The Protein Kinase CK2 Site (Ser\textsuperscript{111/112}) Enhances Recognition of the Simian Virus 40 Large T-antigen Nuclear Localization Sequence by Importin*

Stefan Hübner‡, Chong-Yun Xiao, and David A. Jans§

From the Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, ACT 2601, Australia

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The mechanism by which phosphorylation regulates nuclear localization sequence (NLS)-dependent nuclear protein import is largely unclear. Whereas nuclear accumulation of SV40 large tumor antigen (T-ag) fusion proteins is completely dependent on the T-ag NLS (amino acids 126–132), the rate of nuclear import is increased 50-fold by amino acid residues 111–125 and in particular a site for the protein kinase CK2 (CK2) at serine 111/112. Because the first step of nuclear protein import involves the binding of the NLS by an NLS-receptor complex such as the importin 58/97 heterodimer, we established a novel enzyme-linked immunosorbent assay to test whether NLS recognition is influenced by amino acids amino-terminal to the NLS and the CK2 site. We found that recognition of the T-ag NLS by importin 58/97 was enhanced 10-fold in the presence of amino acid residues 111–125 and strongly dependent on importin 97. A T-ag fusion protein in which the spacer between the CK2 site and the NLS was decreased showed 30% reduced binding by importin 58/97. Maximal nuclear accumulation of this protein was reduced by more than 50%, indicating the physiological importance of the correctly positioned CK2 site. Phosphorylation by CK2 increased the T-ag NLS binding affinity for importin 58/97 by a further 40%. We conclude that flanking sequences and in particular phosphorylation at the CK2 site are mechanistically important in NLS recognition and represent the basis of their enhancement of T-ag nuclear import. This study thus represents the first elucidation of the mechanistic basis of the regulation of nuclear protein import through phosphorylation within a phosphorylation-regulated NLS.

Nuclear protein transport is dependent on specific targeting signals called nuclear localization sequences (NLSs),\textsuperscript{1} defined as the sequences sufficient and necessary for nuclear targeting (1, 2). They are typically short sequences of a single series of basic residues (resembling the NLS of the SV40 large tumor antigen (T-ag)) or of two clusters of basic residues interrupted by a 10–12-amino acid spacer (bipartite NLSs), and they appear to be functional in various protein contexts, suggesting that NLS function is largely independent of secondary/tertiary structure (see Refs. 3–5).

NLS-dependent nuclear protein import can be divided into two steps, the first of which is energy-independent and involves recognition and targeting of the NLS-bearing protein to the nuclear pore complex (NPC) by a heterodimeric protein complex. The NLS is specifically recognized by the smaller subunit of the complex or NLS receptor (6), known variously as importin 58 (7), importin $\alpha$ (8), hSRP1/NP-1 (9), or karyopherin $\alpha$ (10). The larger protein subunit, importin 97 (11), importin $\beta$ (12), karyopherin $\beta$ (13), P97 (14), or Kap95p (15), binds importin 58 specifically but cannot bind the NLS. Its role is to target the importin-NLS carrying protein complex to the NPC through its affinity for NPC components such as nucleoporins (13, 16–18). The second, energy-dependent step of translocation of the import substrate through the NPC into the nucleus (19, 20) requires the GTPase Ran/TC4 (21, 22) and the Ran-interacting factor p10/NTF2 (23, 24).

In addition to the NLS, phosphorylation in the vicinity of NLSs has been shown to play a role in regulating nuclear protein import through modulation of NLS function in either a positive or negative fashion (4, 5). The regulatory modules able to confer regulated nuclear protein import on heterologous proteins, called phosphorylation-regulated NLSs (prNLSs) (4, 5), have been identified for a number of nuclear proteins (25–29). Despite clear evidence that prNLSs regulate nuclear protein import (4, 5), the precise mechanism of the action of phosphorylation in terms of regulating NLS function is largely unclear, especially with respect to prNLSs where phosphorylation enhances transport.

Although nuclear import in the case of T-ag is completely dependent on the T-ag NLS (amino acids 126–132) (30), measurements of nuclear import kinetics at the single cell level have shown that the sequences amino-terminal to the NLS (amino acid residues 111–125) enhance the rate of nuclear transport about 50-fold (31, 32). The sequence primarily responsible for this effect has been demonstrated to be the protein kinase CK2 (previously casein kinase II) site at serine 112 (31, 32), but the mechanism of action of phosphorylation at the site is unknown. The cyclin-dependent kinase site at threonine 124 flanking the NLS also regulates T-ag nuclear import through negative modulation of the maximum level of nuclear accumulation (25), and the prNLSs or regulatory module for T-ag nuclear import is thus called the “CcN motif,” where “C” denotes the CK2 site, “c” denotes the cyclin-dependent kinase site, and “N” denotes the NLS (25, 32).

