were only slightly different (Figs. 1 and 2; Table I), whereby y-IMPα/β bound RB-Bip-β-Gal with significantly ($P < 0.01$) higher affinity (ca. five-fold) than m-IMPα/β (Fig. 1; Table I). y-IMPα is thus a higher affinity NLS-receptor for the RB NLS than m-IMPα, which requires m-IMPβ to achieve high affinity. Whereas the T-ag and N1N2 NLSs show similar binding affinities for m- and y-IMPα/β [Hu and Jans, 1999] RB-Bip-β-Gal showed lower affinity binding by m-IMPα and m-IMPα/β than y-IMPα, and y-IMPα/β implying that the RB NLS may have particular different spatial requirements for high affinity recognition by different IMPs, in contrast to the T-ag and N1N2 NLSs [see Fontes et al., 2003].

Nuclear Import Conferred by the RB Bipartite NLS Requires NTF2; Inhibition by High Levels of NTF2

The ability of m-IMPα/β and y-IMPα/β to mediate RB NLS-dependent nuclear import was tested in vitro (Fig. 3A,B; Table II). Nuclear accumulation was negligible in the absence of either RanGDP or y-IMPα/β with an accumulation at the NE evident in the presence of y-IMPα/β and absence of RanGDP. In the presence of y-IMPα/β and RanGDP but in the absence of NTF2, a lack of nuclear accumulation was also evident with the levels of nuclear accumulation similar to those in the absence of y-IMPα/β (Fig. 3A,B; Table II). Nuclear accumulation was clearly observed in the presence of NTF2, with an Fn/c_{max} of 2.7 and $t_{1/2}$ of 4.8 min ($P < 0.01$). Different results were observed for m-IMPα/β in the presence of RanGDP, no significant nuclear accumulation being observed in the presence or absence of NTF2 (Fig. 3A,B; Table II) with the levels of nuclear accumulation similar to those in the absence of m-IMPα/β or Ran (Table II). The specificity of transport in all cases was demonstrated by the fact that the RB-BipMut-β-Gal protein, mutated in the proximal arm of the bipartite NLS, did not accumulate to any significant extent

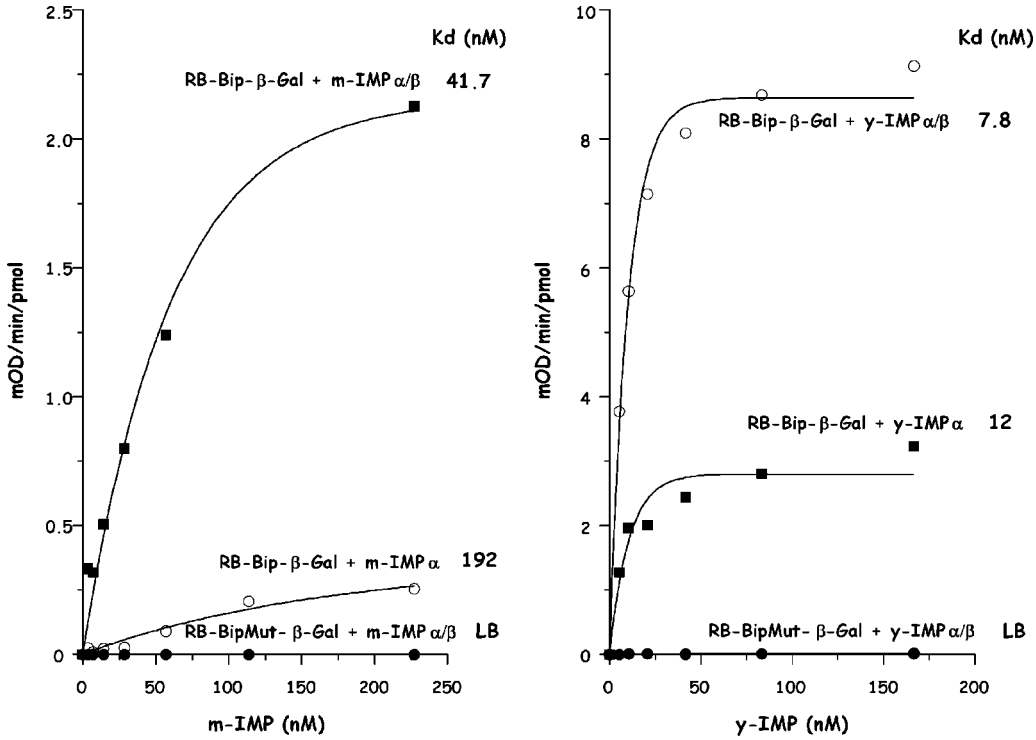

Fig. 1. y-IMPα binds the RB NLS with higher affinity than m-IMPα. Microtiterplates were coated with RB-Bip-β-Gal or the NLS-mutated RB-BipMut-β-Gal, and incubated with increasing concentrations of m-IMPα (**left panel**) or y-IMPα (**right panel**) in the absence or presence of m-IMPβ or y-IMPβ as indicated. Curves were fitted for the function $B(x) = B_{max}(1-e^{-}kB)$, where x is the concentration of IMP, and B is the level of IMP bound, with the apparent dissociation constants (Kds, representing the IMP concentration yielding half maximal binding) indicated. Curves for wild type RB-Bip-β-Gal and mutant derivative RB-BipMut-β-Gal are shown for m-IMPα (left panels) or y-IMPα (right panels) in the absence or presence of m-IMPβ or y-IMPβ as indicated. The results are from a single typical experiment performed in triplicate, with pooled data shown in Table I.

