SV40 Large Tumor Antigen Nuclear Import Is Regulated by the Double-stranded DNA-dependent Protein Kinase Site (Serine 120) Flanking the Nuclear Localization Sequence*

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Nuclear localization sequence (NLS)-dependent nuclear import of SV40 large tumor antigen (T-Ag) fusion proteins is regulated by phosphorylation sites for casein kinase II (CKII) and the cyclin-dependent kinase Cdc2 amino-terminal to the NLS (amino acids 126–132). Between the T-Ag CKII and Cdc2 sites is a site (Ser120) for the double-stranded DNA-dependent protein kinase (dsDNA-PK), which we show here for the first time to play a role in regulating T-Ag nuclear import. We replaced Ser120 by aspartic acid or alanine using site-directed mutagenesis and assessed the effects on nuclear transport kinetics both in vitro (microinjected cells) and in vitro (mechanically perforated cells) in HTC rat hepatoma cells. Maximal nuclear accumulation of the Asp120 and Ala120 protein derivatives was approximately 40% and 70% reduced in vitro, respectively, compared with that of the wild type protein, and similarly reduced in vitro, although to a lesser extent. This implies that the dsDNA-PK site regulates the maximal level of nuclear accumulation, normally functioning to enhance T-Ag nuclear transport; the higher accumulation of the Asp120 protein compared with the Ala120 protein indicates that negative charge at the dsDNA-PK site is mechanistically important in regulating nuclear import. The Asp120 protein accumulated in the nucleus at a faster rate than the wild type protein, implying that phosphorylation at Ser120 may also regulate the nuclear import rate. CKII phosphorylation of the Asp120 protein in cytosol or by purified CKII was approximately 30% higher than that of the Ser120 and Ala120 proteins, while negative charge at the CKII site increased dsDNA-PK phosphorylation of Ser120 by approximately 80% compared with wild type, implying physical and functional interactions between the two phosphorylation sites. Quantitation of NLS recognition by the importin 58/97 subunits using an enzyme-linked immunosorbent assay indicated that while the Ala120 protein derivative had a binding affinity very similar to that of wild type, the Asp120 derivative showed 40% higher affinity. In vitro CKII phosphorylation increased importin binding by about 30% in all cases. These results imply that negative charge at the dsDNA-PK site may enhance nuclear import through increasing both NLS recognition by importin subunits, and phosphorylation at the CKII site, which itself also facilitates NLS recognition by importin 58/97.

Nucleocytoplasmic protein transport is central to eukaryotic signal transduction and gene regulation and thereby to processes such as cell proliferation, differentiation, and transformation. Nuclear protein import is an active process (1, 2) dependent on specific targeting signals (nuclear localization sequences; NLSs)1 (3, 4), and comprises at least two steps. In the first, a heterodimeric complex, consisting of the importin 58 or α (NLS-binding protein or receptor) (5, 6) and β (nuclear pore complex-docking protein) (7, 8) subunits, targets the NLS-containing protein to the nuclear pore complex. In this process, importin 58 specifically binds NLSs while importin 97 recognizes the importin 58 subunit, as well as having affinity for particular nuclear pore complex components (9–11). Subsequent translocation into the nucleus is mediated by the GTP-binding protein Ran/TC4 (12) and interacting factor p10/NTF2 (13–15). The final step of nuclear import may involve some sort of feedback loop, which regulates the maximal level of nuclear accumulation (see Ref. 16).

Nuclear protein import is known to be precisely regulated, with phosphorylation as one of the major mechanisms by which this is brought about (17, 18). While enormous progress has been made in the last 2–3 years in terms of characterizing both the steps and factors involved in nuclear import (see Refs. 17 and 18), little is known of the molecular mechanisms by which phosphorylation regulates nuclear protein import. We have shown previously that nuclear import of simian virus SV40 large tumor antigen (T-Ag) β-galactosidase fusion proteins is regulated by the CcN motif (19), which comprises phosphorylation sites for casein kinase II (CKII) and the cyclin-dependent kinase (cdk) Cdc2 together with the NLS. While the NLS is absolutely necessary for nuclear import (20, 21), the CKII site accelerates the rate of nuclear protein import about 50-fold (19, 21), and phosphorylation at the Cdc2 site reduces the maximal nuclear accumulation about 70% (19). The mechanistic basis of the CKII-site enhancement of T-Ag fusion protein import appears to be through phosphorylation increasing the affinity of interaction of the T-Ag NLS with importin 58/97 (see Ref. 22), while indirect evidence suggests that Cdc2 site phosphorylation may increase T-Ag affinity for a cytoplasmic anchor or inhibitor protein (see Ref. 19).

