# p34$^{cdc2}$-mediated Phosphorylation at T$^{124}$ Inhibits Nuclear Import of SV-40 T Antigen Proteins

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***Abstract.*** The nuclear import of transcription regulatory proteins appears to be used by the cell to trigger transitions in cell cycle, morphogenesis, and transformation. We have previously observed that the rate at which SV-40 T antigen fusion proteins containing a functional nuclear localization sequence (NLS; residues 126–132) are imported into the nucleus is enhanced in the presence of the casein kinase II (CK-II) site S$^{111/112}$. In this study purified p34$^{cdc2}$ kinase was used to phosphorylate T antigen proteins specifically at T$^{124}$ and kinetic measurements at the single-cell level performed to assess its effect on nuclear protein import. T$^{124}$ phosphorylation, which could be functionally simulated by a T-to-D$^{124}$ substitution, was found to reduce the maximal extent of nuclear accumulation whilst negligibly affecting the import rate. The inhibition of nuclear import depended on the stoichiometry of phosphorylation. T$^{124}$ and S$^{111/112}$ could be phosphorylated independently of one another. Two alternative mechanisms were considered to explain the inhibition of nuclear import by T$^{124}$ phosphorylation: inactivation of the NLS and cytoplasmic retention, respectively. Furthermore, we speculate that in vivo T$^{124}$ phosphorylation may regulate the small but functionally significant amount of cytoplasmic SV-40 T antigen. A sequence comparison showed that many transcription regulatory proteins contain domains comprising potential CK-II-sites, cdc2-sites, and NLS. This raises the possibility that the three elements represent a functional unit regulating nuclear protein import.

CLOSE correlations between the nuclear cytoplasmic distribution of certain transcription factors (Lenardo and Baltimore, 1989; Shirakawa and Mizel, 1989; Ghosh et al., 1990; Nasmyth et al., 1990), morphogens (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989) or oncogene products (Van Etten et al., 1989; Roux et al., 1990), and functional states of cell cycle, morphogenesis, and transformation have recently been observed. This suggests that some steps in gene expression and signal transduction may be regulated by a precisely scheduled import of transcriptionally active proteins into the nucleus.

Components involved in nuclear protein import (for reviews see Roberts, 1989; Silver, 1991; Garcia-Bustos et al., 1991) include the nuclear pore complex (Davis and Blobel, 1987; Wozniak et al., 1989; Akey et al., 1989; Reichelt et al., 1990; Starr et al., 1990), which mediates both passive and active transport across the nuclear envelope, and the nuclear localization sequence (NLS)[1] (Kalderon and Smith, 1984; Kalderon et al., 1984; Lanford and Butel, 1984; Hall et al., 1984; Silver et al., 1984). NLS-binding proteins (Yoneda et al., 1988; Adam et al., 1989; Benditt et al., 1989; Li and Thomas, 1989; Lee and Mélèse, 1989; Silver et al., 1989) have also recently been identified, although their specificity and subcellular location remain unclear.

It is well documented that the specificity of nuclear protein import is determined by the NLS (Kalderon et al., 1984). Little is known, however, about the regulation of import kinetics, which may be the critical parameter triggering transitions of cell cycle, morphogenesis, or transformation. Based on an examination of published data (Peters, 1986) and our data concerning the nuclear import of nucleoplasmin (Schulz and Peters, 1987), we have previously suggested that nuclear import of NLS-bearing proteins may be modulated by covalent modifications of the transported proteins. Using hybrid proteins in which fragments of the SV-40 T antigen were fused to the $NH_2$-terminus of *Escherichia coli* β-galactosidase we confirmed (Rihs and Peters, 1989) that the NLS of the SV-40 T antigen (PKKKRKV$^{132}$) is necessary and sufficient to target large tetrameric proteins (~480 kD) to the nucleus. However, the presence of a sequence comprising the NLS together with $NH_2$-terminal flanking sequences greatly enhanced the rate of nuclear protein import. Recent analysis (Rihs et al., 1991; Ackermann et al., 1991)

1. *Abbreviations used in this paper*: CK-II, casein kinase II; CLSM, confocal laser scanning microscopy; HTC, hepatoma tissue culture; IAF, 5-iodacetamido fluorescein; μ, stoichiometry of phosphorylation; NLS, nuclear localization sequence.

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revealed that this effect is elicited by phosphorylation at the CK-II site $S^{111/112}$.

The T antigen sequence contained in our fusion proteins, in addition to the casein kinase II (CK-II) site, bears a second phosphorylation site, $T^{124}$. In authentic SV-40 T antigen this site is specifically phosphorylated by the cell division control (or histone H1) kinase $p34^{cdc2}$ (McVey et al., 1989). The cdc2 kinase has recently been the focus of great interest, both through its central role in initiating mitotic and meiotic M phase (for reviews, see Moreno and Nurse, 1990; Lewin, 1990; Draetta, 1990; Nurse, 1990), and through its intriguing array of protein substrates, relating to both mitotic activation (Peter et al., 1990; Heald and McKeon, 1990; Chou et al., 1990) as well as transformation and oncogenesis (McVey et al., 1989; Kipreos and Wang, 1990; Morgan et al., 1989; Bischoff et al., 1990).

In this study purified $p34^{cdc2}$ from HeLa cells was used to phosphorylate SV-40 T antigen fusion proteins at $T^{124}$ in vitro. The effects of $T^{124}$ phosphorylation on nuclear protein import were measured both in vivo and in vitro. We observed that $T^{124}$ phosphorylation strongly inhibited nuclear protein import, an effect that could be simulated by a $T^{124}$-to-$D^{124}$ substitution. In addition, the effects of sequential phosphorylation at $T^{124}$ and $S^{111/112}$ on nuclear import were studied. The results suggest that cdc2 and CK-II sites act independently of each other and control different steps of the nuclear import process.

## Materials and Methods

### Chemicals and Reagents

5-iodacetamido-fluorescein (IAF) was from Molecular Probes Inc. (Eugene, OR), [$\gamma^{32}$P]ATP from DuPont-New England Nuclear (Bad Homburg, Germany), and the cAMP-PK inhibitor peptide PK-I 5-24 from Bachem (Heidelberg, Germany). All other reagents were from the sources previously described (Ackermann et al., 1991; Rihs et al., 1991).