(Fig. 3A,B; Table II). The results thus demonstrate that y-IMPα/β but not m-IMPα/β can mediate nuclear import of RB NLS-containing transport substrate sufficiently in the presence of NTF2 (Fig. 3A,B; Table II).

Since the RB bipartite NLS appeared to have an absolute requirement for NTF2 for nuclear import comparable to that of N1N2 NLS, but in contrast to the T-ag NLS [see Hu and Jans, 1999], transport measurements were also per-

TABLE I. Binding Affinities of Yeast and Mouse IMPs for RB NLS-Containing β-Gal Fusion Proteins and Peptides as Determined Using an ELISA-Based Assay

Protein/peptide	y-IMPα (A)	y-IMPα/β (B) Kd (nM)[b]	y-IMPβ (C)	A/B	m-IMPα (D)	m-IMPα/β (E) Kd (nM)[b]	m-IMPβ (F)	D/E
A) Fusion proteins[a]								
RB-Bip-β-Gal[c]	22.2 ± 3.9 (4)	7.8 ± 0.5 (3)	LB[d] (2)	2.8	153 ± 38 (2)	38.4 ± 4.2 (3)	LB (2)	4.0
RB-BipMut-β-Gal	LB (2)	LB (2)	LB (1)	—	LB (2)	LB (2)	LB (2)	—
B) Peptides[a]								
pepRB[e]	20.6 ± 2.1 (2)	10.4 ± 0.6 (2)	LB (1)	2.0	59.7 ± 3.3 (2)	11.7 ± 2.1 (2)	LB (1)	5.1
pepRBM	LB (2)	LB (2)	LB (1)	—	LB (2)	LB (2)	LB (1)	—

[a]See Materials and Methods section for amino acid sequence details.
[b]Data represent the mean ± SEM (n in parentheses) for the apparent dissociation constant (Kd) determined as outlined in Materials and Methods.
[c]Significant differences (Student's *t*-test) ($P < 0.05$) were observed between the Kds for m-IMPα compared to y-IMPα (columns A and D, $P = 0.0014$), m-IMPα in the absence or presence of m-IMPβ (columns A and B, $P = 0.0109$), y-IMPα in the absence or presence of y-IMPβ (columns D and E, $P = 0.0016$) and m-IMPα/β compared to y-IMPα/β (columns B and E, $P = 0.0002$).
[d]LB, low binding—Kd not able to be determined.
[e]Significant differences were observed between the Kds for y-IMPα compared to m-IMPα (columns A and D, $P = 0.0097$), m-IMPα in the absence or presence of m-IMPβ (columns A and B, $P = 0.0066$) and y-IMPα in the absence or presence of y-IMPβ (columns D and E, $P = 0.0416$).

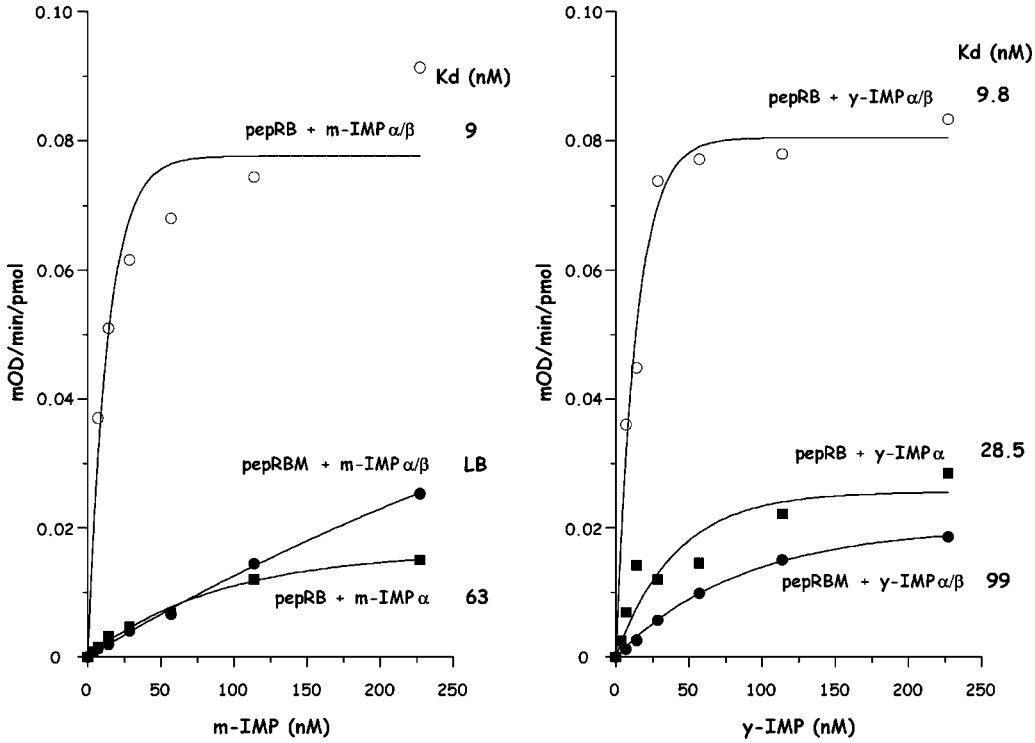

Fig. 2. y-IMPα binds the RB NLS peptide with higher affinity than m-IMPα. Measurements were performed as described in the legend to Figure 1. Curves for wild type pepRB and mutant derivative pepRBM are shown for m-IMPα (**left panels**) or y-IMPα (**right panels**) in the absence or presence of m-IMPβ or y-IMPβ as indicated. The results are from a single typical experiment performed in triplicate, with pooled data shown in Table I.

formed in the presence of increasing concentrations of NTF2 (Fig. 4). y-IMPα/β mediated import was enhanced in the presence of NTF2 concentrations of 0.15–0.6 μM, with the optimal concentration for RB NLS-mediated nuclear accumulation being ca. 0.3 μM. Nuclear accumulation was inhibited essentially completely by NTF2 concentrations >1.5 μM in similar fashion to previous results for the T-ag and N1N2 NLSs [Hu and Jans, 1999]. NTF2 thus appears to play a vital role in RB NLS-mediated nuclear import, in analogous fashion to that mediated by the N1N2 bipartite NLS, its concentration determining whether it has enhancing or inhibitory effects.

