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Negative charge at the casein kinase II site flanking the nuclear localization signal of the SV40 large T-antigen is mechanistically important for enhanced nuclear import

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Nuclear import of SV40 large T-antigen (T-ag) is completely dependent on the T-ag nuclear localization sequence (amino acids 126–132), but the rate of nuclear import is greatly increased by the additional presence of the N-terminal flanking sequence (amino acids 111–125), which includes a site for casein kinase II (CKII) (Ser^{111/112}). The role of this site was investigated by site-directed mutagenesis and analysis of the effects on phosphorylation and on nuclear import at the single cell level using microinjection and quantitative fluorescent techniques. Removal of the CKII site either by substitution of S^{111/112} by nonphosphorylatable amino acid residues, or mutation of the Asp-Asp-Glu^{113/115} CKII recognition sequence to Asn-Asn-Gln, resulted in nuclear import rates less than 4% wild type, demonstrating that the CKII site was responsible for the enhancement of nuclear import conferred by T-ag amino acids 111–125. The substitution of Asp for Ser¹¹², the serine preferentially phosphorylated by purified CKII, enhanced nuclear import to about 45% maximal wild type rates. It is concluded that negative charge at the CKII site, normally provided by phosphorylation, is mechanistically important for nuclear transport enhancement. There was no evidence of a direct role for phosphatases or dephosphorylation in the transport process.

Introduction

The nuclear-cytoplasmic distribution of many transcription factors (Lenardo & Baltimore, 1989; Shirakawa & Mizel, 1989; Nasmyth *et al.*, 1990; Moll *et al.*, 1991), morphogens (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989), or oncogene products (Van Etten *et al.*, 1989; Roux *et al.*, 1990) closely parallels changes in the cell cycle, differentiation or metabolic state of eukaryotic cells. Proteins capable of rapid translocation into the nucleus upon hormonal stimulation include the cAMP-dependent protein kinase catalytic subunit (Nigg *et al.*, 1985; Pearson *et al.*, 1991) and the glucocorticoid hormone receptor (Picard *et al.*, 1988). Regulation of the nuclear entry of transcription activating proteins may represent a key level of control in regulating gene expression and signal transduction.

Proteins larger than 45 kD require a nuclear localization sequence (NLS) (Hall *et al.*, 1984; Kalderon *et al.*, 1984; Lanford & Butel, 1984; Silver *et al.*, 1984) in order to be targeted specifically to the nucleus. Although their specificity and subcellular location is still unclear, NLS-binding proteins (Adam *et al.*, 1989; Benditt *et al.*, 1989; Li & Thomas, 1989; Meier & Blobel, 1990; Lee *et al.*, 1991; Stochaj *et al.*, 1991) have been postulated to play a direct role in active nuclear transport. Recent evidence suggests that the molecular chaperonin and heat shock related HSP70/HSC70 protein is both necessary for NLS-dependent nuclear transport in reconstituted systems and able to bind NLSs specifically (Yoneda *et al.*, 1988; Imamoto *et al.*, 1992; Shi & Thomas, 1992).

Little is known concerning the regulation of import kinetics, which is likely to be the critical parameter in cueing the above-mentioned cell cycle transitions, morphogenesis and transformation. In addition to the NLS, specific signals carried by the transported proteins appear to be capable of functioning in a regulatory fashion, whereby covalent modifications such as phosphorylation play a role (Jans *et al.*, 1991a; Moll *et al.*, 1991; Rihs *et al.*, 1991). The nuclear localization of chimeric proteins in which fragments of the SV40 large T-antigen (T-ag) are fused to the *Escherichia coli* β -galactosidase enzyme is completely dependent on the presence of the T-ag NLS (amino acids 126–132) (Rihs & Peters, 1989). However, import is markedly enhanced by the presence of the N-terminal sequences (amino acids 111–125) adjacent to the NLS (Rihs & Peters, 1989). We hypothesized that this effect might be due to the casein kinase II (CKII) phosphorylation site (Ser^{111/112}), since a deletion (amino acids 111–119) including the CKII site drastically impaired nuclear import (Rihs *et al.*, 1991). This site is conserved in the sequences of the T-antigens of all polyoma virus family members, as well as in the adenovirus E1a and the human papilloma virus E7 proteins (see Pipas, 1992). CKII has recently been the focus of intense interest in terms of its range of physiologically important substrates, including oncogene products and transcription factors (Krebs *et al.*, 1988; Pinna, 1990; Marshak & Carroll, 1991).