### SV-40 T Antigen/β-Galactosidase Fusion Proteins

The construction of plasmids expressing the SV-40 T antigen/β-galactosidase fusion proteins used, in which fragments of the SV-40 T antigen amino acids 111–135 are fused to the $NH_2$-terminus of *E. coli* β-galactosidase (amino acids 9–1,023) has been described previously (Rihs and Peters, 1989; Rihs et al., 1991). The plasmid encoding the fusion protein P13 was derived in similar fashion to those encoding proteins P8 and P10 (Rihs et al., 1991) using oligonucleotide site-directed mutagenesis, according to Taylor et al. (1985). The mutated 108-bp EcoRI fragment carrying the SV-40 sequence amino acids 111–135 was directly inserted into EcoRI partially restricted lacZ-plasmid vector pPR2 (Rihs and Peters, 1989) to yield plasmid pPJ1. Integrity of the construct was confirmed by DNA sequencing (Sanger et al., 1977). 1 mM isopropyl-β-thiogalactoside (IPTG) (Boehringer-Mannheim GMBH, Mannheim, Germany) was used in media to induce expression of T antigen fusion proteins in *E. coli*, and proteins purified by affinity chromatography and labeled with IAF as described (Rihs and Peters, 1989).

### $p34^{cdc2}$ and Phosphorylation of T Antigen Fusion Proteins

Human cdc2 protein kinase complex was purified from HeLa cells as described (Brizuela et al., 1989; Bischoff et al., 1990). The preparation contains both p60-cdc2 and cyclin B-cdc2 (Bischoff et al., 1990).

*In vitro* phosphorylation of IAF-labeled T antigen fusion proteins or histone H1 was routinely performed at 30°C for 4 h in 50 mM Tris-HCl, pH 8.0/10 mM $MgCl_2$/0.1 mM ATP/10 μCi of [$\gamma$-$^{32}$P]ATP (3,000 Ci/mM). Phosphorylation was then either analyzed by SDS gel electrophoresis (Laemmli, 1970) and autoradiography of dried gels (routine exposure time of 1–5 h at −70°C); or monitored by spotting onto Whatman P81 paper (Botterell et al., 1987), washing with orthophosphoric acid (Glass et al., 1978), and scintillation counting (Bischoff et al., 1990). Phosphorylation of IAF-labeled fusion proteins was always performed in parallel under identical conditions in the presence or absence of [$\gamma$-$^{32}$P]ATP, with the latter used in the microinjection or perforated hepatoma tissue culture (HTC) cells assays for nuclear import. The stoichiometry of phosphorylation, derived from quantitation of the radiolabeled reaction, was assumed to reflect that in the parallel nonradiolabeled phosphorylation.

To check for the possibility that $p34^{cdc2}$-mediated phosphorylation might denature T antigen fusion proteins (thereby inhibiting their nuclear import) two types of control experiments were performed. (*a*) It is well known that only the tetrameric, enzymatically active form of β-galactosidase fusion proteins binds to a p-aminobenzyl-1-thio-β-galacto-pyranoside-agarose column. Fusion proteins were phosphorylated by incubation with $p34^{cdc2}$ in the presence of [$\gamma^{32}$P]ATP and then assayed for binding to the column. Yields were >93%, and the proteins could subsequently be eluted with pH 10.05 buffer. cdc2-phosphorylated histone H1 was used as a control. (*b*) The histochemical X-gal assay for β-galactosidase activity (cf. Rihs and Peters, 1989) was used to study the intracellular distribution of $p34^{cdc2}$-phosphorylated fusion proteins 30 min and some 12 h after microinjection into HTC cells. β-Galactosidase activity could be detected both in the cytoplasm and nucleus of injected cells (not shown) indicating a native tetrameric enzyme.

*Xenopus* egg extract used with perforated cells (see below) did not seem to contain phosphatase activity. This was shown by phosphorylation of T antigen proteins with $p34^{cdc2}$ in the presence of [$\gamma$P$^{32}$]ATP and subsequent incubation with *Xenopus* egg extract for 90 min at 23°C. Results, analyzed by SDS gel electrophoresis and autoradiography, provided no evidence for dephosphorylation of $T^{124}$.

### *Xenopus* Egg Extract and Phosphorylation of T Antigen Fusion Proteins

*Xenopus* egg extract was prepared and T antigen fusion proteins phosphorylated at $S^{111/112}$ by incubation with egg extract as described (Ackermann et al., 1991).

### Cell Culture, Microinjection, Cell Perforation, and Laser Microscopy

Cells of the rat HTC cell line, a derivative of Morris hepatoma 7288C (Flow Laboratories, Bonn, Germany) were propagated in DMEM supplemented with 10% FCS (Lang et al., 1986). HTC heterokaryons were fused with polyethyleneglycol as described previously (Rihs and Peters, 1989), ~1 h before microinjection. Microinjection and microfluorimetric measurements were performed as described (Rihs and Peters, 1989; Rihs et al., 1991).

HTC cells were grown on coverslips, mechanically perforated and nuclear protein import measurements performed by confocal laser scanning microscopy (CLSM) as described (Ackermann et al., 1991).

The experimental data for the kinetics of nuclear import were fitted by the equation

$$Fn/c(t) = Fn/c(\infty) \cdot [1 - e^{-kt}], \quad (1)$$

using the Marquardt-Levenberg algorithm. Here $Fn/c(t)$ and $Fn/c(\infty)$ are nuclear cytoplasmic fluorescence ratios at time $t$ and at very long times, respectively, and $k$ is the import rate.

## Results

### $p34^{cdc2}$ from HeLa Cells Specifically Phosphorylates $T^{124}$ in SV-40 T Antigen Fusion Proteins

We have previously constructed a series of fusion proteins in which small fragments of the SV-40 T antigen comprising the NLS are fused to the $NH_2$-terminus of *E. coli* β-galactosidase (Rihs and Peters, 1989; Rihs et al., 1991). The structure of these proteins is given in Table I emphasizing residues phosphorylated by CK-II ($S^{111/112}$) or cdc2 kinase ($T^{124}$) and the critical residue 128 of the NLS. P13 (described for the first time in this study, see Materials and Methods for details