Nuclear Import Conferred by the RB Bipartite NLS Is Dependent on the IMP α/β Heterodimer

To confirm that the nuclear import pathway of RB NLS is IMPα/β mediated, the nuclear import of RB NLS-containing fusion protein as well as T-ag- and N1N2 NLS-containing proteins was assayed in the absence and presence of exogenously added anti-IMPα5, anti-IMPα1, or anti-IMPβ1 antibodies in the in vitro assay using cytosolic extract as the source of transport factors. In all cases, the nuclear import of T-ag NLS-containing fusion protein was inhibited significantly, with the maximal level of nuclear accumulation significantly ($P < 0.05$) reduced (ca. 30%) by anti-IMPα antibodies, whilst anti-IMPβ antibody abolished nuclear accumulation completely (Fn/c_{max} ca. 0.8) ($P < 0.01$) (Fig. 5A,B; Table III). Very similar results were observed for N1N2 NLS- and RB NLS-mediated nuclear import (Fig. 5A,B; Table III). The results indicate that nuclear import conferred by all three different NLSs occurs predominantly through IMPα/β.

DISCUSSION

This study represents the first quantitative examination of nuclear import conferred by the RB NLS in vitro using a defined system, together with quantitative comparison of the ability of two conventional NLS-binding IMPαs to bind the RB NLS and mediate RB NLS-dependent nuclear import. One of our important

findings is that the IMPα subunits from mouse and yeast appear to be different in their requirement for IMPβ to achieve NLS high binding affinity. This is highlighted by the results here, where y-IMPβ does enhance y-IMPα binding, but not to such a great extent as the enhancement of m-IMPβ on m-IMPα binding. Consistent with our previous study of the NLSs of T-ag and the N1N2 [Hu and Jans, 1999], y-IMPα thus appears to resemble IMPα from the plant *A. thalania* to some extent, the latter being able to bind a variety of NLS types in the absence of IMPβ1 with high affinity [Hubner et al., 1999]. There would thus appear

