Negative charge at the protein kinase CK2 site enhances recognition of the SV40 large T-antigen NLS by importin: effect of conformation

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Abstract SV40 large tumor-antigen (T-ag) nuclear import is enhanced by the protein kinase CK2 (CK2) site ($Ser^{111}Ser^{112}$) flanking the nuclear localization sequence (NLS). Here we use site-directed mutagenesis to examine the influence of negative charge and conformation at the site on T-ag nuclear import and recognition by the NLS-binding importin subunits. Negative charge through aspartic acid in place of Ser^{111} simulated CK2 phosphorylation in enhancing nuclear accumulation to levels well above those of proteins lacking a functional CK2 site. This was shown to be through enhancement of T-ag NLS recognition by importin using an ELISA-based assay. Asp^{112}-substituted mutants containing proline at positions 109, 110 (wild-type position) or 111 were compared to assess the role of conformation at the CK2 site. Maximal nuclear import of the protein with Pro^{109} was lower than that of the Pro^{110} derivative, with the Pro^{111} variant even lower, these differences also being attributable to effects on importin binding. All results indicate a correlation of the initial nuclear import rate with the importin binding affinity, demonstrating that NLS recognition by importin is a key rate-determining step in nuclear import.

Key words: Nuclear import kinetics; Confocal laser scanning microscopy; Microinjection; Phosphorylation; SV40 large tumor-antigen

1. Introduction

The first step of nuclear localization sequence (NLS)-dependent nuclear protein import involves recognition of the NLS-containing protein by a heterodimeric complex comprising importin (karyopherin) α and β (importin 58 and 97) [1,2]. The NLS is bound specifically by importin α [3], followed by targeting to the nuclear envelope-localized nuclear pore complex (NPC) through importin β's ability to bind to specific NPC components [4,5]. The subsequent step of translocation into the nucleus through the NPC is an energy-dependent process requiring the monomeric GTP-binding protein Ran [6,7] and other modifying factors.

Although the NLS is an essential determinant for the nuclear entry of most proteins of 45 kDa or greater [8–10], NLS-dependent nuclear import of many proteins has been shown to be regulated by phosphorylation [11,12]. The protein kinase CK2 (CK2) site ($Ser^{111}Ser^{112}$) flanking the NLS of simian virus SV40 large tumor-antigen (T-ag), for example, enhances the rate of nuclear import of T-ag fusion proteins 50-fold [13]. That CK2 may have a more general role in regulating protein nuclear transport is indicated by the fact that many other proteins contain consensus CK2 sites in the vicinity of their respective NLSs [12]; of these, the *Xenopus* nuclear phosphoprotein nucleoplasmin has also been reported to exhibit CK2 enhancement of the nuclear import rate in microinjected oocytes [14]. Using an enzyme-linked immunosorbent assay (ELISA) assay to determine importin-NLS binding, we have recently demonstrated that the enhancement of T-ag nuclear import by the CK2 site is through modulation of importin binding to the T-ag NLS [15]. Whilst phosphorylation at the CK2 site increases the affinity of importin binding [15,16], altering the position of the CK2 site relative to the T-ag NLS reduces importin binding [15].

In this study, we use site-directed mutagenesis to examine the importance to T-ag nuclear import of negative charge and conformation at the CK2 site. Ser^{111} was substituted by aspartic acid, resulting in maximal nuclear accumulation about 50% that of the wild-type protein, much higher than that for proteins lacking a functional CK2 site [17]. Negative charge at position 111 can thus simulate phosphoserine at this site to enhance T-ag nuclear import in similar fashion to negative charge at position 112 [17]. Using an ELISA-based assay [15,16], this effect was demonstrated for the first time to be through modulation of importin binding to the T-ag NLS. To assess the effects of conformation at the CK2 site on T-ag importin binding and nuclear import, a series of mutants was constructed containing proline at either position 109, 110 (wild-type position) or 111 in the context of negative charge at the CK2 site through Asp^{112}. Maximal nuclear accumulation of the Pro^{109} derivative was lower than that of the protein with Pro^{110}, whilst that of the Pro^{111} variant was even lower. These effects were shown to be through effects on the binding of importin to the T-ag NLS. All results indicate a correlation between the nuclear import rate and affinity of NLS recognition by importin, demonstrating that the latter is a key rate-determining step in nuclear import.