This study undertakes site-directed point mutational analysis of the role of the T-ag CKII site (Ser^{111/112}) in regulating the nuclear import of T-ag fusion proteins, as well as characterizing the latter's ability to be phosphorylated by CKII, demonstrating that the CKII site is indeed responsible for the enhanced nuclear transport conferred by T-ag amino acids 111–125. We

show that Ser¹¹² is the serine residue that is preferentially phosphorylated by CKII in our T-ag fusion proteins, and examine the effect on nuclear transport kinetics of substituting this residue with an aspartic acid residue. We have previously demonstrated that Asp can functionally mimic phosphothreonine in terms of an effect on nuclear transport (Jans *et al.*, 1991a) whereby Asp in place of Thr¹²⁴ resembles *in vitro* p34^{cdc2}-kinase phosphorylation at this site in reducing maximal nuclear import levels. Proteins containing Asp¹¹² are found to simulate those possessing an intact (phosphorylatable) CKII site, being accumulated in the nucleus at up to 45% maximal wild type rates, implying that negative charge at the CKII site, normally brought about by phosphorylation, plays a mechanistic role in nuclear transport. An active role for dephosphorylation at Ser¹¹² in the nuclear transport process can be discounted.

Results

Site-directed mutagenesis of the CKII-site N-terminal to the SV40 T-ag nuclear localization sequence

We have previously described plasmids encoding fusion proteins which contain T-ag sequences in N-terminal to *E. coli* β -galactosidase sequence (residues 9–1023) (Jans *et al.*, 1991a; Rihs *et al.*, 1991). The sequences of the T-ag(WT126–135), T-ag(WT111–135), T-ag(CKII Del) and T-ag(Gly¹¹¹) fusion proteins (Rihs & Peters, 1989; Jans *et al.*, 1991a; Rihs *et al.*, 1991) are included in Table 1, together with those of the proteins described for the first time in this study (see Materials and methods for details of plasmid constructions and mutagenesis). Protein T-ag(WT111–135) (Rihs *et al.*, 1991) contains wild type T-ag sequence amino acids 111–135, including the CKII site (Ser-Ser-Asp-Asp-Glu^{111–115}) and NLS (Pro-Lys-Lys-Lys-Arg-Lys-Val^{126–132}). Protein T-ag(CKII Del) contains T-ag amino acids 120–135, thereby lacking the CKII site

(though retaining the NLS). The T-ag(Asn-Asn-Gln^{113/115}) and T-ag(Gly-Ala^{111/112}) fusion proteins contain T-ag amino acids 111–135, but possess non-functional CKII-phosphorylation sites, whereby the latter contains the nonphosphorylatable Ala-Gly residues in place of Ser^{111/112}, whilst T-ag(Asn-Asn-Gln^{113/115}) retains Ser^{111/112} but the CKII recognition sequence Asp-Asp-Glu^{113/115} is mutated to Asn-Asn-Gln (Table 1). A further CKII Site mutant derivative was constructed containing Asp at position 112, with Gly in place of Ser¹¹¹ (Table 1).

Mutation of the casein kinase II site impairs nuclear import of T-ag fusion proteins

In vivo nuclear import kinetics of the fluorescently labelled T-ag(Asn-Asn-Gln^{113/115}) and T-ag(Ala-Gly^{111/112}) fusion proteins were compared to those for the wild type T-ag(WT111–135) and CKII-site-deleted T-ag(CKII Del) proteins in microinjected HTC polykaryons or Vero cells (Figures 1–3, Table 1). Results for both cell lines were completely comparable (Figures 1 and 3, and not shown) in that both fusion proteins exhibited greatly reduced nuclear accumulation rates compared to the wild type protein T-ag(WT111–135) with import kinetics similar to those for T-ag(CKII Del) (Figure 2, Table 1).