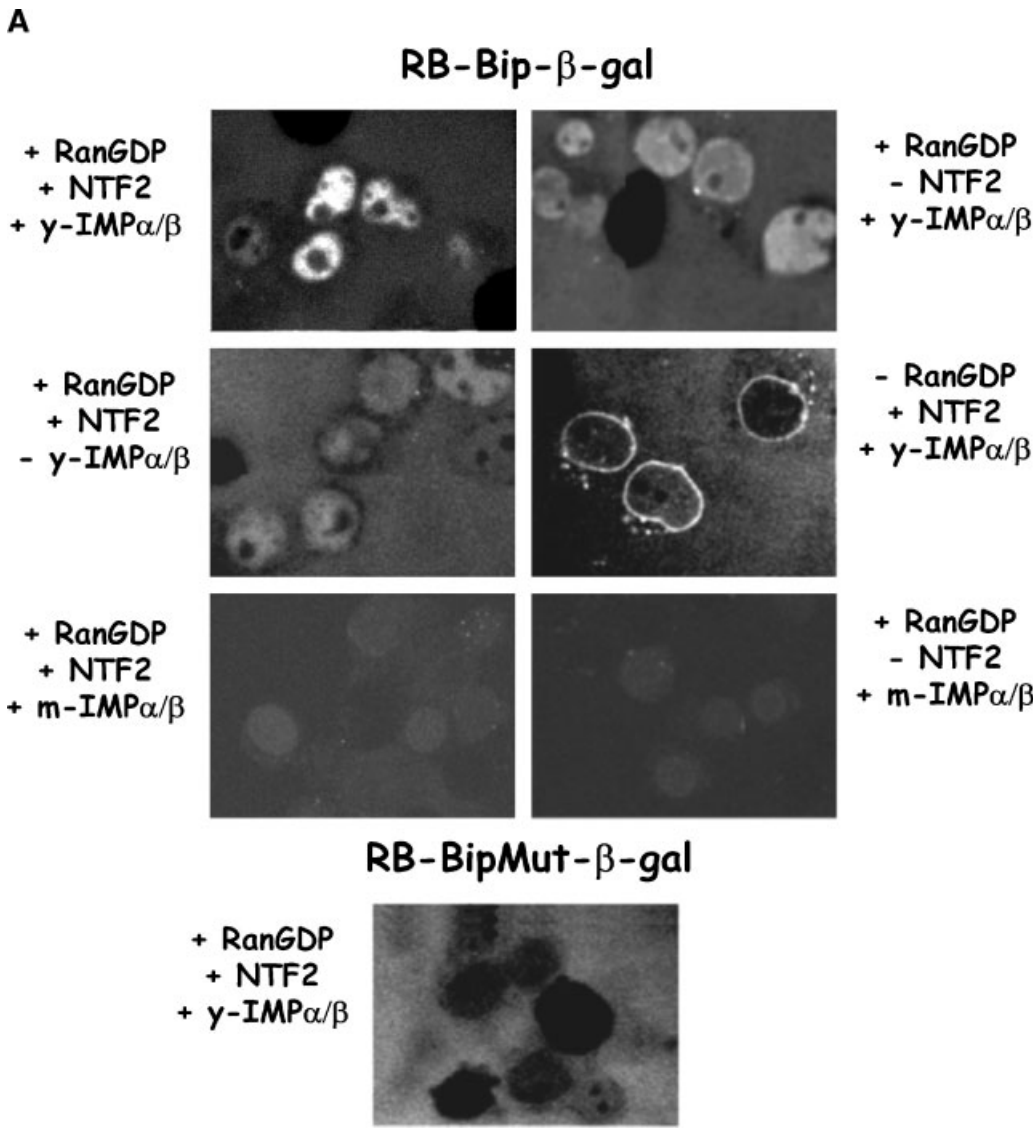

Fig. 3. **A**: Visualization of binding at the NE or nuclear accumulation of RB NLS-containing fusion proteins. Visualization of binding at the NE or nuclear accumulation of the RB NLS-containing fusion protein RB-Bip-β-Gal, or lack of nuclear accumulation of the NLS-mutant derivative RB-BipMut-β-Gal mediated by IMPα/β as indicated in the absence or presence of RanGDP and NTF2. **B**: Reconstitution of the nuclear import of the RB NLS-containing fusion proteins in vitro using purified components in the absence or presence of NTF2. Nuclear import was reconstituted in mechanically perforated HTC cells as described in Materials and Methods. Nuclear import kinetics are shown for RB-Bip-β-Gal and RB-BipMut-β-Gal mediated by y-IMPα/β in the presence of RanGDP and NTF2, or for RB-Bip-β-Gal in the absence or presence of y-IMPα/β, RanGDP or NTF2 (**left panel**). Nuclear import kinetics are also shown for RB-Bip-β-Gal in the presence of m-IMPα/β and RanGDP, in the presence or absence of NTF2 (**right panel**). Results shown are from a single typical experiment where each data point represents at least five separate measurements for each of Fn, Fc and background fluorescence (Materials and Methods). Curves were fitted as described in Materials and Methods. Pooled data are presented in Table II.

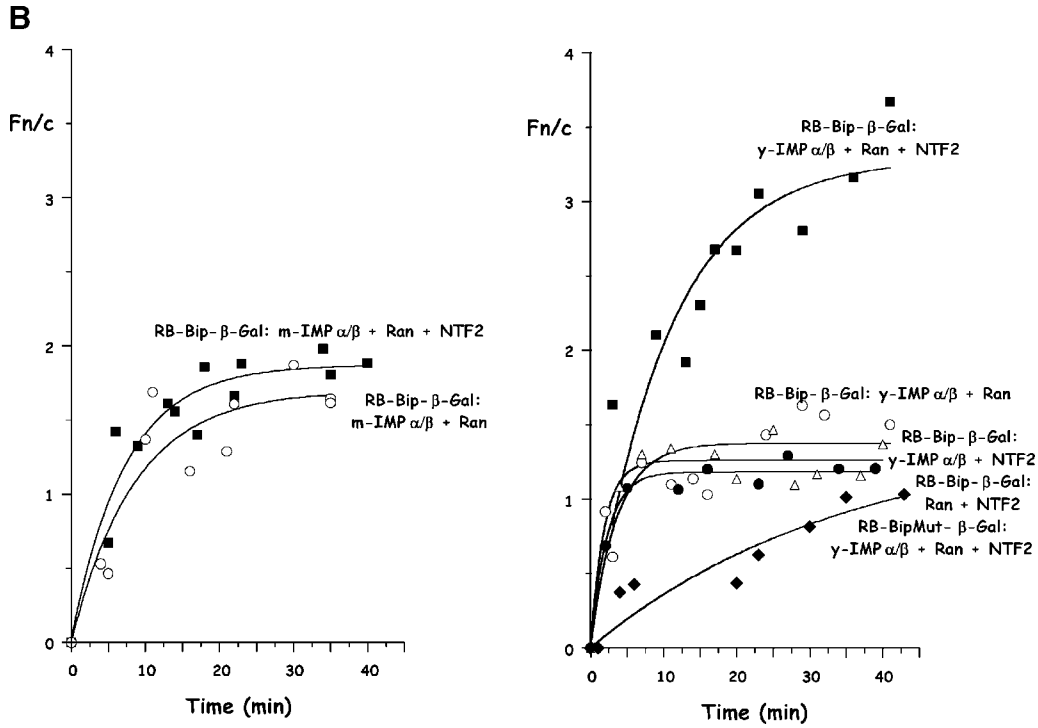

Fig. 3. (*Continued*)

to be a gradation through evolution in the properties of IMPα, the vertebrate forms (of which there are multiple forms in mammals, compared to only one in yeast) being much more strongly dependent on the additional presence of IMPβ to achieve high NLS binding affinity. Another finding is that binding of m-IMPα and m-IMPα/β, but not y-IMPα or y-IMPα/β, to the RB NLS is significantly affected by the presentation of the RB NLS in the context of the β—Gal fusion protein, in contrast to the N1N2 and T-ag NLSs. This implies that the RB NLS may have particular spatial requirements for high affinity recognition by different IMPs, in contrast to the N1N2 NLS, consistent with differences at the "P5" position residue of the NLS (R in RB, compared to K in N1N2) in interacting with the major NLS binding site on IMPα, and the fact that the 11 amino acid spacer of RB is more extended, compared to the 12 amino acid spacer of N1N2 [see Fontes et al., 2003].