Interestingly, there is a phosphorylation site for the double
dsDNA-PK Regulates SV40 T-antigen Nuclear Import

stranded DNA dependent protein kinase (dsDNA-PK) at Ser^{120} (23, 24) between the CcN motif CKII and Cdc2 sites. The dsDNA-PK is a mostly nuclear localized serine/threonine protein kinase (25, 26), which has been shown to phosphorylate substrates such as the heat shock protein HSP90 (27), Ku autoantigen (26), and the tumor suppressor p53 (26, 28, 29). It is involved in the repair of DNA strand breaks (30, 31), DNA replication (32, 33), transcription (34–36), and D/VJ recombination of immunoglobulin genes (37, 38). To assess whether the dsDNA-PK site of T-Ag regulates its nuclear import, we set out to replace Ser^{120} by either aspartic acid or alanine using site-directed mutagenesis. Nuclear import measurements of the mutant derivatives both in vivo and in vitro indicate for the first time that Ser^{120} is important for maximal nuclear accumulation, as well as affecting the nuclear import rate. The basis of this appears to be due to Ser^{120} modulating T-Ag NLS recognition by the importin 58/97 dimer. Significantly, the CKII and dsDNA-PK phosphorylation sites appear to interact at the level of both phosphorylation and enhancement of T-Ag nuclear import, and we propose a model where the sites are in close proximity to one another and participate directly in binding to importin. Since many nuclear proteins contain dsDNA-PK sites in the vicinity of their NLSs, the dsDNA-PK may have a more general role in modulating nuclear protein import.

MATERIALS AND METHODS

Chemicals and Reagents—Isopropyl-1-thio-β-D-galactopyranoside, β-galactosidase (EC 3.2.1.23.37), and polyethylene glycol 1500 were from Boehringer Mannheim, and 5-iodacetamidofluorescein was from Molecular Probes. p-Aminobenzyl-1-thio-β-D-galactopyranoside-agarose was from Sigma. Purified rat liver CKII, the peptide substrate specific for dsDNA-PK (Glu-Pro-Pro-Leu-Ser-Glu-Glu-Ala-Asp-Leu-Trp-Lys-Val), and dsDNA-PK from HeLa nuclear extracts were from Promega. Other reagents were from the sources described previously (19, 21, 39, 40).

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% fetal calf serum as described previously (19, 21).

β-Galactosidase Fusion Proteins—The T-Ag sequences of the β-galactosidase fusion proteins used in this study are described in Fig. 1. All contain T-Ag amino acids 111–135 or variants thereof, encompassing the CcN motif (comprising CKII (Ser^{113/117}) and ckd) (Thr^{124}) sites and the NLS (Pro-Lys-Lys-Arg-Lys-Val^{132})), fused NH_2-terminal to the Escherichia coli β-galactosidase enzyme sequence (amino acids 9–1023). Of these, the CKII site mutants CN-β-Gal and DcN-β-Gal and Cdc2 site mutant CN-β-Gal have been described previously (19, 21, 40). Plasmid pPR16 encoding the CcN-β-Gal fusion protein (20) was used as a template to substitute the dsDNA-PK site Ser^{120} with either Ala or Asp using oligonucleotide site-directed mutagenesis (CLONTECH transformer kit; see Refs. 22 and 40).

Fusion Protein Expression—1 mm isopropyl-1-thio-β-D-galactopyranoside was used in media to induce fusion protein expression in E. coli strain MC1060 (41). Proteins were purified by affinity chromatography and labeled with 5-iodacetamidofluorescein as described (20).

Nuclear Import Kinetics—Analysis of nuclear import kinetics at the single cell level using either microinjected (in vivo) or mechanically perforated (in vitro) HTC cells in conjunction with confocal laser scanning microscopy (CLSM; Bio-Rad MRC-600) was described as previously (19, 21, 39, 42). In the case of microinjection (Narshige IM-200 pneumatic microinjector and Leitz micromanipulator), HTC cells were fused with polyethylene glycol about 1 h prior to microinjection to produce polycaryons (21). Reticulocyte lysate (Promega) was used as the source of the cytosol for the in vitro assay (42). Image analysis of CLSM files (using the NIH Image public domain software) and curve fitting were performed as described (29, 40).