Results indicated that point mutations at the CKII site, either of Ser^{111/112} or of the CKII Asp-Asp-Glu^{113–115} recognition site, drastically reduced the nuclear import rate, conclusively confirming the previous hypothesis (Rihs *et al.*, 1991) that the CKII site is responsible for the nuclear transport enhancement conferred by T-ag amino acids 111–125. The result for the Asn-Asn-Gln^{113–115} fusion protein strongly implies that CKII is indeed the kinase responsible for phosphorylation at the site *in vivo* since mutation of the recognition sequence alone, leaving Ser^{111/112} intact, eliminates nuclear import enhancement. Nuclear accumulation of fusion proteins T-ag(Asn-Asn-Gln^{113/115}) and T-ag(Ala-Gly^{111/112}) in both cell lines was complete within 10 h, as revealed by histochemical

Table 1 Nuclear import parameters and SV40 T-antigen sequence of casein kinase II site mutated derivatives of the T-ag/ β -galactosidase fusion proteins in this study

Fusion protein	CKII-site	SV40 T-ag sequence*	Nuclear import rate† k (10 ⁻³ /min)
		NLS	
T-ag(WT126-135)		¹²⁶ P KKKRKVEDP ¹³⁵	1
T-ag(CKII Del) (WT120-135)		¹²⁰ SQHSTPPKKKKRKVEDP ¹³⁵	2
T-ag(WT111-135)	¹¹¹ SSDDEATADSQHSTPPKKKKRKVEDP ¹³⁵		88
T-ag(Gly ¹¹¹)	gSDDEATADSQHSTPPKKKKRKVEDP ¹³⁵		100
T-ag(Asn-Asn-Gln ¹¹³⁻¹¹⁵)	SSnnqATADSQHSTPPKKKKRKVEDP ¹³⁵		2
T-ag(Ala-Gly ^{111/112})	agDDEATADSQHSTPPKKKKRKVEDP ¹³⁵		3
T-ag(Gly ¹¹¹ Asp ¹¹²)	gADDEATADSQHSTPPKKKKRKVEDP ¹³⁵		40

*All fusion proteins contain SV40 T-ag sequences fused N-terminal to *E. coli* β -galactosidase (amino acids 9–1023) (Jans *et al.*, 1991a; Rihs & Peters, 1989; Rihs *et al.*, 1991). The single amino acid code is used, whereby the NLS is underlined, bold letters represent the CKII phosphorylation site, and small letters indicate mutated T-ag sequence. Fusion proteins T-ag(WT126-135), T-ag(WT111-135), T-ag(Gly¹¹¹) and T-ag(CKII Del) are from Rihs & Peters (1989) and Rihs *et al.* (1991); the other proteins are described for the first time in this study (see Materials and methods for details of expression plasmid construction). †Raw data (see Figure 2) were fitted for the function $F_n/c(t) = 0.45 + 16 \cdot (1 - e^{-kt})$ (Jans *et al.*, 1991a), where t is time in min. The correlation coefficient was greater than 0.92 in all cases. The value of 16 for $F_n/c_{\max}$ is based on data for T-ag(WT111-135) and T-ag(Gly¹¹¹) fitted for the function $F_n/c(t) = 0.45 + F_n/c_{\max} \cdot (1 - e^{-kt})$, which yield $F_n/c_{\max}$ values of 15.6 and 16.0 respectively (k values of 94 and $101 \times 10^{-3}/\text{min}$ respectively). The $F_n/c(t=0)$ value of 0.45 is the $F_n/c_{\max}$ value for NLS-deficient proteins (Jans *et al.*, 1991a; Rihs & Peters, 1989; Rihs *et al.*, 1991).