We show here that RB NLS-conferred nuclear import was reduced >30% by anti-IMPα antibodies and abolished by anti-IMPβ antibody, compared to in its absence, in similar fashion to observations for the T-ag and N1N2 NLSs. This confirms previous observations that RB NLS-mediated nuclear import occurs through IMPα/β [Efthymiadis et al., 1997]. The extent of the inhibition of transport effected by the anti-IMPα and anti-IMPβ antibodies presumably relates to the fact that the RB bipartite NLS is recognized by different isoforms of IMPα, not just IMPα1 or α5 [Kohler et al., 1997; Seki et al., 1997; Nachury et al., 1998]. Based on its relatively poor ability both to bind and transport RB-Bip-β-Gal to the nucleus, IMPα5 (Srp1/NPI) rather than IMPα1 (Rch1) may be the IMPα form specialized for RB NLS-mediated nuclear import.

As shown here, y-IMPα/β but not m-IMPα/β can mediate the nuclear import of proteins carrying the RB NLS in combination with human Ran and NTF2. Nuclear import mediated by the RB NLS requires NTF2 absolutely. Varying the concentration of NTF2 changes the nuclear import efficiency dramatically, high levels of NTF2 inhibiting nuclear accumulation. We previously found that the role of NTF2 in in vitro nuclear import of T-ag is different from that for N1N2 [Hu and Jans, 1999]. The former does not require NTF2 absolutely, whereas transport mediated by the N1N2 NLS is completely dependent on NTF2. The fact that

TABLE II. Nuclear Import Kinetics of T-ag-CcN-β-Gal, N1N2-β-Gal and RB-Bip-β-Gal Fusion Proteins In Vitro Using Purified Transport Factors

Addition[b]	Nuclear import parameter[c]			*P*-value[a]
	Fn/c_{max}	$t_{1/2}$ min	N	
T-ag NLS containing fusion proteins				
T-ag-CcN-β-Gal + y-IMPα + RanGDP + NTF2	0.9 ± 0.4	2.5 ± 0.6	2	
T-ag-CcN-β-Gal + y-IMPβ + RanGDP + NTF2	0.8 ± 0.1	3.7 ± 0.2	2	
T-ag-CcN-β-Gal + y-IMPα/β + RanGDP + NTF2	9.4 ± 0.8^{a1}	1.8 ± 1.2^{c1}	2	
T-ag-CcN-β-Gal + y-IMPα/β + RanGDP	6.3 ± 1.5^{b1}	4.0 ± 1.8^{d1}	4	0.0251(a1:b1), 0.0490(c1:d1)
T-ag-CcN-β-Gal + y-IMPα/β + NTF2	1.7 ± 0.9	2.6 ± 1.8	2	
T-ag-CcN-β-Gal + RanGDP + NTF2	0.9 ± 0.3	ND	2	
T-ag-CcN-β-Gal + y-IMPα/β	1.7 ± 0.2	2.7 ± 0.5	2	
T-ag-CcN-β-Gal alone	0.8 ± 0.06	ND	5	
T-ag-CcN-β-Gal + m-IMPα + RanGDP + NTF2	1.3 ± 0.3	ND	2	
T-ag-CcN-β-Gal + m-IMPβ + RanGDP + NTF2	1.2 ± 0.2	ND	1[d]	
T-ag-CcN-β-Gal + m-IMPα/β + RanGDP + NTF2	5.0 ± 0.9^{e}	2.6 ± 1.1^{g}	3	
T-ag-CcN-β-Gal + m-IMPα/β + RanGDP	4.8 ± 1.2^{f}	6.3 ± 2.2^{h}	3	0.9061(e:f), 0.0474(g:h)
T-ag-CcN-β-Gal + m-IMPα/β	1.3 ± 0.2	6.9 ± 0.2	2	
T-ag-Cc-β-Gal + y-IMPα/β + RanGDP + NTF2	0.6 ± 0.1	7.1 ± 0.1	1[d]	
T-ag-Cc-β-Gal + m-IMPα/β + RanGDP + NTF2	1.4 ± 0.1	55 ± 0.3	1[d]	
N1N2 bipartite NLS containing fusion proteins				
N1N2-β-Gal + y-IMPα/β + RanGDP + NTF2	4.2 ± 0.3^{i}	4.1 ± 0.5	2	
N1N2-β-Gal + y-IMPα/β + RanGDP	1.5 ± 0.3^{j}	6.2 ± 1.4	2	0.0183(i:j)
N1N2-β-Gal + RanGDP + NTF2	1.4 ± 0.1^{k}	3.7 ± 0.6	2	0.0125(i:k), 0.7818(j:k)
N1N2-β-Gal alone	1.4 ± 0.2^{m}	7.6 ± 3.8	2	0.0162(i:m), 0.8075(j:m)
N1N2-β-Gal + m-IMPα/β + RanGDP + NTF2	3.3 ± 0.5^{n}	7.8 ± 0.8^{o}	2	
N1N2-β-Gal + m-IMPα/β + RanGDP	1.2 ± 0.1^{p}	2.5 ± 0.1^{q}	2	0.047(n:p), 0.031(o:q)
N1N2-β-Gal + m-IMPα/β + NTF2	1.6 ± 0.2	7.2 ± 0.03	1[d]	
N1N2A-β-Gal + y-IMPα/β + RanGDP + NTF2	1.0 ± 0.1	7.3 ± 2.1	2	
N1N2A-β-Gal + y-IMPα/β + RanGDP	1.0 ± 0.3	9.0 ± 3.2	2	
N1N2A-β-Gal alone	1.1 ± 0.1	4.8 ± 1.1	2	
N1N2A-β-Gal + m-IMPα/β + RanGDP + NTF2	0.9 ± 0.2	4.8 ± 1.4	2	
N1N2A-β-Gal + m-IMPα/β + RanGDP	1.0 ± 0.1	32 ± 2.7	2	
N1N2B-β-Gal + y-IMPα/β + RanGDP + NTF2	2.8 ± 0.1	13.9 ± 1.6	2	
N1N2B-β-Gal + y-IMPα/β + RanGDP	1.6 ± 0.3	7.3 ± 2.1	2	
N1N2B-β-Gal alone	1.2 ± 0.2	10 ± 0.02	1[d]	
N1N2B-β-Gal + m-IMPα/β + RanGDP + NTF2	2.6 ± 0.04	8.4 ± 5.7	2	
N1N2B-β-Gal + m-IMPα/β + RanGDP	1.5 ± 0.1	6.4 ± 0.3	2	
RB Bipartite NLS containing fusion proteins				
RB-Bip-β-Gal + RanGDP + y-IMPα/β + NTF2	2.68 ± 0.17^{r}	4.76 ± 0.68	5	
RB-Bip-β-Gal + RanGDP + y-IMPα/β	1.71 ± 0.16^{s}	4.03 ± 0.15	4	0.0048 (r:s)
RB-Bip-β-Gal + RanGDP + NTF2	1.38 ± 0.11^{t}	2.39 ± 1.07	2	0.0065 (r:t), 0.2561 (s:t)
RB-Bip-β-Gal + y-IMPα/β + NTF2	1.44 ± 0.26^{w}	2.49 ± 0.54	2	0.0111 (r:w), 0.4016 (s:w)
RB-Bip-β-Gal + RanGDP + m-IMPα/β + NTF2	1.89 ± 0.18	5.55 ± 1.3	3	
RB-Bip-β-Gal + RanGDP + m-IMPα/β	1.78 ± 0.10	5.80 ± 0.2	2	
RB-Bip-β-Gal alone	1.25 ± 0.33	1.32 ± 0.34	2	
RB-BipMut-β-Gal + RanGDP + y-IMPα/β + NTF2	1.12 ± 0.3	ND	2	