2. Materials and methods

2.1. Chemicals

Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Boehringer Mannheim, *p*-nitrophenyl phosphate from Sigma, and the sulfhydryl labelling reagent 5-iodoacetamidofluorescein (IAF) from Molecular Probes. Other reagents were from the sources described previously [15,18–20].

2.2. Plasmid constructs encoding T-ag fusion proteins

All of the T-ag β-galactosidase (β-gal) fusion proteins used in this study contain T-ag amino acid residues 111–135 (see Table 1) or variants thereof, fused N-terminal to the *Escherichia coli* β-galactosidase sequence (amino acids 9–1023; EC 3.2.1.23.37) [13,19,21]. Plasmids pPR16 and pDAJ10, encoding the wild-type T-ag fusion protein CcN-β-Gal and its derivative D2cN-β-Gal which contains $Gly^{111}Asp^{112}$ in place of $Ser^{111/112}$ (see Table 1), have been described previously [13,17,19]. Plasmid pCYX1 encoding T-ag fusion protein

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D1cN-β-Gal with $Asp^{111}Ala^{112}$ in place of the CK2 site $Ser^{111/112}$ of the wild-type T-ag fusion protein was generated from plasmid pDAJ10 [17] using oligonucleotide site-directed mutagenesis (Clontech Transformer Kit) [15,22]. Plasmid M13DAJ2 [17] was used for site-directed mutagenesis to generate plasmids pDAJ11 and pDAJ13, encoding T-ag fusion proteins D3cN-β-Gal and D4cN-β-Gal which contain Pro^{109} and Pro^{111}, respectively, together with Asp^{112} (see Table 1 for amino acid sequences). In the case of plasmid pDAJ13, a *Sau*3A restriction site was inserted at amino acids 110/111, enabling isolation of the mutated 101-bp *Sau*3A fragment, which was then filled-in with Klenow, followed by the addition of 10-mer *Eco*RI linkers into *Eco*RI-partially digested plasmid pPR2 [17,19]. In the case of plasmid pDAJ11, the 5′-overhanging ends were digested with Mung bean nuclease, followed by the ligation of 10-mer *Sma*I linkers into the *Sma*I site of plasmid pPR2 [17,19]. The integrity of all constructs was confirmed by DNA sequencing.

2.3. Fusion protein expression, purification and labelling

Induction of fusion protein expression in *E. coli* using IPTG, protein purification by affinity chromatography, and IAF labelling were all performed as described previously [13,17].

2.4. Cell culture

Cells of the HTC rat hepatoma tissue culture cell line (a derivative of Morris hepatoma 7288C) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum [13,21].

2.5. Nuclear import kinetics

Analysis of nuclear import kinetics at the single cell level using microinjected HTC cells in conjunction with confocal laser scanning microscopy (CLSM; Bio-Rad MRC-600) was as described previously [13,17,19,21]. HTC cells were fused with polyethylene glycol at least 1 h prior to microinjection to produce polykaryons [13,19]. Image analysis of CLSM files using the NIH Image public domain software, and curve fitting were performed as described [18,22].

2.6. Expression of mouse importin 58- and 97-fusion proteins

Expression in *E. coli* of mouse importin 58-glutathione-*S*-transferase (GST) or 97-GST [2] was induced with IPTG, and GST fusion proteins purified and GST-free mouse importin 58 prepared by thrombin cleavage as described previously [15].