Phosphorylation of Reticulocyte Lysate or by Purified CKII or dsDNA-PK—Phosphorylation of T-Ag fusion proteins in reticulocyte lysate or HTC cytosolic and nuclear extracts (prepared as described; Refs. 40 and 42) was performed as described previously (40, 42). In vitro phosphorylation using purified CKII was as described previously (40, 42), while phosphorylation using purified dsDNA-PK was performed essentially as described by Lees-Miller et al. (26, 28) using 0.5 unit/μl dsDNA-PK for 1 μg/μl SV40 T-Ag β-Gal fusion protein and 100 ng/μl salmon sperm DNA in the assay buffer: 12.5 mM Hepes-KOH (pH 7.5), 1 mM spermidine, 6.5 mM MgCl_2, 10% glycerol, 0.05% Nonidet P-40, 25 mM KCl, 0.5 mM dithiothreitol, 0.2 mM ATP containing 0.5 μM [γ-^32P]ATP at 30 °C.

dsDNA-PK activity in cell extracts was measured using the dsDNA-PK specific peptide substrate (see above) in the presence of 1 μM cAMP-dependent protein kinase inhibitor 5–24 (42) and 100 ng/μl salmon sperm DNA in the assay buffer: 50 mM Hepes (pH 7.5), 100 mM KCl, 10 mM MgCl_2, 2 mM EGTA, and 0.1 mM EDTA for 30 min at 30 °C. Analysis was performed as described previously (40, 43) through spotting onto Whatman P-81 filters, washing with orthophosphoric acid, and scintillation counting. In the case of fusion proteins, the stoichiometry of phosphorylation was quantitated, subsequent to SDS-gel electrophoresis, using a Molecular Dynamics PhosphorImager, where exposure values were converted to absolute values through identical analysis of in vitro phosphorylated samples of predetermined stoichiometry of phosphorylation (40, 43).

Whereas CKII peptide phosphorylation was exclusively cytosolic (16.7 units/μg), a low but significant level of dsDNA-PK peptide phosphorylation activity was found in HTC cytosol (4.7 units/μg), about 14% of that found in nuclear extracts (34.3 units/μg). Cdc2 peptide (Pro-Lys-Thr-Pro-Lys-Ala-Lys-Lys-Leu; Upstate Biotechnology Inc., Lake Placid, NY) phosphorylating activity was exclusively nuclear (15.4 units/μg) in HTC extracts.

ELISA-based Binding Assay—An ELISA-based binding assay was used to examine the binding affinity between importin subunits (mouse importin 58/97-glutathione S-transferase (GST) fusion proteins, expressed as described; Refs. 5, 7, and 22) and unphosphorylated and CKII prephosphorylated T-Ag fusion proteins as described (22). Briefly, T-Ag fusion proteins were coated onto 96-well microtiter plates, blocked with BSA, hybridized with various dilutions of precomplexed importin 58/97 (the latter as a GST fusion protein), and then successive incubations carried out with goat anti-GST primary (Pharmacia Biotech Inc.) and alkaline phosphatase-coupled rabbit anti-goat secondary (Sigma) antibodies. Quantitation was performed using the substrate p-nitropheno- nyl phosphate (Sigma), and the change of absorbance at 405 nm followed on a plate reader (Molecular Devices), with values corrected by subtracting both the absorbance at 0 min, and the absorbance in wells incubated without importin 58/97/GST complex.

To correct for differences in coating, T-Ag fusion proteins were subjected to a parallel β-galactosidase ELISA assay (22) using a β-galac- tosidase specific monoclonal antibody (Promega) together with an anti-mouse alkaline phosphatase-conjugated secondary antibody and p-nitrophenoylethylamine (Sigma) as substrate. Measurements for importin binding were ultimately corrected for any differences in coating efficiencies quantified in the β-galactosidase-based ELISA assay, to enable a true esti- mate of bound importin to be made.