Because the CK2 phosphorylation event enhancing T-ag
transport clearly occurs in the cytoplasm prior to nuclear import (32), we hypothesized that phosphorylation may modulate the affinity of the interaction of the NLS receptor with the T-ag NLS. In the present study we address this possibility directly by using a novel ELISA-based binding assay to quantitate NLS recognition by importin subunits. We find that NLS binding by importin 58 was strongly dependent on importin 97. Significantly, sequences flanking the T-ag NLS enhance recognition by importin 58/97 by about 10-fold, whereas CK2 phosphorylation increases binding by a further 40%. This modulation of NLS recognition through CK2 phosphorylation presumably constitutes the basis of enhancement of the rate of nuclear import by the CK2 site in vivo.

EXPERIMENTAL PROCEDURES

Table I

<table>
<thead>
<tr>
<th>Fusion protein or peptide</th>
<th>SV40 T-antigen sequence</th>
<th>Presence of functional CK2 site</th>
<th>B_{max} relative to CcN-β-Gal</th>
<th>K_{D} relative to CcN-β-Gal</th>
<th>Initial nuclear import rate relative to CcN-β-Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CcN-β-Gal</td>
<td>SSDEATADSQHSTPPKKKRKV</td>
<td>+</td>
<td>100 (8)</td>
<td>1</td>
<td>100</td>
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<tr>
<td>CN-β-Gal</td>
<td>SSDEATADSQHSTPPKKKRKV</td>
<td>+</td>
<td>104 ± 1.2 (2)</td>
<td>1.17 ± 0.24</td>
<td>100</td>
</tr>
<tr>
<td>sh-CN-β-Gal</td>
<td>SSDEATADSQHSTPPKKKRKV</td>
<td>+</td>
<td>70 ± 5.7 (4)</td>
<td>1.7 ± 0.10</td>
<td>42</td>
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<tr>
<td>sh-CN-β-Gal</td>
<td>SQRTTRPV</td>
<td>+</td>
<td>53 ± 5.2 (5)</td>
<td>2.4 ± 0.18</td>
<td>10.5</td>
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<tr>
<td>CN-β-Gal</td>
<td>agDDEATADSQHSTPPKKKRKV</td>
<td>-</td>
<td>72 ± 1.6 (8)</td>
<td>2.3 ± 0.26</td>
<td>11.9</td>
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<tr>
<td>CN-β-Gal</td>
<td>SSNqATADSQHSTPPKKKRKV</td>
<td>-</td>
<td>74 ± 4.0 (2)</td>
<td>2.4 ± 0.52</td>
<td>11.6</td>
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<tr>
<td>N-β-Gal</td>
<td>SSDEATADSQHSTPPKKKRKV</td>
<td>+</td>
<td>10 ± 1.8 (2)</td>
<td>ND</td>
<td>6.4</td>
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<tr>
<td>Cc-β-Gal</td>
<td>SSDEATADSQHSTPPKKKRKV</td>
<td>+</td>
<td>1.9 ± 1.9 (2)</td>
<td>ND</td>
<td>ND</td>
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<td>Peptides</td>
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<td>pep101Lys</td>
<td>PgSDDEAAdaAQhPaaPPKKKRKV</td>
<td>+</td>
<td>100 (2)</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>pep11Lys</td>
<td>PgSDDEAAdaAQhPaaPPKKKRKV</td>
<td>-</td>
<td>7.3 (1)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pep101Thr</td>
<td>PgSDDEAAdaAQhPaaPPKKKRKV</td>
<td>+</td>
<td>1.2 ± 1.2 (2)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table I—Binding of importin 58/97 to T-ag fusion proteins and peptides as quantified using an ELISA-based binding assay

a All proteins contain T-ag sequences fused amino-terminal to E. coli β-galactosidase (amino acids 9–1023). The single letter amino acid code is used, the NLS is double underlined, and the CK2 site is in bold letters. Small letters indicate deviations from the T-ag sequence.

b Data represent the mean ± S.E., with n in parentheses.

b The initial rates of nuclear import are calculated from Refs. 31, 33 and this study (Fig. 2B).

c ND, not able to be determined.