2.7. ELISA-based binding assay

Binding of importin subunits to T-ag fusion proteins was quantitated using an ELISA-based assay [15,16]. Briefly, fusion proteins were coated into microtiterplates and incubated with increasing dilutions of precomplexed importin 58/97-GST complex. Bound importin was detected using GST-specific primary and alkaline phosphatase-conjugated secondary antibodies, followed by incubation with the colorimetric substrate *p*-nitrophenyl phosphate. The change of absorbance at 405 nm was followed for 90 min using a plate reader (Molecular Devices), with values corrected by subtracting both the absorbance at 0 min, and the absorbance in wells incubated without importin. T-ag fusion proteins were also subjected to a parallel β-galactosidase ELISA assay (see [15,16,23]) to correct for any differences in coating efficiencies and enable a true estimate of bound importin using a β-galactosidase specific monoclonal antibody as described [15,16,23].

3. Results

3.1. Aspartic acid at position 111 within the CK2 site enhances T-ag nuclear import

Site-directed mutagenesis was used to substitute Ser^{111} of the T-ag CK2 site with aspartic acid in the context of the non-phosphorylatable Ala residue at position 112 (see Section 2), and the kinetics of nuclear import measured (Fig. 1, Table 1). Aspartic acid at position 111 (protein D1cN-β-Gal; see Table 1 for the T-ag sequences of the fusion proteins) enhanced nuclear accumulation compared to proteins lacking a functional CK2 site [17]. Maximal nuclear accumulation of D1cN-β-Gal was about 50% that of the wild-type protein with a functional CK2 site (Fig. 1 and Table 1), comparable to that of D2cN-β-Gal containing aspartic acid in place of Ser^{112} at the CK2 site (Fig. 1; see also [17]). In the case of both D1cN-β-Gal and D2cN-β-Gal [17], the introduced negative charge simulates phosphoserine at the CK2 site in the wild-type protein meaning that negative charge at either Ser^{111} or Ser^{112} can function to enhance nuclear import, consistent with the ability of both to be phosphorylated by CK2 [17], and facilitate nuclear import in the absence of the other (see [13]). The fact that the initial nuclear import rate of both D1cN-β-Gal and D2cN-β-Gal is comparable to that of the wild-type protein (Fig. 1 and Table 1) is consistent with the idea that negative charge at the CK2 site increases the T-ag transport rate [13].

3.2. Conformation at the CK2 site affects T-ag nuclear import kinetics

To assess the importance of conformation at the CK2 site, site-directed mutagenesis was used to generate a series of mutants containing proline at either position 109, 110 (the wild-type position) or 111, in the context of negative charge at the CK2 site through Asp^{112} (see Table 1 for sequence details). The nuclear import kinetics of D2cN-β-Gal (Pro^{110}), D3cN-β-Gal (Pro^{109}) and D4cN-β-Gal (Pro^{111}) were compared, maximal accumulation of all three being higher than that of proteins with non-functional or deleted CK2 sites (see [13,17]), again consistent with the idea that Asp at the CK2 site can enhance T-ag nuclear import (Fig. 1). The maximal nuclear accumulation of the fusion proteins D3cN-β-Gal and D4cN-β-Gal, however, was about 30 and 50% lower than that of

Table 1
In vivo nuclear import kinetics and NLS binding by mouse importin 58/97 of T-ag fusion proteins

Fusion protein[a]	T-ag sequence[b] (CK2 site–NLS)	Nuclear import parameter[c] Initial rate[e]		Binding affinity[d]	
		$F_{n/c}$/min (n)	F_n/c_{max}	B_{max} relative to wild type	K_D
CcN-β-Gal	P^{110}**SSDDE**ATADSQHSTPPKKKRKV^{132}	1.14 ± 0.27 (4)	5.9 ± 0.20	100	6.55 ± 0.2
D1cN-β-Gal	P^{110}**d**aDDEATADSQHSTPPKKKRKV^{132}	0.95 ± 0.18 (8)	2.8 ± 0.10	94 ± 4	12.20 ± 1.1
D2cN-β-Gal	P^{110}g**d**DDEATADSQHSTPPKKKRKV^{132}	1.07 ± 0.30 (8)	3.6 ± 0.20	90 ± 2	12.20 ± 0.1
D3cN-β-Gal	p^{109}gg**d**DDEATADSQHSTPPKKKRKV^{132}	0.77 ± 0.08 (5)	2.4 ± 0.10	110 ± 19	16.65 ± 1.9
D4cN-β-Gal	p^{111} **d**DDEATADSQHSTPPKKKRKV^{132}	0.66 ± 0.06 (4)	1.9 ± 0.02	100 ± 21	24.85 ± 7.1