RESULTS

The dsDNA-PK Site at Ser^{120} Is Phosphorylated in T-Ag Fusion Proteins and Regulates Their Nuclear Import—To assess the role of the dsDNA-PK phosphorylation site (Ser^{120}) between the CcN motif CKII and cdk sites in nuclear protein import, site-directed mutagenesis of existing T-Ag fusion protein expression plasmid constructs was performed to substitute Ser^{120} by either alanine or aspartic acid (see “Materials and Methods” for construct details; see Fig. 1 for sequence details). Phosphorylation of the proteins was tested using purified dsDNA-PK from HeLa nuclear extracts. In contrast to the Ala^{120} and Asp^{120} fusion protein derivatives, those containing an intact Ser^{120} were phosphorylated to a marked extent, implying that phosphorylation by dsDNA-PK was specific to Ser^{120} (Fig. 2, and data not shown). Interestingly, the Dcn-β-Gal (52S) protein, which contains aspartic acid in place of Ser^{120} and hence possesses negative charge at the CKII site (see Fig. 1) exhibited about 75% increased phosphorylation by dsDNA-PK compared with the wild type protein CcN-β-Gal (Ser^{120}) (Fig. 2). Increased dsDNA-PK phosphorylation was also exhibited by a protein containing aspartic acid at position 111 (data not shown), supporting the idea that negative charge at the CKII site enhanced phosphorylation of Ser^{120}. Like both CcN-β-Gal (Ala^{120}) and CcN-β-Gal (Asp^{120}), T-Ag fusion pro-
tein CN-β-Gal (Ala120), which lacks Ser120 (as well as Ser123-Thr124 of the Cdc2 site; Ref. 21), was phosphorylated to a negligible extent by dsDNA-PK (data not shown).

The kinetics of nuclear import of the Asp120 and Ala120 T-Ag CcN-β-Gal (Ser120) fusion protein derivatives were compared with those of the wild type CcN-β-Gal (Ser120) protein both in vivo and in vitro (Figs. 3 and 4; Table I). The maximal nuclear accumulation of the Asp120 and Ala120 proteins was about 60 and 30% that of wild type, respectively, in vivo, and was also reduced in vitro (by 30 and 40%, respectively, relative to wild type; see Fig. 4 and Table I). The transport rate of the Asp120 protein was between 2 and 2.5 times faster than that of the wild type CcN-β-Gal T-Ag fusion protein (Ser120) (see Table I). The dsDNA-PK site thus appeared to enhance T-Ag nuclear import, affecting both the maximal level and rate of nuclear accumulation.

The dsDNA-PK Site Influences Phosphorylation at the T-Ag CKII Site—Since the results for dsDNA-PK phosphorylation (Fig. 2) implied that negative charge at the CKII site influenced phosphorylation at Ser120, we decided to test whether the effects of mutations at the dsDNA-PK site on nuclear import might be the direct or indirect result of inhibition of phosphorylation at the CKII site. In vitro phosphorylation experiments were accordingly carried out both using cytosolic extracts and purified rat liver CKII and a variety of T-Ag fusion proteins (Fig. 5). Significantly, CcN-β-Gal (Asp120) was found to be phosphorylated to a 30% higher extent in cytosol than both CcN-β-Gal (Ser120) and CcN-β-Gal (Ala120) (Fig. 5, left panel). The CKII site-mutated fusion protein cN-β-Gal (Ser120) exhibited negligible phosphorylation, implying that, as observed previously (40, 42), more than 90% of the observed cytosolic phosphorylation was attributable to CKII. Results using purified CKII (Fig. 5, right panel) were essentially identical to those using cytosol, CcN-β-Gal (Asp120) exhibiting about 30% increased phosphorylation compared to wild type. The results clearly indicated that negative charge at position 120 did not inhibit phosphorylation at the CKII site, but instead increased it markedly, suggesting that dsDNA-PK phosphorylation at 120 could potentially regulate nuclear import through increasing CKII-site phosphorylation. Asp111/112 enhancement of Ser120 phosphorylation (previous section), strongly implying close proximity and functional interaction of the CKII and dsDNA-PK sites.

The dsDNA-PK Site Participates in NLS Binding by Importin 58/97—One possibility to explain the observed faster nuclear import kinetics of CcN-β-Gal (Asp120) compared to CcN-β-Gal (Ser120) is that the dsDNA-PK site is involved in binding

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**FIG. 1.** Sequence of the SV40 T-Ag fusion proteins used in this study. All fusion proteins contain SV40 T-Ag sequences fused NH2-terminal to E. coli β-galactosidase (amino acids 9–1023). The single-letter amino acid code is used, whereby the NLS is underlined and the phosphorylation sites for CKII, Cdc2, and the dsDNA-PK shaded. **Up**ercase letters indicate T-Ag sequence. The cN-, CN-, CcN-, and DcN-β-Gal fusion proteins have been described previously (19, 21, 40).