RESULTS AND DISCUSSION

High Affinity Binding of the T-ag NLS by Importin 58/97 Requires the CK2 Site—Initial experiments to investigate binding of the importin 58/97 subunits to the T-ag NLS (Table I for T-ag sequences of the fusion proteins) using the dot blot technique indicated poor binding of importin 58 to the T-ag NLS in the absence of importin 97, which alone did not bind the T-ag NLS at all (Fig. 1A). This was consistent with the results of others that the importin 58/97 complex constitutes the high affinity NLS receptor (37, 38). Interestingly, importin 58/97 only showed strong binding to the T-ag NLS in the presence, compared with in the absence, of T-ag amino acids 111–125 flanking the NLS (amino acids 126–132) (Fig. 1A). To evaluate this further, the ligand-Western blot technique was used to assess binding of the importin 58/97 complex to various T-ag fusion proteins differing in the T-ag sequences amino-terminal...
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Figure 1. Visualization of binding of importin subunits to T-ag fusion proteins using dot blot (A) and ligand-Western blot (B) analysis. For sequences of the T-ag fusion proteins see Table I. A, immobilized T-ag fusion proteins were probed with 150 nM importin 58-GST/97-GST complex (top), importin 58-GST (middle), or importin 97-GST (bottom), with bound importin subunits or complex detected using a GST-specific antibody in conjunction with an alkaline phosphatase-labeled secondary antibody. B, T-ag fusion proteins were electrophoresed, transferred to nitrocellulose, and probed with 200 nM importin 58/97-GST. Detection of bound importin 58/97-GST was performed as described for the dot blot assay.

Figure 2. Visualization (after 30 min by CLSM) (A) and time course (B) of nuclear import of CcN-β-Gal and sh-CcN-β-Gal using microinjected HTC cells. The measurements are from a single typical experiment from a series of three similar experiments, where each point represents the average of at least five separate measurements for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence, respectively, with autofluorescence subtracted. Curves were fitted for the function \( F(t) = F_{n,\text{max}} - F_{c,\text{max}} \left(1 - e^{-kt}\right) \) (26, 33), where \( F_{n,\text{max}} \) is the maximal level of nuclear accumulation, \( k \) is the rate constant, and \( t \) is time in minutes (25, 31, 32). \( F_{n,\text{max}} \) was 6.72 and 2.37 for CcN-β-Gal and sh-CcN-β-Gal, respectively, whereas the initial rate of nuclear accumulation was 0.57 and 0.87 \( F_{n}/\text{min} \), respectively.

CK2, where sh-CcN-β-Gal was phosphorylated to an identical extent to CcN-β-Gal (stoichiometry of phosphorylation of 2.04 and 2.02 mol P_i/mol tetramer for sh-CcN-β-Gal and CcN-β-Gal, respectively).

Quantitation of T-ag Fusion Protein Binding to the Importin 58/97 Complex—To define the role of the T-ag amino-terminal sequences in NLS binding by importin 58/97 in quantitative terms, a novel ELISA-based binding assay was established (see “Experimental Procedures” for details) whereby T-ag fusion proteins coated onto microtiterplates were incubated with increasing amounts of importin 58 and 97 or importin 58/97 complex. Negligible association of importin 97 with CcN-β-Gal was observed, while only low efficiency binding was evident when importin 58 alone was used compared with that when the importin 58/97 complex was used (not shown), consistent with dot blot analysis (Fig. 1A). The apparent dissociation constant \( K_D \) of CcN-β-Gal for importin 58 alone was 10 times that for the importin 58/97 complex.

The role of the different regions of the CcN motif in recognition of the T-ag NLS by importin 58/97 was assessed using the CN-β-Gal, sh-CcN-β-Gal, CcN-β-Gal, CN-β-Gal, sh-cN-β-Gal, and N-β-Gal T-ag fusion protein derivatives (Fig. 3A and Table I). The amino-terminal sequences flanking the NLS enhanced binding by importin 58/97 whereby binding to wild type protein (CcN-β-Gal) was about 10-fold higher than that to the fusion protein containing the T-ag NLS alone (N-β-Gal) (Table I). Cc-β-Gal bound negligible amounts of importin 58/97, indicating the specificity of the assay (Table I). The sh-cN-β-Gal protein, which lacks the CK2 site, bound importin 58/97 to levels about 50% that of CcN-β-Gal, implying that the CK2 site was necessary for efficient importin binding. The CN-β-Gal, con-
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level of T-ag fusion protein nuclear accumulation (25) by alanine residues (the CN-β-Gal protein) had no significant effect on importin recognition relative to CcN-β-Gal (Table I), further underlining the central importance of the CK2 phosphorylation site in importin binding. Taking the results from Table I (the rightmost data columns), the $K_D$ relative to wild type for the respective fusion proteins correlated very well (regression coefficient of 0.99) with their initial rates of nuclear import in vivo determined either previously (31, 32) or in this study, implying that the apparent dissociation constant is critical in determining the initial rate of nuclear import.