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*Table I. Phosphorylation of the SV-40 T Antigen/β-Galactosidase Fusion Proteins Used in This Study by Xenopus Egg Extract (CK-II Specificity) or Purified HeLa p34$^{cdc2}$*

| Fusion protein | T antigen residue* | | | | | NLS | Phosphorylation‡ | |
|---|---|---|---|---|---|---|---|---|
| | CK-II site | | | cdc2 site | | | $S^{111}/S^{112}$ (egg extract) | $T^{124}$ (p34$^{cdc2}$) |
| | 111 | 112 | 120 | 123 | 124 | | | |
| β-gal | – | – | – | – | – | K | – | 0 |
| P3 | – | – | – | – | (N) | K | – | 0.03 |
| P2 | – | – | – | (S) | (S) | K | – | 0.02 |
| P1 | – | – | – | (S) | (S) | K | – | 0.02 |
| P7 | (R) | (N) | S | S | T | K | – | 1.33 |
| P6 | G | S | S | S | T | K | + | 2.51 |
| P9 | S | G | S | S | T | K | + | 2.41 |
| P4K | S | S | S | S | T | K | + | 0.82 |
| P4T | S | S | S | S | T | T | + | 0.81 |
| P8 | S | S | S | A | A | K | + | 0.03 |
| P10 | S | S | A | A | A | K | + | 0.04 |
| P13 | S | S | S | S | D | K | + | 0.04 |

* The single amino acid code is used, whereby residues in parentheses are derived from the DNA linkers used.
‡ Data for the phosphorylation of $S^{111/112}$ by incubation with *Xenopus* egg extract are from Ackermann et al. (1991), whereby + represents an average stoichiometry of phosphorylation between 0.6 and 1.5, and – that of <0.02. For $T^{124}$ phosphorylation, the stoichiometries (mol $P_i$/mol tetramer) are from a single typical experiment and applicable only to the specific reaction conditions used ($\mu$ of histone H1 under the same conditions was 1.21). $\mu$ of the P4K, P4T, P7, P6, and P9 fusion proteins could be increased or decreased by altering the concentration of ATP (0.1–0.4 mM) and amount of p34$^{cdc2}$ (varied by a factor of 2.5) added (see Table II).

of the construction) contains a wild type CK-II site, but an aspartic acid residue substituted for $T^{124}$.

In vitro phosphorylation of the fusion proteins by human p34$^{cdc2}$ was analyzed both qualitatively (by SDS gel electrophoresis; Fig. 1) and quantitatively (binding to P81 phosphocellulose paper according to Bischoff et al. [1990]; Tables I and II). Essentially, only proteins carrying an intact cdc2 site (i.e., $T^{124}$ in P4K, P4T, P6, P7, and P9) were phosphorylated to any significant extent. Phosphorylation of P4T (not shown) in which the critical NLS residue $K^{128}$ was substituted by threonine was indistinguishable from that of P4K carrying the wild-type NLS. We conclude that p34$^{cdc2}$ specifically phosphorylated $T^{124}$ in our fusion proteins as it does in authentic T antigen (McVey et al., 1989) and that phosphorylation was independent of a functional NLS.

The stoichiometry of phosphorylation $\mu$ is given in Tables I and II. Since the fusion proteins have a tetrameric structure the theoretical maximum of $\mu$ is 4 mol of $P_i$ per mole of protein. In practice, $\mu$ depended on the concentrations of ATP (0.1–0.4 mM) and enzyme and could be varied between 0 and ~3, under conditions at which fusion proteins lacking the cdc2 site (P8, P10) were not phosphorylated to any significant degree (<0.05 mol $P_i$/mol tetramer) and histone H1 phosphorylation stoichiometries did not exceed 1.5. In-

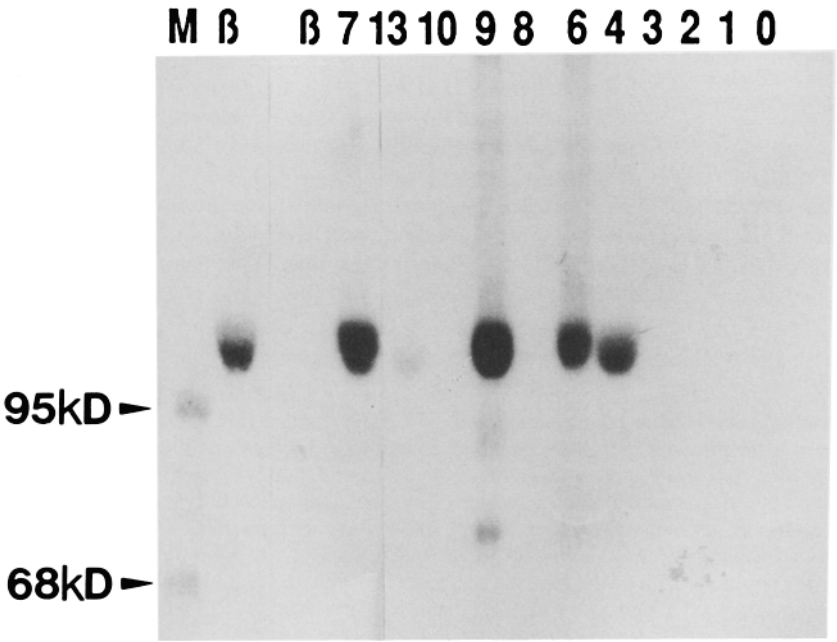


*Figure 1.* p34$^{cdc2}$ from HeLa cells specifically phosphorylates $T^{124}$ in SV-40 T antigen fusion proteins. The T antigen fusion proteins P1, P2, P3, P4K, P6, P7, P8, P9, P10, P13 (see Table I for sequence details), or β-galactosidase were incubated with p34$^{cdc2}$ for 4 h at 30°C in the presence of [$\gamma^{32}$P]ATP, followed by SDS-gel electrophoresis and autoradiography of the dried gel. The autoradiograph is shown on the right, whereby lanes *1–13* correspond to fusion proteins P1 through P13; *ß*, *E. coli* β-galactosidase; *0*, no fusion protein added. Coomassie blue staining of molecular mass markers (lane *M* showing gelantine [95 kD] and BSA [68 kD]) and β-galactosidase (116 kD) is shown in the left two lanes. The figure shows that those and only those fusion proteins were phosphorylated that bear a threonine residue in position 124.