ND, not able to be determined.
[a]*P*-values (Student's *t*-test) for the statistical significance of the comparisons indicated in parenthesis for Fn/c_{max} or $t_{1/2}$ are shown.
[b]Results are shown for transport in the presence or absence of particular exogenously added recombinant nuclear protein import components (RanGDP, y-IMPα/β, m-IMPα/β and NTF2), and in the presence of GTP and an ATP-regenerating system.
[c]Raw data (see Figs. 3B and 4) were fitted for the function $Fn/c\ (t) = Fn/c_{max} \times (1 - e^{-}kt)$ (see Materials and Methods), where Fn/c_{max} is the maximal level of accumulation at steady state in the nucleus, and t is time in minutes. An Fn/c_{max} of 1.0 indicates nuclear entry and equilibration between nucleus and cytoplasm, with values below 1 indicating exclusion from the nucleus. NTF2 was used at the optimal concentration for nuclear accumulation (see Fig. 4). Results represent the mean ± SEM.
[d]SE from curve fit.

NTF2 is required for nuclear import mediated by the RB-NLS is consistent with the idea that it plays a central regulatory role in nuclear transport mediated by bipartite NLSs [Tachibana et al., 1996; Paschal et al., 1997; Wong et al., 1997], but may not be critical for nuclear transport mediated by monopartite NLSs, at least in vitro [Hu and Jans, 1999]. The exact mechanism of this is not clear, but the obvious implication is that NTF2 here may have roles in nuclear import addition to that of nuclear import of RanGDP.

The results presented here indicate a close relationship between the IMP binding affinity and rate of nuclear import, where the highest affinity conformation of NLS and NLS receptor leads to the most efficient nuclear accumulation. This is illustrated most poignantly by the comparison between y-IMPα/β and m-IMPα/β which exhibit quite different RB-Bip-β-Gal binding