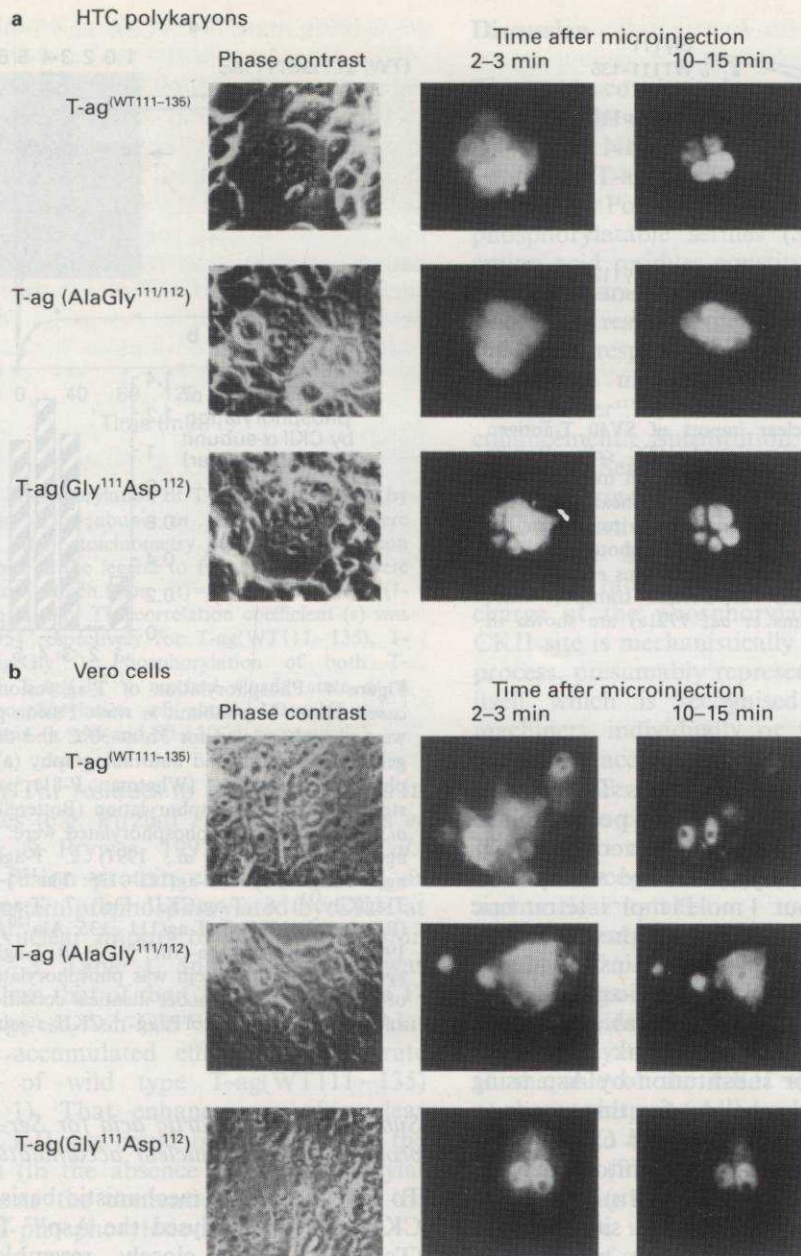


Figure 1 Fluorescent visualization of short term nuclear import of SV40 T-antigen fusion proteins *in vivo* in microinjected HTC polykaryons (a) and Vero (b) cells. Phase contrast is shown on the left

staining of fixed cells for β -galactosidase activity (Materials and methods) (Figure 3 and not shown), kinetics similar to those of T-ag(CKII Del) (Rihs *et al.*, 1991). T-ag(WT111-135) (wild type) protein accumulation is maximal within 10-15 min (Figures 1-3) (Rihs & Peters, 1989; Jans *et al.*, 1991a; Rihs *et al.*, 1991).

Phosphorylation of SV40 large tumour-antigen fusion protein derivatives by casein kinase II is predominantly at serine-112