[a]All fusion proteins contain T-ag sequences fused amino-terminal to *E. coli* β-galactosidase (amino acids 9–1023).
[b]The single letter amino acid code is used, with wild-type T-ag residues shown in capital letters. The CK2 site is bold and underlined, and the NLS double underlined; the CK2 site aspartic acid residues are shown in bold type.
[c]Raw data (see Fig. 1B and not shown) from at least four experiments were fitted as described in Section 2.
[d]Values represent the mean ± S.E.M. for four separate experiments (see Fig. 2).
[e]Initial nuclear import rates were calculated from data (see Fig. 2) up to 3 min after microinjection [22].

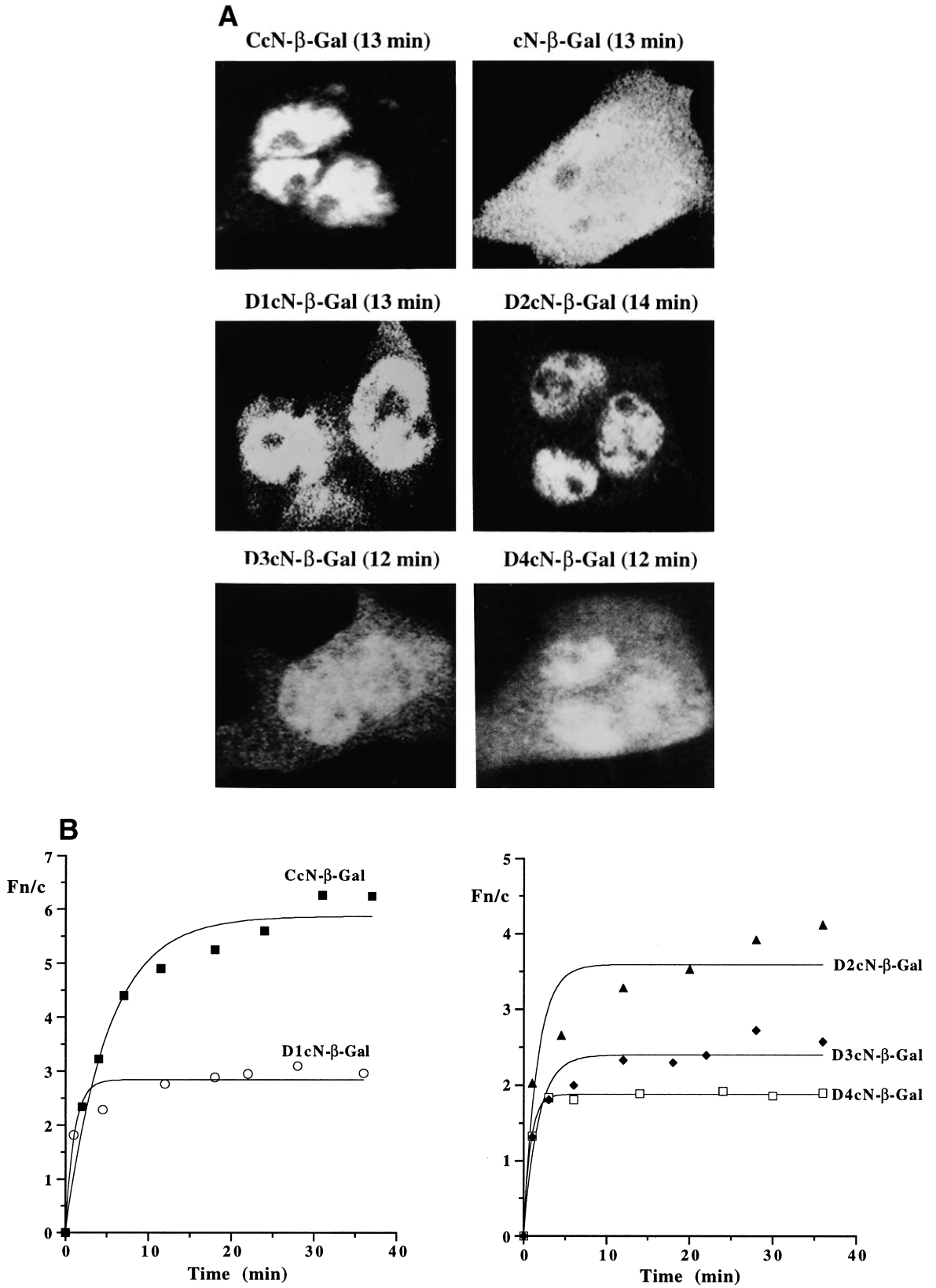

Fig. 1. Influence of mutations at the CK2 site on nuclear import kinetics of T-ag fusion protein derivatives in vivo. A: CLSM images show HTC polykaryons about 13 min after microinjection with T-ag fusion proteins. B: Nuclear import was measured in vivo in HTC cells using CLSM and image analysis as described in Section 2. The measurements represent the average of at least four separate experiments, where each point represents the average of 6–10 separate measurements for each of nuclear (F_n) and cytoplasmic (F_c) fluorescence, respectively, with autofluorescence subtracted. Curves were fitted for the function $F_{n/c}(t)=F_n/c_{max}$ $(1-e^{-kt})$ [13,15,18], where F_n/c_{max} is the maximal nuclear accumulation level, k is the nuclear import rate constant, and t is time in minutes.