Peptides comprising T-ag amino acids 112–132 (pep101Lys), T-ag amino acids 112–132 with a nonfunctional NLS (pep101Thr), and T-ag amino acids 126–132 (pep11Lys) (Table I) were also tested. Consistent with the studies using the T-ag fusion proteins, binding of the importin 58/97 complex to pep101Lys ($K_D$ of 17 nM) was significantly higher than that to pep11Lys, underlining the importance of the flanking regions to T-ag NLS recognition by importin 58/97. Peptide pep101Thr showed negligible binding (Fig. 3B and Table I), again confirming the specificity of the ELISA-based binding assay.

CK2 Site Phosphorylation Increases the Affinity of Binding of Importin 58/97 to the T-ag NLS—Our previous work had demonstrated that nuclear accumulation of CcN-β-Gal is much more rapid than that of cn-β-Gal$_1$, cn-β-Gal$_2$, and sh-cN-β-Gal, all of which lack a functional CK2 site (Refs. 31 and 32 and Table I). To test whether phosphorylation at the CK2 site influences binding of importin 58/97 to the T-ag NLS, CcN-β-Gal was phosphorylated with CK2 to a stoichiometry of 1.0–1.5 mol P/mol tetrameric T-ag fusion protein, and importin 58/97 binding was quantified using the ELISA-based binding assay. The clear result was that phosphorylation at the CK2 site increased binding of importin 58/97, with about a 40% decrease in the value of the $K_D$ compared with unphosphorylated CcN-β-Gal (Table II). Differences between non- and phosphorylated T-ag proteins were much more marked (up to 2.5-fold differences in the amount of importin 58/97 complex bound) at lower importin 58/97 concentrations (data not shown); it should also be stressed that the quantitative differences here almost certainly underestimate the effect of CK2 phosphorylation in terms of increasing the affinity for importin 58/97 because CK2 phosphorylated CcN-β-Gal tetramers represent a mixed population of unphosphorylated and phosphorylated CK2 sites as a result of the stoichiometry of phosphorylation (i.e. 2.5 mol of CK2 sites per tetramer are not phosphorylated). This almost certainly results in underestimation of the $K_D$ for importin association of CK2-phosphorylated protein.

To confirm that the effects on importin binding did not result from trace amounts of CK2 in the protein samples in the case of phosphorylated protein, control experiments were carried out using sh-cN-β-Gal, N-β-Gal, and Cc-β-Gal preincubated
with CK2. Phosphorylation of sh-cN-β-Gal and N-β-Gal was undetectable, as expected (Table II). There was no increase in the maximal binding of the CK2-preincubated proteins sh-cN-β-Gal and N-β-Gal compared with the nonpreincubated T-ag fusion proteins (Table II). We also found no increased binding of importin 58/97 to CK2 preincubated β-Gal (not shown). Significantly, a 3-fold increase in binding of importin 58/97 to CK2 prephosphorylated Cc-β-Gal was observed relative to nonphosphorylated Cc-β-Gal (Table II). This enhancing effect of CK2 site phosphorylation on binding by importin 58/97 even in the absence of a functional NLS is a further indication that the CK2 site probably participates directly in binding to importin 58.

In conclusion, this study establishes that the mechanistic basis of CK2 site-mediated enhancement of T-ag nuclear import is through the CK2 site and CK2 site phosphorylation increasing the affinity of interaction of the NLS receptor importin 58/97 with the T-ag NLS. As supported by the correlation between the $K_D$ and initial nuclear import rate in vivo (see above), the higher affinity of binding presumably results in more rapid kinetics of association of the NLS receptor with the T-ag transport substrate and faster docking at the NPC ultimately leading to an accelerated rate of transport, which has

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REFERENCES

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