Both Ser¹¹¹ and Ser¹¹² can be predicted on the basis of the consensus CKII phosphorylation site sequence (Ser/Thr-X-X-Asp/Glu) (Krebs *et al.*, 1988; Litchfield *et al.*, 1990; Pinna, 1990; Marshak & Carroll, 1991) to be phosphorylated by CKII. To determine whether our T-ag fusion protein derivatives are specifically phosphorylated by CKII, we carried out *in vivo* phosphor-

ylation experiments using recombinant human casein kinase II α -subunit. Only proteins possessing a functional CKII site (based on the CKII site consensus) were phosphorylated (Figure 4), whether or not they possessed an intact NLS or *cdc2*-K site (at Thr¹²⁴; Jans *et al.*, 1991a); e.g. protein T-ag (111-135: Thr¹²⁸) lacks a functional NLS but is phosphorylated to the same extent as T-ag(WT111-135) - Figure 4 lane 4 - as are the *cdc2*-K site deficient proteins T-ag(111-135: Ala^{123/124}) and T-ag(111-135: Ala¹²⁰Ala^{123/124}) - Figure 4 lanes 7 and 8. The protein T-ag(Gly¹¹²), which contains T-ag amino acids 111-135 with glycine in place of Ser¹¹² (Rihs *et al.*, 1991) was not phosphorylated as strongly as protein T-ag(Gly¹¹¹) (containing Ser¹¹²) or fusion proteins containing both CKII site serines. This was confirmed by examining the time course of phosphorylation by CKII α -subunit (Figure 5). T-ag(Gly¹¹¹) (containing Ser¹¹²) was phosphorylated at a rate and level comparable to that of wild type

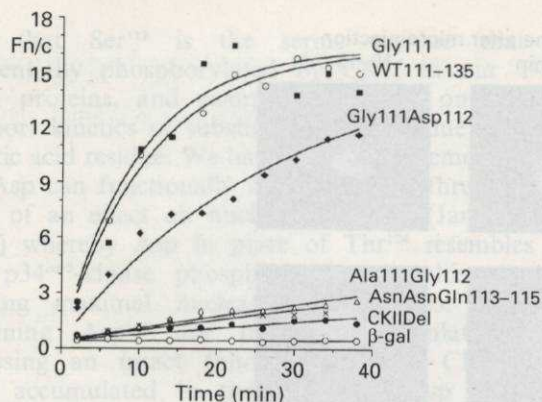


Figure 2 Time course of nuclear import of SV40 T-antigen fusion proteins *in vivo* in microinjected HTC cells. Results represent the average of at least eight single cell measurements (SD not greater than 28% the value of the mean) for Fn/c (fluorescence quantitated in the nucleus, relative to that in the cytoplasm, as determined by fluorescence microphotolysis – see Materials and methods) (Rihs & Peters, 1989; Jans *et al.*, 1991a; Rihs *et al.*, 1991). The results for nuclear transport rates determined by curve-fitting (Jans *et al.*, 1991a) are shown in Table 1 (see legend)

protein (carrying both Ser^{111/112}), whereas T-ag(Gly¹¹²), containing only Ser¹¹¹, was relatively poorly phosphorylated (Figure 5). Phosphorylation of both T-ag(WT111–135) and T-ag(Gly¹¹¹) reached steady state at a stoichiometry of about 1 mol Pi mol⁻¹ tetrameric fusion protein (Figure 5). Clearly, CKII predominantly phosphorylated the T-ag fusion proteins at Ser¹¹², consistent with *in vivo* results for T-ag in SV40 infected cells (Scheidtmann *et al.*, 1991a), as well as studies with synthetic peptides (Marshak & Carroll, 1991). Ser¹¹² was chosen for substitution by Asp using site-directed mutagenesis (see below) for this reason.

In vitro phosphorylation of the various CKII site T-ag fusion protein derivatives by the mitotic p34^{cdc2}-kinase (see above, Jans *et al.*, 1991a) was also examined (results not shown). All CKII site mutants, including the Asp¹¹² derivative (below), were phosphorylated to extents comparable to that for T-ag(WT111–135).