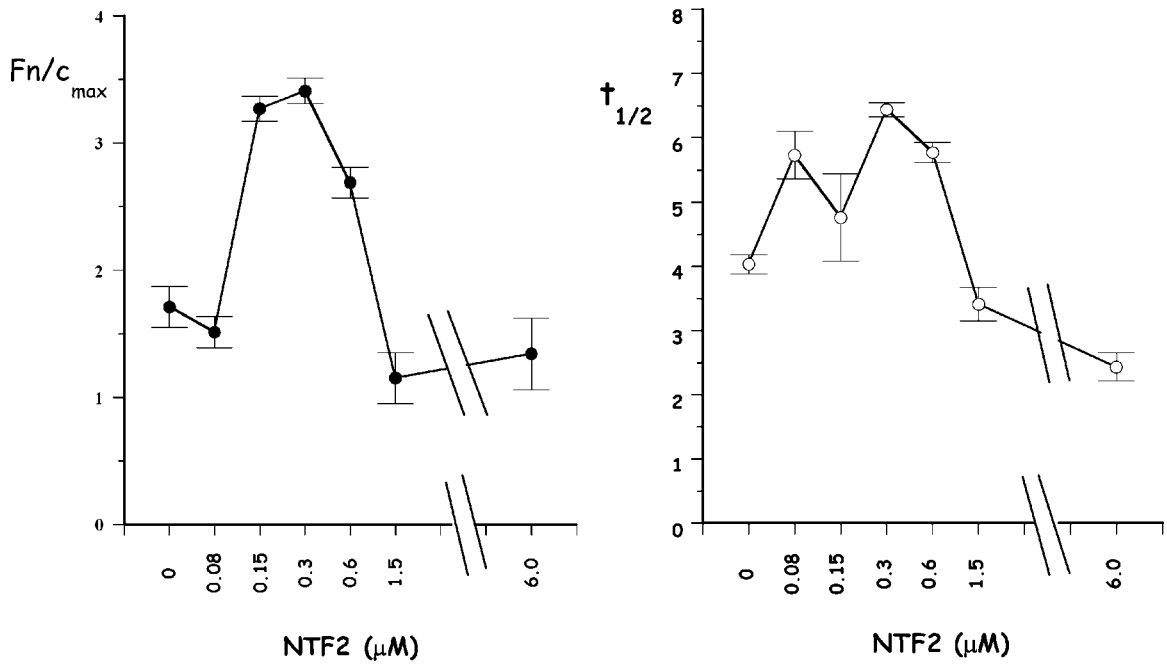

Fig. 4. Relationship between NTF2 concentration and RB NLS-mediated nuclear import efficiency. Fn/c_{max} (**left panel**) and $t_{1/2}$ (**right panel**) are plotted against the NTF2 concentration. Nuclear transport experiments in the presence of by y-IMPα/β, RanGDP, an ATP regenerating system and increasing of NTF2 were performed for RB-Bip-β-Gal, and transport kinetics determined as described in legend to Figure 3B. Results are for the mean ± SEM from a series of 3 separate experiments.

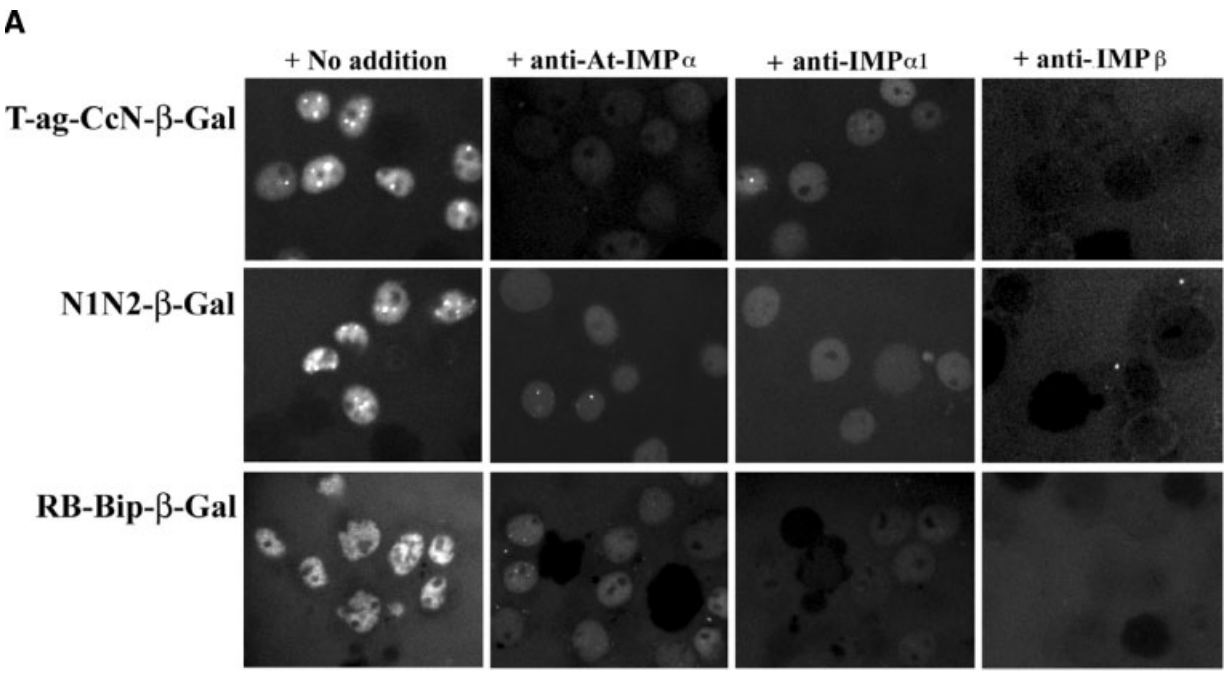

Fig. 5. **A**: Visualization of nuclear accumulation of T-ag-, N1N2- and RB-containing fusion proteins in vitro, in the presence of cytosol, in the presence or absence of anti-IMP antibodies. CLSM images (after 30 min incubation at room temperature) of nuclear accumulation in vitro in the presence of cytosol. Inhibition of nuclear import of the T-ag-, N1N2- and RB- NLS containing fusion proteins (T-ag-CcN-β-Gal, N1N2-β-Gal and RB-Bip-β-Gal) by anti-At-IMPα (anti-IMPα5), anti-IMPα1 and anti-IMPβ antibodies are shown. Quantitative data are presented in Figure 5B and Table III. **B**: Effect of anti-IMP antibodies on nuclear import of T-ag-CcN-β-Gal, N1N2-β-Gal and of RB-Bip-β-Gal. Nuclear transport of T-ag-CcN-β-Gal (**top panel**), N1N2-β-Gal (**middle panel**) and RB-Bip-β-Gal (**bottom panel**) was measured in the presence of cytosolic extract, an ATP-regenerating system, and in the absence or presence of the antibodies indicated. The measurements represent the average of at least three separate experiments, where each point represents the average of 6–10 separate measurements for each of Fn and Fc and background fluorescence (Materials and Methods). Curves are fitted as described in Materials and Methods. Collated data are presented in Table III.