**FIG. 2.** Specific phosphorylation of T-Ag fusion proteins at Ser120 by purified dsDNA-PK from HeLa nuclear extracts. Proteins (see Fig. 1 for T-Ag sequence details) were phosphorylated for 10 (A) or 20 min as indicated at 30 °C prior to analysis after gel electrophoresis and autoradiography (A), or analysis using a PhosphorImager (B), as described under “Materials and Methods.” Results are from a single typical experiment from a series of similar experiments. dsDNA-PK possessed 12 ± 3 pmol of P/min of phosphorylation activity for the dsDNA-PK-specific peptide substrate Glu-Pro-Pro-Leu-Ser-Gln-Glu-Ala-Phe-Ala-Asp-Leu-Trp-Lys-Lys in the same experiment.

**FIG. 3.** Visualization of T-Ag fusion protein nuclear accumulation in vivo and in vitro. CLSM images of CcN-β-Gal (Ser120), CcN-β-Gal (Asp120), and CcN-β-Gal (Ala120) are from in vivo and in vitro nuclear transport assays after 22 (steady state time point) and 9 min (early time point), respectively, as indicated. Scale bars are as indicated.
The dsDNA-PK site within the CcN motif regulates T-Ag nuclear import, and the first demonstration of the possible mechanism of action of phosphorylation at the site. The dsDNA-PK site of T-Ag is important for nuclear import is shown by the fact that maximal nuclear accumulation is reduced in Asp120 and Ala120 mutants both in vivo and in vitro. Nuclear accumulation of the Asp120 mutant is higher than that of the Ala120 mutant, implying that negative charge at position 120 (normally supplied by phosphorylation of wild type Ser120) is mechanistically important in the effect on nuclear import, thus being quite comparable to similar findings for the T-Ag CKII and cdk CcN motif sites (see Refs. 19 and 40). An intriguing result was that the Asp120 mutant shows a faster import rate than the wild type Ser120 protein, indicating that negative charge at position 120 also regulates the nuclear import rate.

Phosphorylation at the CKII site either in reticulocyte lysate or using purified CKII was elevated in the case of the Asp120 mutant, compared with wild type and the Ala120 mutant, meaning that the increased import rate on the part of the Asp120 mutant may in part be due to increased phosphorylation at the CKII site which is known to accelerate nuclear import (21, 40).

**TABLE I**

| Fusion protein\(^a\) | In vivo | Importin 58/97 binding parameter\(^b\) | | In vitro | Importin 58/97 binding parameter\(^b\) |
|----------------------|---------|----------------------------------------|-----------------|---------|----------------------------------------|
|                      | \(k\) \(\times 10^{-3}\) | \(F_{n/c_{max}}\) | \(k\) \(\times 10^{-3}\) | \(F_{n/c_{max}}\) | \(B_{max}\) | \(K_{D}\) | \(B_{max}\) | \(K_{D}\) |
| CcN-β-Gal (Ser\(^{120}\)) | 145 ± 54 | 7.99 ± 0.78 | 54 ± 14 | 5.82 ± 0.27 | 100 | 1 | 101 ± 17 | 0.74 ± 0.04 |
| CcN-β-Gal (Ala\(^{120}\)) | 905 ± 359 | 2.60 ± 0.14 | 76 ± 10 | 3.32 ± 0.22 | 91 ± 12 | 0.92 ± 0.11 | 117 ± 17 | 0.80 ± 0.20 |
| CcN-β-Gal (Asp\(^{120}\)) | 428 ± 120 | 5.02 ± 0.22 | 108 ± 17 | 4.01 ± 0.21 | 96 ± 4.2 | 0.66 ± 0.02 | 93 ± 9.4 | 0.81 ± 0.06 |

\(^a\) The T-Ag β-galactosidase fusion protein sequences are shown in Fig. 1.  
\(^b\) Raw data (see Fig. 4, and data not shown) were fitted for the function \(F_{n/c} (t) = F_{n_{max}}/c_{max} \times (1 - e^{-k_{t}})\) (19, 22, 42), where \(t\) is time in minutes. Results shown are averaged over two separate experiments with the S.E. (derived from the curve fits) indicated.