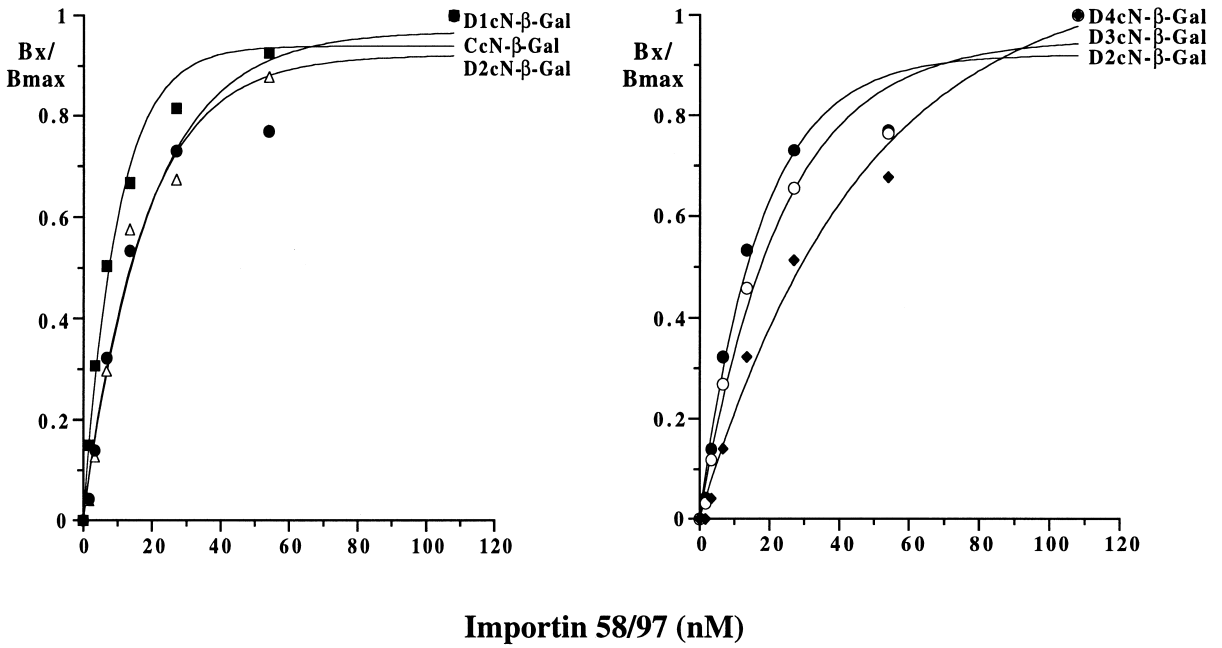

Fig. 2. Influence of mutations at the CK2 site on T-ag NLS binding by importin 58/97. Microtiterplates coated with fusion proteins (0.5 μg/well) were hybridised with increasing amounts of mouse importin 58/97 as described in Section 2. Curves were fitted for the function $B(x) = B_{max}\ (1-e^{-kB})$, where x is the concentration of importin [15,16]. The results are from a single typical experiment representative of four separate experiments. Pooled data are shown in Table 1.

D2cN-β-Gal, respectively, and the initial import rates of D3cN-β-Gal and D4cN-β-Gal about 70 and 60% lower, respectively (Fig. 1B and Table 1). The different position of proline relative to the Asp^{112} presumably alters conformation at the CK2 site suggesting that the precise position of the CK2 site is important for NLS recognition [15].

3.3. *Importin binding properties of proteins containing Asp at the CK2 site*

To test whether the above results could be understood in terms of effects on NLS recognition, the Asp containing proteins were tested for recognition by the NLS-binding importin 58/97 heterodimer using an ELISA-based assay [15,16]. The K_Ds (apparent dissociation constants [15]) of D1cN-β-Gal and D2cN-β-Gal with Asp^{111} or Asp^{112}, respectively, were about two times higher than that of the wild-type protein (Fig. 2, left panel, and Table 1), implying that decreased affinity of importin for the T-ag NLS is the basis of their reduced nuclear import compared to wild type. Interestingly the K_D of the protein derivatives with either Pro^{109} or Pro^{111} was further increased compared to D2cN-β-Gal with proline at the wild-type position (110) (Fig. 2, right panel, and Table 1). This implies that alteration of the position of proline relative to Asp^{112} modulates T-ag nuclear protein import (Fig. 1 and Table 1) through effects on NLS recognition by importin 58/97; proline presumably changes precise conformation at the CK2 site, thereby impairing T-ag interaction with importin 58/97. This is consistent with the idea [15,16] that the CK2 site participates directly in T-ag NLS/importin recognition [16] by interacting directly with the importin 58 subunit.

4. Discussion

This study is the first investigation of the influence on T-ag nuclear import of conformation in the context of negative charge at the CK2 site. Substitution of Ser^{111} by aspartic acid results in nuclear import well above that of proteins lacking a functional CK2 site [13,17,19], similar to the effect