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*Table II. Effect of In Vitro Phosphorylation by HeLa p34$^{cdc2}$ of T Antigen Fusion Proteins on Their Nuclear Import In Vitro (Perforated HTC Cells) and In Vivo (Microinjected HTC Cells)*

| Fusion protein | Phosphorylation experiment | $\mu$* | Nuclear import‡ In vitro $Fn/c_{max}$ | % | In vivo $Fn/c_{max}$ | % |
|---|---|---|---|---|---|---|
| P4K | | – | 14.7 | 100 | 15.5 | 100 |
| P4K | IV | 0.51 | ND | | 9.9 | 64 |
| P4K | V | 1.51 | 1.5 | 10 | 3.6 | 23 |
| P4K | VII | 0.85 | 7.4 | 50 | ND | |
| P4T | | – | 0.6 | | 0.5 | |
| P4T | VII | 0.84 | 0.6 | | ND | |
| P6 | | – | 9.8 | 100 | 15.2 | 100 |
| P6 | VI | 0.99 | 1.7 | 17 | 5.4 | 36 |
| P6 | VII | 1.67 | 4.2 | 43 | ND | |
| P7 | | – | 1.4 | | 1.5 | |
| P7 | VII | 0.98 | 2.2 | | ND | |
| P8 | | – | 11.2 | | 14.0 | |
| P8 | III | 0.03 | 13.7 | | 13.2 | |
| P8 | IV | <0.01 | 11.5 | | 13.5 | |
| P9 | | – | 11.9 | 100 | 13.5 | 100 |
| P9 | II | 0.30 | 7.2 | 61 | 9.8 | 72 |
| P9 | III | 1.07 | 2.8 | 24 | 3.5 | 26 |
| P9 | IV | 1.40 | 2.5 | 21 | 1.8 | 13 |
| P9 | V | 2.75 | 2.4 | 20 | 1.4 | 10 |
| P9 | VII | 2.10 | 2.3 | 19 | ND | |
| P10 | | – | 15.3 | | 21.2 | |
| P10 | VII | 0.03 | 14.8 | | ND | |
| P13 | | – | 4.2 | | 6.5 | |
| P13 | VII | 0.06 | 4.8 | | ND | |

*ND*, not determined.

* $\mu$ is the stoichiometry of T$^{124}$ phosphorylation (mol $P_i$/mol fusion protein tetramer). Phosphorylation of IAF-labeled T antigen fusion proteins (2 mg/ml) was performed as described in Materials and Methods (4 h, 300°C). Histone H1 (100 $\mu$g/ml) phosphorylation performed under the same conditions gave stoichiometries of phosphorylation of 0.1, 0.2, 0.7, 0.8, 0.74, and 1.21 mol $P_i$/mol for the phosphorylation reaction series II, III, IV, V, VI, and VII, respectively (see also legend to Table I).

‡ Nuclear import is given in terms of the maximal nuclear cytoplasmic fluorescence ratio $Fn/c_{max}$, measured after a transport time of 120 min at 23°C (in vitro) or 30 min at 37°C (in vivo), respectively. Measurements were performed at 0.2 mg/ml T antigen fusion protein, as described in Materials and Methods. Results represent the means for at least seven separate determinations of $Fn/c_{max}$ (SD ≤30% of the mean).

terestingly, the P6 and P9 fusion proteins, containing altered but functional CK-II sites, consistently served as better substrates for p34$^{cdc2}$ than the wild-type P4K (Tables I, II, and not shown), the significance of which is unclear.

### *Phosphorylation of T$^{124}$ Inhibits Nuclear Import of T Antigen Fusion Proteins*

T antigen fusion proteins were fluorescently labeled with the sulfhydryl-reactive fluorochrome IAF and then phosphorylated by incubation with p34$^{cdc2}$. $\mu$ was quantitated for each preparation using [$\gamma^{32}$P]ATP and fusion proteins P8 and P10 (which cannot be phosphorylated by p34$^{cdc2}$) were used as negative controls. Proteins were then injected into living HTC cells (Rihs and Peters, 1989) or added to mechanically perforated HTC cells, a recently established in vitro system (Ackermann et al., 1991), in the presence of *Xenopus* egg extract and ATP. Nuclear import was visualized and quantitated by CLSM, as described (Ackermann et al., 1991).

Fig. 2 shows perforated cells incubated with fluorescently labeled protein P4 until a final constant level of nuclear accumulation had been reached (2 h). The nuclear fluorescence intensity, depended inversely on the degree of phosphorylation (Fig. 2 *a–c*). In the case of fusion proteins not carrying a phosphorylatable residue in position 124 (P10, Fig. 2, *d* and *e* and P8, not shown) nuclear fluorescence was very strong irrespective of preincubation with p34$^{cdc2}$. For P13, in which phosphorylation of T$^{124}$ is simulated by an aspartic acid residue (see below), nuclear fluorescence was reduced (Fig. 2, *f* and *g*) irrespective of preincubation with p34$^{cdc2}$.

Examples of nuclear import kinetics are displayed in Fig. 3 whilst Table II contains the phosphorylation and nuclear import data of all experiments performed. Qualitatively, import kinetics were very similar in vivo and in vitro. However, because different temperatures were used (37 or 23°C, respectively), the $k$ values were about three times larger in vivo than in vitro whilst $Fn/c_{max}$, i.e., the experimentally determined maximum extent of nuclear accumulation, was about equal for identical proteins and comparable phosphorylation conditions. The data revealed in particular that T$^{124}$ phosphorylation strongly reduced $Fn/c_{max}$. In contrast, the import rate $k$ appeared to be independent of T$^{124}$ phosphorylation amounting on average to (36.8 ± 8.6) $10^{-3}$ $min^{-1}$ (mean ± SD, $n$ = 9) in perforated and (100.6 ± 18.3) $10^{-3}$ $min^{-1}$ (mean ± SD, $n$ = 8) in microinjected cells.

The dependence of $Fn/c_{max}$ on the degree of phosphorylation is shown in Fig. 4. A stoichiometry of 1 mol $P_i$ per mole tetramer was sufficient to cause an 80–90% reduction of $Fn/c_{max}$.

Since trace amounts of p34$^{cdc2}$ were present in the transport assay it might have interfered with nuclear import by phosphorylating sites other than T antigen residue T$^{124}$.

*Figure 2.* Phosphorylation of T$^{124}$ inhibits nuclear import of T antigen fusion proteins. T antigen fusion proteins were fluorescently labeled with 5-iodacetamido-fluorescein and then phosphorylated in vitro by incubation with p34$^{cdc2}$. Perforated HTC cells were incubated (90 min, 23°C) with the phosphorylated fusion proteins in the presence of transport medium containing 60% (vol/vol) *Xenopus* egg extract and 0.4 mg/ml rhodamine-labeled 70 kD dextran (see Materials and Methods). Nuclear protein accumulation was visualized by confocal laser scanning microscopy. (*a–c*) Nuclear accumulation of P4K decreased with increasing p34$^{cdc2}$-mediated phosphorylation, phosphorylation stoichiometries were 0 (*a*), 0.51 (*b*), and 1.5 ($c_1$) mol $P_i$ per mole of protein tetramer, respectively; $c_2$ shows the rhodamine fluorescence of $c_1$, indicating nuclear envelope integrity by exclusion of the 70-kD dextran. (*d* and *e*) Presence of p34$^{cdc2}$ in the transport assay had no effect on nuclear accumulation of T antigen fusion proteins which do not have the cdc2 site; P10 without (*d*) or with (*e*) preincubation with p34$^{cdc2}$. (*f* and *g*) Substitution of T$^{124}$ by an aspartic acid residue mimicks p34$^{cdc2}$-mediated phosphorylation; P13 without (*f*) or with (*g*) preincubation with p34$^{cdc2}$.