**DISCUSSION**

This study represents the first demonstration that the dsDNA-PK site within the CcN motif regulates T-Ag nuclear transport, and the first determination of the possible mechanism of action of phosphorylation at the site. The dsDNA-PK site of T-Ag is important for nuclear import is shown by the fact that maximal nuclear accumulation is reduced in Asp\(^{120}\) and Ala\(^{120}\) mutants both in vivo and in vitro. Nuclear accumulation of the Asp\(^{120}\) mutant is higher than that of the Ala\(^{120}\) mutant, implying that negative charge at position 120 (normally supplied by phosphorylation of wild type Ser\(^{120}\)) is mechanistically important in the effect on nuclear import, thus being quite comparable to similar findings for the T-Ag CKII and cdk CcN motif sites (see Refs. 19 and 40). An intriguing result was that the Asp\(^{120}\) mutant shows a faster import rate than the wild type Ser\(^{120}\) protein, indicating that negative charge at position 120 also regulates the nuclear import rate.

Phosphorylation at the CKII site either in reticulocyte lysate or using purified CKII was elevated in the case of the Asp\(^{120}\) mutant, compared with wild type and the Ala\(^{120}\) mutant, meaning that the increased import rate on the part of the Asp\(^{120}\) mutant may in part be due to increased phosphorylation at the CKII site which is known to accelerate nuclear import (21, 40).
The initial step of nuclear import involves binding by the importin heterodimer (5–11). Results from our ELISA-based binding assay, which enables direct measurement of the affinity of NLS recognition by importin (see also Ref. 22), indicate that the Asp120 mutant has a higher affinity for importin 58/97. This implies that Ser 120 may be directly involved in contacting importin 58 in conjunction with the NLS and that negative charge at the site increases the binding affinity. Importin binding can be further increased by CKII phosphorylation, consistent with our previous observations (see Ref. 22). The clear implication is that the amino acids flanking the T-Ag NLS, including the dsDNA-PK and CKII sites, determine the affinity of NLS binding by importin 58/97. Negative charge at position 120 is thus able to enhance the nuclear protein import rate through directly increasing the affinity of binding of the NLS by importin 58/97, as well as through facilitating phosphorylation at the CKII site and thereby further increasing the affinity of binding between the NLS and importin 58/97. This suggests that phosphorylation at Ser120 could also regulate T-Ag nuclear import through modulation of NLS recognition. Although the dsDNA-PK is known to be largely nuclear (see also Ref. 44) and phosphorylation at Ser120 may therefore mostly take place in the nucleus, we observed significant phosphorylation of a specific dsDNA-PK peptide substrate in cytosol (see “Materials and Methods”), implying that Ser120 phosphorylation prior to nuclear entry, thereby enhancing CKII site phosphorylation and interaction with importin 58/97, may occur under physiological conditions. Consistent with this, T-Ag has been shown to be phosphorylated at Ser120 to some extent in the cytosol in SV40 virus infected cells (see Refs. 45 and 46 with respect to data for phosphopeptides 7 and 11). Although we are formally unable at this stage to assert that the cytosolic phosphorylation activity is due to dsDNA-PK rather than to another kinase of similar properties and specificity (e.g., a dsDNA-PK of a different subunit make-up; see Ref. 44), what is clear from this study is that the dsDNA-PK site positively regulates both T-Ag NLS recognition by importin and T-Ag nuclear import. Nuclear
phosphorylation of Ser\(^{120}\) by dsDNA-PK may also play a role in nuclear import in the light of the fact that, based on our results here, CKII site phosphorylation in the cytoplasm would make Ser\(^{120}\) a better phosphorylation site for dsDNA-PK in the nucleus. This might constitute the feedback communication between nucleus and cytoplasm indicated by our previous study (16). The state of dsDNA-PK site phosphorylation may conceivably be important for the import step of translocation into the nucleus, during which the NLS-containing protein enters the nucleus complexed to the importin 58 subunit alone (10, 47) rather than to the higher affinity importin 58/97 dimer; in this case, higher affinity binding to importin 58 through negative charge at Ser\(^{120}\) may be critical. Dephosphorylation at the dsDNA-PK site seems unlikely to be mechanistically important in regulating T-Ag nuclear import (see also Refs. 19 and 40), since the nuclear import properties of the Asp\(^{120}\) fusion protein, in the absence of c-Jun and VirE2, are in close proximity to consensus CKII sites (see legend to Table II). Whether these sites indeed regulate nuclear import of their respective proteins will require direct experimentation.