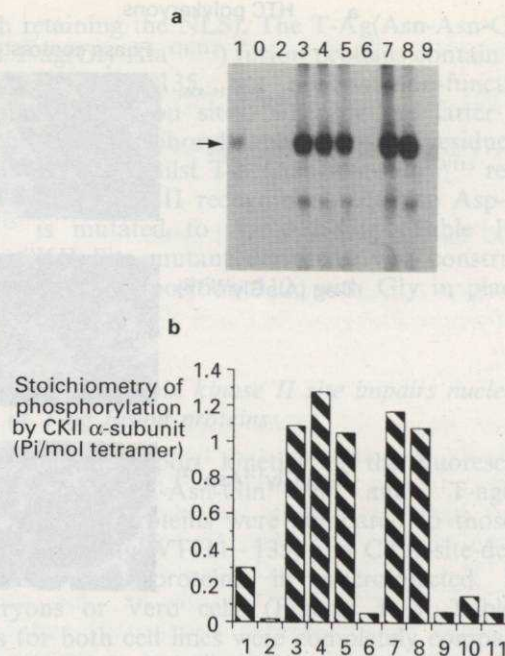


Figure 4 Phosphorylation of T-ag fusion proteins by human casein kinase II α -subunit *in vitro*. Fusion proteins (6 μ g protein) were phosphorylated for 3 h at 30°C and then analysed either by gel electrophoresis and autoradiography (a) or by spotting onto phosphocellulose (b) (Whatman P-81) paper to estimate the stoichiometry of phosphorylation (Botterell *et al.*, 1987; Jans *et al.*, 1991a). Proteins phosphorylated were: 0. no substrate 1. T-ag(Gly¹¹²) (Rihs *et al.*, 1991) 2. T-ag(WT126–135) 3. T-ag(WT111–135) 4. T-ag(111–135: Thr¹²⁸) (Rihs *et al.*, 1991) 5. T-ag(Gly¹¹¹) 6. T-ag(CKII Del) 7. T-ag(111–135: Ala^{123/124}) (Rihs *et al.*, 1991) 8. T-ag(111–135: Ala¹²⁰ Ala^{123/124}) (Rihs *et al.*, 1991) 9. T-ag(Asn-Asn-Gln^{113–115}) 10. T-ag(Ala-Gly^{111/112}) 11. T-ag(Gly¹¹¹Asp¹¹²). Casein was phosphorylated to a stoichiometry of 0.34 mol Pi mol⁻¹ under identical conditions, corresponding to an activity of 33.5 pmol Pi μ g⁻¹ CKII α -subunit per min

Substitution of aspartic acid for Ser-112 in T-ag fusion proteins enhances nuclear accumulation

To investigate the mechanistic basis of the role of the CKII site, we analysed the Asp¹¹² T-ag fusion protein (Table 1). Asp closely resembles phosphoserine/phosphothreonine with regard to size and charge characteristics, and has been shown to simulate

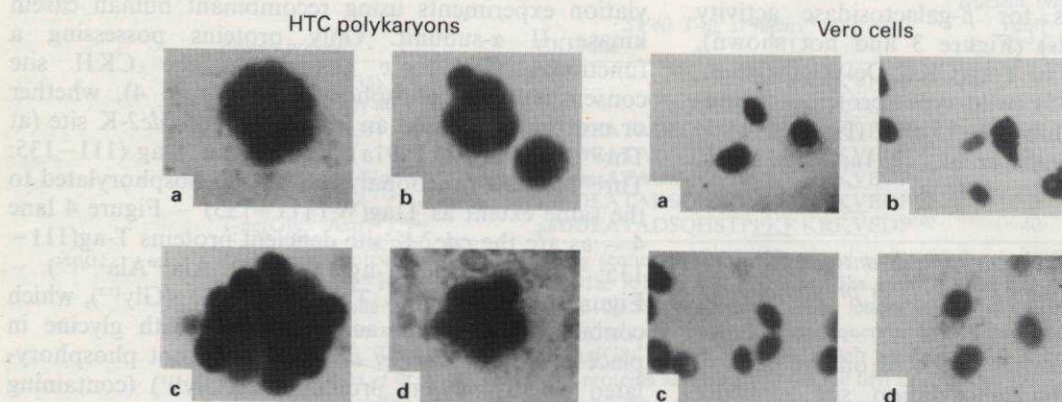


Figure 3 Nuclear accumulation in HTC polykaryons (left) and Vero cells (right) as shown by histochemical staining for β -galactosidase activity. At various times after microinjection, cells were fixed and stained as described in Materials and methods (Rihs & Peters, 1989). A. T-ag(WT111–135); cells fixed after 10 min; B. T-ag(Asn-Asn-Gln^{113–115}); cells fixed after 8 h; C. T-ag(Ala-Gly^{111/112}); cells fixed after 8 h; D. T-ag(Gly¹¹¹Asp¹¹²); cells fixed after 15 min