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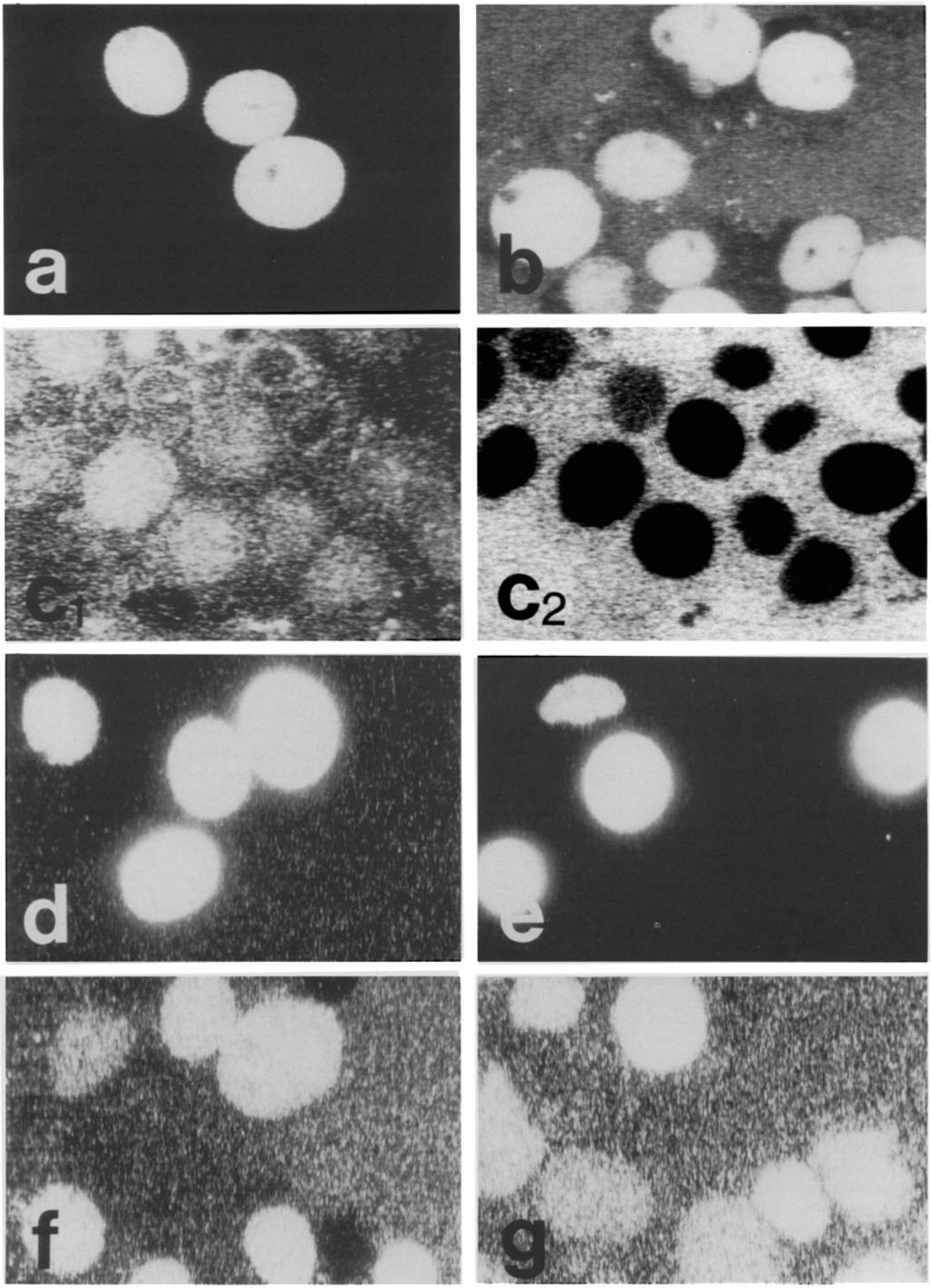

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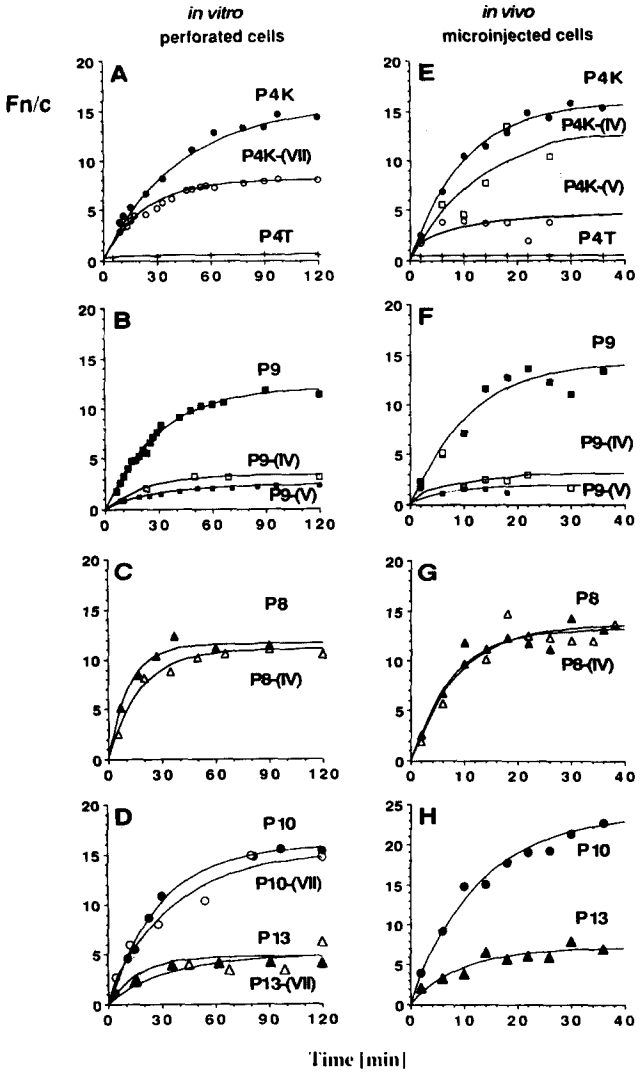