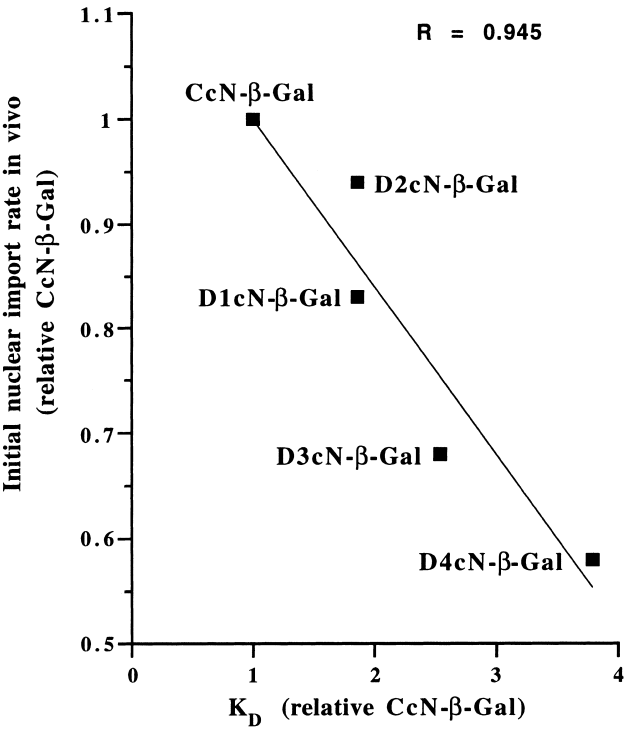

Fig. 3. Correlation between the affinity of NLS recognition by importin 58/97 and initial nuclear import rate of T-ag fusion proteins. Data were calculated from the data in Table 1; R is the regression coefficient.

of Asp^{112} [17]. This indicates that negative charge at either 111 or 112 can simulate phosphoserine at the CK2 site in terms of enhancement of nuclear import. The fact that the initial nuclear import rate of these two mutant proteins is comparable to that of the wild-type fusion protein supports the importance of negative charge at the CK2 site in accelerating T-ag nuclear import. That the affinity of T-ag NLS recognition by the importin subunits in the case of D1cN-β-Gal or D2cN-β-Gal is much higher than that of proteins lacking a functional CK2 site (see [15]) demonstrates directly that negative charge at the CK2 site, normally produced by phosphoserine, is important in NLS binding by importin 58/97 (see [16]). This study shows that the position of proline relative to the CK2 site also influences T-ag nuclear import by affecting the binding of importin to the T-ag NLS. Proline imposes strong restraints on protein conformation [24] through its cyclic structure, which limits the angle of rotation about the α-carbon and nitrogen within a peptide bond and introduces a fixed bend into the peptide chain [25,26]. This study shows that moving proline either closer to or further away from the CK2 site alters recognition of the T-ag NLS by importin and in turn influences the nuclear import kinetics. Clearly, precise conformation at the CK2 site affects interaction with importin, supporting the idea that the CK2 is directly involved in importin binding (see [16]).

All of the results of this study strongly imply a correlation between the nuclear import rate and importin binding affinity. This is borne out by plotting the K_Ds of the various T-ag derivatives described here against their initial rates of nuclear import, relative to that of the wild-type protein (CcN-β-Gal) (Fig. 3), to reveal a direct correlation ($R = 0.945$). The initial import rate is thus directly dependent on the affinity of the NLS interaction with importin (see [15]), with the results of our recent study comparing the T-ag NLS and the bipartite NLS of Rb (retinoblastoma protein) with respect to NLS binding affinity and nuclear import kinetics [23] consistent with this idea. The affinity of binding to importin 58/97 is thus the major determinant of the initial transport rate, implying that NLS recognition and binding by importin in the cytoplasm is a key limiting event for nuclear import. The high importin binding affinity in the case of T-ag with negative charge at the CK2 site presumably results in more rapid kinetics of association of importin with T-ag, and faster docking at the NPC, leading to an accelerated rate of transport.

Aspartic acid-containing phosphorylation-regulated NLSs (prNLSs; [11,12]), such as those described here with intrinsic, high affinity for importin, can be regarded as constitutively active prNLSs where phosphorylation is not required to enhance nuclear import. Since they can confer efficient nuclear entry on other molecules, they may be of use as targeting signals to facilitate the directed transport of DNA molecules encoding a gene of interest to the nucleus of relevant cells in gene therapy or other applications [27]. Alternatively, engineered prNLSs can be used in drug delivery where the therapeutic agent in question has its specific target site of action within the nucleus (see [28]). Work to test the use of such constitutive prNLSs in molecular targeting is currently in progress.

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