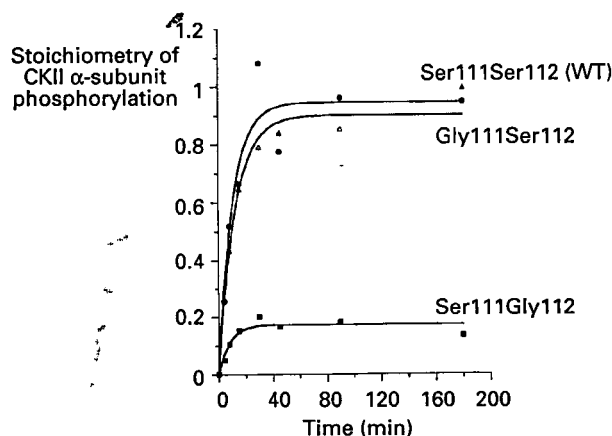


Figure 5 Kinetics of phosphorylation of T-ag fusion proteins by human casein kinase II α -subunit *in vitro*. Proteins were phosphorylated and the stoichiometry of phosphorylation quantitated as described in the legend to Figure 4. Results were fitted for the function $\text{Stoich.Phos.}(t) = \text{Max.Stoich.Phos.} \cdot (1 - e^{-kt})$, where t is time in min. The correlation coefficient (r) was 0.966, 0.992 and 0.954 respectively for T-ag(WT111–135), T-ag(Gly¹¹¹) and T-ag(Gly¹¹²). Phosphorylation of both T-ag(WT111–135) and T-ag(Gly¹¹¹) reached steady state at a stoichiometry of phosphorylation of about 1.0 mol Pi mol⁻¹ tetrameric fusion protein ($k = 94$ and 90×10^{-3} , respectively)

phosphorylated Ser/Thr residues in functional terms in a number of studies (e.g. Firzlaft *et al.*, 1991; Jans *et al.*, 1991a; Manak & Prywes, 1991; Mosialos *et al.*, 1991). The Asp¹¹²-fusion protein can be regarded as approximating a protein prephosphorylated by CKII at amino acid 112. Nuclear import of the Asp¹¹² fusion protein (Figures 1–3, Table 1, and not shown) was much more rapid than that of the CKII-site mutants T-ag(CKII Del), T-ag(Asn-Asn-Gln^{113–115}) and T-ag(Ala-Gly^{111/112}). It was accumulated efficiently, at a rate about 45% that of wild type T-ag(WT111–135) (Figure 2, Table 1). That enhancement of nuclear transport by the CKII site could be simulated by the Asp¹¹² substitution (in the absence of a phosphorylatable Ser¹¹¹) supports the contention that S¹¹² is the major site of CKII-phosphorylation.

CKII α -subunit and phosphorylation activity in cytosol from HTC cells

The fact that Asp¹¹²-substituted proteins microinjected into the cytoplasm of HTC or Vero cells migrated rapidly to the nucleus implied that the phosphorylation event at the CKII site which is important for nuclear transport probably occurs in the cytoplasm. We confirmed this by examining phosphorylation in cytosolic and nuclear extracts made from HTC cells (Ackerman & Osheroff, 1989) using our array of phosphorylation site mutated fusion proteins (results not shown). Western blots indicated the presence of CKII α -subunit in both cytosolic and nuclear extracts, whilst phosphorylation activity in cytosolic extracts showed CKII site specificity since only proteins with a functional CKII site were phosphorylated and those lacking it were not. In contrast, nuclear extracts appeared to possess little CKII-specific phosphorylation activity, but contained double stranded DNA-dependent protein kinase (phosphorylates T-ag at Ser¹²⁰ – Chen *et al.*, 1991) and *cdc2*-K protein kinase site activities.