*Figure 3.* Phosphorylation of $T^{124}$ reduces the maximum extent of nuclear accumulation rather than the relative import rate. The kinetics of nuclear protein import were measured in vitro (mechanically perforated HTC cells; *A–D*) or in vivo (microinjected HTC cells; *E–H*) by confocal laser scanning microscopy or microfluorimetry. The nuclear cytosolic fluorescence ratio *Fn/c* is plotted versus time. Each entry is the mean value of at least five separate measurements (the SD was always <25% of the mean value). The T antigen fusion proteins are indicated in the panels where Roman numerals refer to the phosphorylation experiment specified in Table II. Lines are single exponential functions (Eq. 1) fitted to the data by a least squares routine. The measurements show that $p34^{cdc2}$-mediated phosphorylation decreased $Fn/c_{max}$ both in vitro and in vivo P8. (*C* and *G*) and P10 (*D* and *H*) bearing an alanine residue in position 124 are not affected by preincubation with $p34^{cdc2}$. In P13 (*D* and *H*) $p34^{cdc2}$-mediated phosphorylation was mimicked by substituting $T^{124}$ by an aspartic acid residue.

This possibility, however, could be ruled out by identical experiments with P8 and P10 (Fig. 2, *d* and *e*; Fig. 3, *c*, *d*, *g*, and *h*; Table II). These proteins are not phosphorylated by $p34^{cdc2}$ and their nuclear import was not significantly affected by the presence of $p34^{cdc2}$. That transport was NLS specific was shown by the fact that nuclear import of cdc2-phosphorylated or nonphosphorylated P4T (Fig. 3, *a* and *e*) was negligibly small.

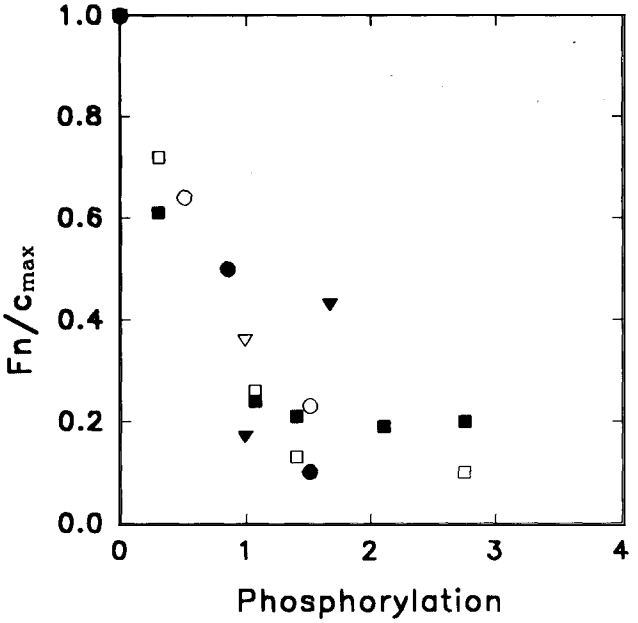


*Figure 4.* Inhibition of nuclear import depends sensitively on the stoichiometry of $T^{124}$ phosphorylation. The relative maximal nuclear accumulation $Fn/c_{max}$ is plotted versus the stoichiometry of phosphorylation (data from Table II). (●) P4K in vitro; (○) P4K in vivo; (▼) P6 in vitro; (▽) P6 in vivo; (■) P9 in vitro; (□) P9 in vivo.

### *Phosphorylation of $T^{124}$ Can Be Simulated by a $T^{124}$-to-$D^{124}$ Substitution*

Aspartic acid closely resembles phosphothreonine in terms of size and charge characteristics. We constructed a T antigen fusion protein (P13, Table I) by site-directed mutagenesis containing aspartic acid in the place of $T^{124}$. Nuclear import of P13 (Fig. 2, *f* and *g*; Fig. 3, *D*, *H*; Table II) resembled that of cdc2-phosphorylated P4K, P6, and P9. $Fn/c_{max}$ was reduced by ~60–70% compared to nonphosphorylated P4K.

### *$T^{124}$ and $S^{111/112}$ Can Be Phosphorylated Independently of Each Other*

As shown previously (Ackermann et al., 1991) residues $S^{111}$ and $S^{112}$ are specifically phosphorylated by incubation of T antigen fusion with *Xenopus* egg extract. We found that P13 (in which $T^{124}$ phosphorylation is simulated by an aspartic acid residue in position 124) could also be phosphorylated by incubation with *Xenopus* egg extract in identical fashion to other fusion proteins containing the CK-II site (not shown) suggesting that phosphorylation of $S^{111/112}$ is not influenced by prior phosphorylation of $T^{124}$. This was tested directly by sequentially phosphorylating $T^{124}$ and $S^{111/112}$ (Fig. 5 and Table III). Thus, phosphorylation of P4K at $S^{111/112}$ increased $k$ about fivefold without much affecting $Fn/c(\infty)$ whilst phosphorylation at $T^{124}$ reduced $Fn/c(\infty)$ by ~40–50% without significantly affecting $k$. Phosphorylation at $T^{124}$ and subsequent phosphorylation at $S^{111/112}$ resulted in both a reduction of $Fn/c(\infty)$ and an increase of $k$. A very similar effect was observed when the order of phosphorylation was reversed. Similar results were obtained for P9 which also bears both the CK-II site and the cdc2 site (data not shown).