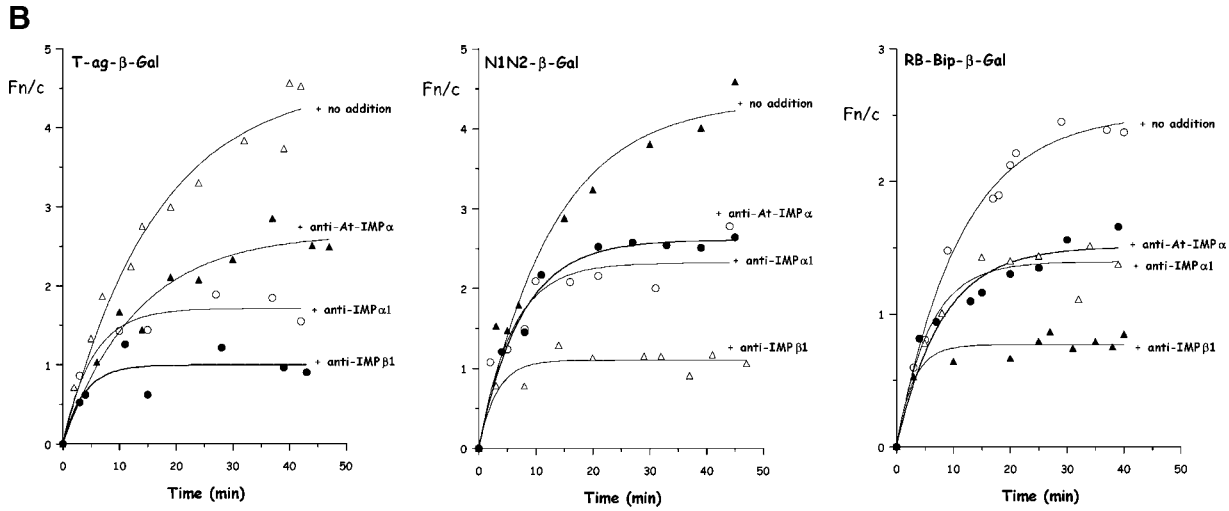

Fig. 5. (*Continued*)

activities; the RB-Bip-β-Gal is recognized by y-IMPα/β with five-fold higher affinity, and in consequence is able to be transported to the nucleus efficiently, whereas RB-Bip-β-Gal is recognized by m-IMPα/β with much lower affinity and is not able to be transported to the nucleus significantly. This is consistent with the idea that the affinity of NLS recognition by IMPs is a critical determinant of the rate and maximal extent of NLS-dependent nuclear import [Efthymiadis et al., 1997; Hubner et al., 1997; Xiao et al., 1997; Hu and Jans, 1999]. The affinity of the recognition of the RB-NLS by different IMPαs may be the key to Rbs subcellular location during the cell cycle in normal cells and during oncogenesis. That a specific form of IMPα is specialized to mediate Rb nuclear import is the focus of future work in this laboratory.

TABLE III. In vitro Nuclear Import Kinetics of T-ag-, N1N2- and RB-NLS-β-Gal Fusion Proteins in the Presence of Cytosolic Extract and Exogenously Added Antibodies or Proteins

	Nuclear import parameter[c]			
Protein[b]	Fn/c_{max}	$t_{1/2}$ min	N	*P*-value[a]
T-ag-CcN-β-Gal	4.1 ± 0.3^{a1}	9.2 ± 0.7	4	
T-ag-CcN-β-Gal + anti-At-IMPα	2.8 ± 0.3^{b1}	10.7 ± 0.2	3	0.0409 (a1:b1)
T-ag-CcN-β-Gal + anti-IMPα1	2.0 ± 0.5^{c1}	3.6 ± 1.2	3	0.0108 (a1:c1)
T-ag-CcN-β-Gal + anti-IMPβ	1.0 ± 0.1^{d}	2.4 ± 0.9	3	0.0005 (a:d)
T-ag-Cc-β-Gal	0.7 ± 0.1	3.0 ± 0.5	2	
N1N2-β-Gal	4.2 ± 0.3^{k}	8.9 ± 0.2	4	
N1N2-β-Gal + anti-At-IMPα	2.1 ± 0.4^{l}	3.6 ± 2.1	2	0.0104 (k:l)
N1N2-β-Gal + anti-IMPα1	2.5 ± 0.1^{m}	3.8 ± 0.5	3	0.0030 (k:m)
N1N2-β-Gal + anti-IMPβ	1.2 ± 0.03^{n}	3.2 ± 0.6	3	0.002 (k:n)
N1N2A-β-Gal	0.9 ± 0.1	4.2 ± 0.67	3	
RB-Bip-β-Gal	2.2 ± 0.1^{r}	6.2 ± 1.8	4	
RB-Bip-β-Gal + anti-At-IMPα	1.5 ± 0.1^{s}	4.1 ± 0.09	2	0.0144 (r:s)
RB-Bip-β-Gal + anti-IMPα1	1.5 ± 0.02^{t}	3.4 ± 0.8	3	0.0025 (r:t)
RB-Bip-β-Gal + anti-IMPβ	0.8 ± 0.01^{u}	2.1 ± 0.03	2	0.0007 (r:u)
RB-BipMut-β-Gal	0.8 ± 0.04	3.6 ± 0.12	3	

[a]*P*-values for the statistical significance of the comparisons indicated for Fn/c_{max} in parenthesis (denoted by superscript letters) are shown.
[b]Results are shown for transport in the presence of exogenously added cytosolic extracts (36 mg/ml), GTP and an ATP-regenerating system, unless otherwise indicated.
[c]Raw data (see Fig. 5B) were fitted for the function $Fn/c\ (t) = Fn/c_{max} \times (1 - e^{-}kt)$, where Fn/c_{max} is the maximal level of nuclear accumulation at steady state, and t is time in minutes. Data represent the mean ± SEM.

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