Based on our results here and our previous study demonstrating the role of the CKII site in NLS binding by importin (22), it is possible to speculate that the dsDNA-PK and CKII sites are both very close to one another in spatial terms and are directly involved in importin recognition, as shown schematically in Fig. 7. Close proximity of the sites as depicted in Fig. 7A explains the fact that negative charge at Ser\(^{120}\) increases phosphorylation at the CKII site; the concentration of negative charge through both the dsDNA-PK site and the CKII site substantially raises the affinity of binding. Additionally, CKII phosphorylation, increasing negative charge at the site, markedly increases the affinity of binding. Consistently, CKII phosphorylation increases importin binding in the case of a T-Ag fusion protein containing the complete NH\(_2\)-terminal flanking region but retaining a mutated (non-functional) NLS (22), implying that the phosphorylated CKII site in the presence of the dsDNA-PK site, may have low but significant affinity for im-

| Protein                  | dsDNA-PK site\(^{a}\) | NLS\(^{b}\)           |
|-------------------------|------------------------|-----------------------|
| SV40 T-Ag               | DS\(^{111}\)QH (23)    | PKKKRKC\(^{132}\)      |
|                         | DS\(^{108}\)Q8\(^{132}\)Q (23) |                        |
|                         | QSS\(^{117}\)Q (23)    |                        |
| p53 (human)             | ES\(^{108}\)Q1 (26, 28) | QFPPKKKPL\(^{132}\)    |
|                         | QLS\(^{108}\)Q (26, 28) |                        |
| c-Fos (human)           | QSS\(^{108}\)Q (26)    | KRRRRR-(12-aa spacer)- |
|                         | SS\(^{108}\)NEPSS\(^{98}\)S (48) | KRRK\(^{160}\)        |
| c-Jun (human)           | MES\(^{110}\)S\(^{98}\)Q (49) | RRKR-(10-aa spacer)-RKKK\(^{74}\) |
| serum response factor    | NAFS\(^{110}\)Q\(^{98}\)A (50) | RRGLKR\(^{140}\)      |
| (human)                 | SHS\(^{146}\)Q\(^{98}\)Q (50) |                        |
| progestosterone receptor | T\(^{108}\)Q\(^{98}\)D (25) | RK-(10-aa spacer)-RFFK\(^{895}\) |
| (human)                 | S\(^{121}\)T\(^{98}\)G (25) |                        |
| Rb (human)              | ET\(^{110}\)Q\(^{98}\)E | KR-(11-aa spacer)-KKLR\(^{577}\) |
| Lamin C (human)         | ET\(^{108}\)Q\(^{98}\)E |                        |
| Agrobacterium tumefaciens\(^d\) | QT\(^{110}\)E | SVTKKRK\(^{652}\) |
| VirE2 protein           | IQ\(^{110}\)T\(^{98}\)E |                        |
|                         | SD\(^{106}\)T\(^{98}\)E | KLR-(12-aa spacer)- |
|                         | E/D\(^{106}\)Q/T\(^{98}\)E | RREIQKR\(^{150}\)    |
|                         | E/D\(^{106}\)Q/T\(^{98}\)E | RAIKTYQSDTEIKLKSK\(^{209}\) |
| Consensus               |                        |                      |

\(^{a}\) References refer to confirmed sites (ST in bold type) (see also Ref. 44). The single-letter amino acid code is used, numbers indicating the residue number within the amino acid sequence of the respective proteins; numbered S/Ts are the putative or confirmed phosphorylated residues.

\(^{b}\) For confirmed NLSs, refer to Refs. 17 and 18. aa, amino acid.

\(^{c}\) Proteins possessing consensus CKII sites in close proximity to their dsDNA-PK sites are indicated: S\(^{111}\)S\(^{112}\)DE (T-Ag); S\(^{7}\)Q\(^{S}\) (p53); S\(^{363}\)S\(^{368}\)N\(^{E}\) (c-Fos); and S\(^{108}\)D\(^{109}\) TE (VirE2), where the CKII site serine is underlined.

\(^{d}\) Bacterial sequence capable of nuclear targeting in plant cells (see Refs. 17 and 18).
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