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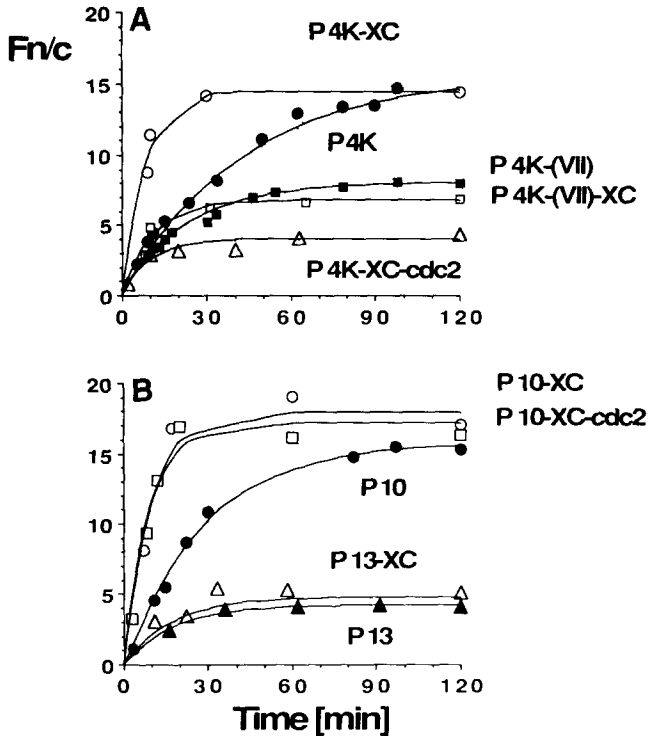


*Figure 5.* $T^{124}$ and $S^{111/112}$ can be phosphorylated independently of one another and influence different parameters of nuclear import kinetics, the maximal extent of accumulation and the relative import rate, respectively. Fluorescently labeled T antigen fusion proteins were specifically phosphorylated at $T^{124}$ by preincubation with $p34^{cdc2}$ (indicated by Roman numerals according to Table II) or/and at $S^{111/112}$ by preincubation with Xenopus egg extract (indicated by *-XC*). The kinetics of nuclear import were measured in vitro (perforated cells) by confocal laser scanning microscopy plotting the nuclear cytosolic fluorescence ratio *Fn/c* versus transport time. (*A*) P4K, not prephosphorylated P4K. *P4K-(VII)*, P4K prephosphorylated at $T^{124}$, experiment VII. *P4K-XC*, P4K prephosphorylated at $S^{111/112}$. *P4K-(VII)-XC*, P4K, first prephosphorylated at $T^{124}$ (experiment VII), then at $S^{111/112}$. *P4K-XC-cdc2*, P4K, first prephosphorylated at $S^{111/112}$, then at $T^{124}$. (*B*) *P10*, nonprephosphorylated P10 (which has no cdc2 site). *P10-XC*, P10, prephosphorylated at $S^{111/112}$. *P10-XC-cdc2*, P10, prephosphorylated at $S^{111/112}$, then incubated with p34cdc2. *P13*, nonprephosphorylated P13 (in which $p34^{cdc2}$-mediated phosphorylation is simulated by a $T^{124}$-to-$D^{124}$ substitution). *P13-XC*, P13, prephosphorylated at $S^{111/112}$.

Control experiments (Fig. 5 *b*, Table III) showed that phosphorylation of P10 (no cdc2-site) at $S^{111/112}$ increased *k* without changing $Fn/c_{max}$, independently of incubation with $p34^{cdc2}$. On the other hand, phosphorylation of P13 (where phosphorylated $T^{124}$ is simulated by an aspartic acid residue) at $S^{111/112}$ did not much affect $Fn/c(\infty)$.

## Discussion

The SV-40 T antigen has been extensively studied with much attention concentrated on its phosphorylation (for review, see Butel and Jarvis, 1986; Prives, 1990). It is therefore perhaps surprising that the effects of phosphorylation on nuclear protein import described here have not been noted previously. This may be due mainly to technical reasons. With our methods, import kinetics can be measured and the parameters *k* and $Fn/c(\infty)$ resolved. Furthermore, the use of T antigen fusion proteins enabled us to study determinants of nuclear transport independent of the extreme functional complexity of the complete T antigen.

*Table III. Effects of Dual Phosphorylation of T Antigen Fusion Proteins at $T^{124}$ and $S^{111/112}$ on Their Nuclear Import In Vitro (Perforated Cells)**

| Protein and phosphorylation sequence | $\mu$ | k | $Fn/c(\infty)$ |
|---|---|---|---|
| | | $10^{-3}$/min | |
| P4K | | 23 | 15.4 |
| P4K-XC | | 128 | 14.4 |
| P4K-(VII) | 0.85 | 32 | 8.1 |
| P4K-(VII)-XC | 0.85 | 95 | 6.8 |
| P4K-XC-cdc2 | | 97 | 4.0 |
| P10 | | 36 | 15.8 |
| P10-XC | | 110 | 17.9 |
| P10-XC-cdc2 | | 114 | 17.1 |
| P13 | | 58 | 4.8 |
| P13-XC | | 68 | 4.1 |

* -XC refers to phosphorylation at $S^{111/112}$ by incubation with *Xenopus* egg extract; roman numerals refer to phosphorylation with $p34^{cdc2}$; -cdc2 indicates addition of $p34^{cdc2}$ to the incubate of fusion proteins with *Xenopus* egg extract. $\mu$ is the stoichiometry of $T^{124}$ phosphorylation (mol $P_i$/mol fusion protein tetramer). The transport rate *k* and the nuclear cytoplasmic fluorescence ratio at infinite time $Fn/c(\infty)$ were derived by fitting eq. 1 to the experimental data.

A key observation of the present paper is the inhibition of nuclear protein import by $T^{124}$ phosphorylation. To account for this observation two alternative mechanisms may be considered: an inactivation of the NLS by electrostatic interaction with the nearby phosphate group at $T^{124}$ or an induction of cytoplasmic anchoring by $T^{124}$ phosphorylation.

The inactivation mechanism has been recently suggested (Moll et al., 1991) to apply in case of the yeast transcription factor SWI5 whose NLS is flanked by three cdc2 phosphorylation sites. With regard to T antigen fusion proteins it may be recalled that these are tetramers carrying four NLSs and four cdc2 sites per molecule. The tetrameric structure of the fusion proteins is conserved during cytoplasmic injection and nuclear import, as indicated by histochemical assays of $\beta$-galactosidase activity (Rihs and Peters, 1989; Rihs et al., 1991, this study). Studies with the pentameric protein nucleoplasmin (Dingwall et al., 1982) showed that a single NLS per pentamer was sufficient to induce nuclear import although the import rate depended sensitively on the signal number. Thus, in the case of T antigen fusion proteins one functional NLS per tetramer may be sufficient to induce nuclear import and only those tetramers in which all of the four $T^{124}$ residues are phosphorylated may be excluded from the nucleus. This would imply that relatively large phosphorylation stoichiometries would be necessary to effect a strong inhibition of nuclear import.

Cytoplasmic retention seems to apply, for instance, to the transcription factor NF-$\kappa$B. It apparently forms a complex with the inhibitor protein I-$\kappa$B in the cytoplasm which cannot be imported into the nucleus. Upon phosphorylation of I-$\kappa$B the complex dissociates and NF-$\kappa$B is imported (Lenardo and Baltimore, 1989; Shirakawa and Mizel, 1989; Zabel and

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*Table IV. Relationship between NLS and Potential and Confirmed CK-II and cdc2-kinase Phosphorylation Sites in Selected Nuclear Proteins*

| Protein‡ | CK-II-site(s)* | cdc2-site(s)* | NLS |
|---|---|---|---|
| SV-40 T antigen | $S^{111}S^{112}$DDE (Grässer et al., 1988) | $ST^{124}$PPK (McVey et al., 1989) | PKKKRKV[132] (Kalderon et al., 1984) |
| Polyoma T antigen | $SS^{156}$PTD<br>$ES^{261}$ENE | $PRT^{187}$PVS<br>$AT^{278}$PPK | VSRKRPRP[196]<br>PPKKARED[286] (Richardson et al., 1986) |
| Human p53 | $T^{284}$EEE | $SS^{315}$PQP (Bischoff et al., 1990) | PQPKKKP[319] (Chelsky et al., 1989) |
| Human c-myc | | $PST^{315}$RKP | PAAKRVKLD[328] (Dang and Lee, 1988) |
| Lamin A/C | $SS^{384}$DTEE<br>$S^{458}$NED | $TS^{344}$PRS<br>$SPS^{392}$PTS (Ward and Kirschner, 1990) | RQRRNELKRS[373]<br>SVTKKRKLE[412] (Loewinger and McKeon, 1988) |
| Mouse c-abl IV | $ESS^{322}$ISDE | $APDT^{566}$PEL (Kipreos and Wang, 1990)<br>$PAVS^{588}$PLL (Kipreos and Wang, 1990) | SALIKKKKKMAP[631] (Van Etten et al., 1989) |
| SWI5 (*Schizosaccharomyces cerevisiae*) | | $KRS^{646}$PRK (Moll et al., 1991)<br>$SSS^{664}$PIK (Moll et al., 1991) | KKYENVVIKRSPRKRGRPRK[655] (Moll et al., 1991) |
| Nucleoplasmin | $EDES^{177}S^{178}$EED | $S^{182}$PTKK<br>$S^{149}$PPK | RPAATKKAGQAKKKKLD[172] (Dingwall et al., 1988) |

* The phosphorylated/phosphorylatable S/T residues are numbered.
‡ References apply to confirmed NLSs, CK-II- and cdc2-sites.

Baeuerle, 1990). Analogous mechanisms have been postulated for the glucocorticoid receptor where the 90-kD heat shock protein, HSP90, may serve as the cytoplasmic anchor (Picard et al., 1988), and for the *Drosophila* morphogen dorsal (Gilmore, 1990; Roth et al., 1989; Steward, 1989). In the case of the cAMP-dependent protein kinase the regulatory subunit may functionally serve as the cytoplasmic anchor of the catalytic subunit, whose nuclear translocation is triggered by cAMP-mediated hormonal stimulation (Nigg et al., 1985; Pearson et al., 1991). If the cytoplasmic retention mechanism applies to T antigen fusion proteins one might expect that only unphosphorylated tetramers can be imported into the nucleus implying that relatively small phosphorylation stoichiometries can induce a significant inhibition of nuclear import.

Our data indicate that relatively small phosphorylation stoichiometries are able to effect a substantial inhibition of nuclear import. Thus, an 80% reduction of $Fn/c_{max}$ was observed at a phosphorylation stoichiometry of one (Fig. 4). This seems to support the cytoplasmic anchoring mechanism, but a definitive conclusion requires a rigorous theoretical analysis of the dependence of $Fn/c_{max}$ on $\mu$. Also, alternative mechanisms, which cannot be discounted at this stage, can be envisaged. For instance, since the T antigen fusion proteins are very large (480 kD) not a single functional NLS but a certain threshold number of NLSs might be required for nuclear import. Thus, further theoretical and experimental studies will be necessary to answer questions concerning the precise mechanism(s) of nuclear import inhibition by phosphorylation.

Before considering the physiological implications of $T^{124}$ phosphorylation, some basic facts of the subcellular distribution, phosphorylation state and oligomeric composition of the SV-40 T antigen may be recalled. The T antigen can be considered as a nuclear oncoprotein. However, a small but distinct fraction (~10%) of the T antigen is nonnuclear (Walser et al., 1989) and, interestingly, has been shown to be phosphorylated at $T^{124}$ (Scheidtmann et al., 1984). The nonnuclear T antigen has important functions because cytoplasmic T antigen mutants retain transforming activity (Lanford et al., 1985; Pinkert et al., 1987). A predominantly cytosolic $p34^{cdc2}$ isoform (Egl) has recently been characterized at the molecular and biochemical level, whilst the bulk of $p34^{cdc2}$ seems to be localized to the nucleus (Riabowol et al., 1989; Booher et al., 1989). In the light of this, and our results here, we suggest that phosphorylation of $T^{124}$ in the cytoplasm may constitute a means to precisely regulate the small but functionally important magnitude of the cytoplasmic T antigen function. According to our data (Fig. 4) a relatively small stoichiometry of phosphorylation (~0.3 mol $P_i$ per mole tetramer) is sufficient to keep ~10% of the T antigen fusion proteins in the cytoplasm. These values approximate well both the in vivo phosphorylation state of the T antigen and its subcellular distribution. Independently of the minor T antigen fraction which is phosphorylated at $T^{124}$ in the cytoplasm and thus retained, the bulk of the T antigen may enter the nucleus and be phosphorylated there to activate its replication stimulating function (McVey et al., 1989).

Is the dual regulation of nuclear protein import by CK-II and cdc2 phosphorylation a special feature of the T antigen

or does it have general implications? Although further studies will clearly be necessary to answer this question, a hint comes from perusal of the sequence of some of important nuclear-localized proteins (Table IV). It shows that the consensus sequence for cdc2-mediated phosphorylation S/T-P-X-K or S/T-P-K (Draetta, 1990; Moreno and Nurse, 1990) is frequently found immediately $NH_2$-terminal to the NLS. In particular human p53, c-abl (two sites) and the lamin A/C proteins resemble T antigen in being phosphorylated very close to their respective NLSs (Bischoff et al., 1990; McVey et al., 1989; Kipreos and Wang, 1990; Ward and Kirschner, 1990). Putative CK-II sites are similarly frequent (Table VI), as pointed out previously (Rihs et al., 1991). Thus, although largely hypothetical at this stage, the "CcN-motif" consisting of CK-II site, cdc2-site, and NLS may be a general element of nuclear import regulation.

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