Discussion

This study conclusively confirms our previous hypothesis that the CKII phosphorylation site flanking the SV40 T-ag NLS has a central role in enhancing nuclear import of T-ag proteins (Rihs & Peters, 1989; Rihs *et al.*, 1991). Point mutations at the site, either of the phosphorylatable serines (Ser^{111/112}) or of the acidic amino acid residues constituting the CKII recognition sequence, drastically reduce the nuclear import rate. The latter result strongly implies that CKII is indeed the kinase responsible for phosphorylation at the site *in vivo*, since mutation of the recognition site alone, leaving Ser^{111/112} intact, eliminates nuclear import enhancement. Substitution by Asp of the CKII-preferred Ser¹¹² of the CKII site to simulate a phosphoserine residue results in enhanced nuclear import, further supporting the importance of the CKII site in accelerating T-ag protein nuclear transport rates. The results indicate that negative charge at the phosphorylated (or Asp¹¹²-substituted) CKII site is mechanistically important in the transport process, presumably representing a regulatory signal in itself which is recognised by the nuclear import machinery individually or in concert with the NLS, to confer accelerated NLS-dependent entry into the nucleus. Alternatively, negative charge at the CKII site may elicit a conformational change which exposes the NLS in a more effective fashion and thereby accelerates both its recognition by the nuclear transport system and nuclear uptake.

Functional studies have been carried out on a number of phosphorylation site SV40 T-ag mutants (e.g. Kalderon & Smith, 1984; Schneider & Fanning, 1988; Scheidtmann *et al.*, 1991a) which relate quite interestingly to the results presented here with respect to the role of the CKII site in T-ag fusion protein nuclear transport. Although T-ag nuclear localization is known to be required for viral infection and DNA replication (SV40 T-ag-NLS mutants are not viable – Kalderon *et al.*, 1984; Kalderon & Smith, 1984), no quantitative analysis has been made at the rate of transport of wild type T-ag to the nucleus. Whilst T-ag CKII-site mutants are definitely nuclear at steady state in viral infected cells (Kalderon & Smith, 1984; Schneider & Fanning, 1988) – as are CKII-site mutant T-ag fusion protein derivatives 8 h after injection into the cytoplasm (see Figure 3; Rihs *et al.*, 1991) – no kinetic measurements of their nuclear uptake have been made. Exact details of the kinetics of infection/viral DNA replication of SV40 T-ag-CKII site phosphorylation mutants in comparison to wild type are also not available; however, substitution of the CKII site Ser¹¹² (SV40 mutant K5) and Ser¹¹¹Ser¹¹² (mutant SV111/112) has been shown to reduce both viral viability (to 44% that of wild type in both cases, with up to 50% slower kinetics of plaque formation – see Kalderon & Smith, 1984; Schneider & Fanning, 1988) and DNA replication activity (to less than 50% wild type – see Schneider & Fanning, 1988). These effects would appear to be consistent with the results presented here in terms of a role of the CKII site in determining the rate of T-ag nuclear localization and ultimately thereby the kinetics of viral infection. SV40 T-ag CKII site mutants comparable to the Asn-Asn-Gln^{113–115} CKII recognition site and Asp¹¹²-substituted

derivatives described here for the first time have not been isolated, but it would obviously be interesting to compare the kinetics of infection of such mutants to wild type virus in the light of the results of this study.

The fact that an Asp¹¹²-substituted fusion protein microinjected into the cytoplasm of HTC or Vero cells migrates rapidly to the nucleus has a number of implications in terms of the mechanism of action of the CKII site in accelerating nuclear import of T-ag proteins. The hypothesis that the CKII site exerts its influence on nuclear transport through phosphorylation occurring in the nucleus in order to prevent transport out of the nucleus seems unlikely. On the contrary, the results imply that the phosphorylation event at the CKII site probably occurs in the cytoplasm, which is consistent with the requirement for cytosol for nuclear transport activity in reconstituted systems (e.g. Jans *et al.*, 1991a) (although CKII is clearly not the only component required for nuclear transport which is supplied by cytosol – unpublished; see Adam *et al.*, 1990, 1991; Shi & Thomas, 1992; Stochaj & Silver, 1992). Experiments demonstrating CKII-specific phosphorylation activity in HTC cytosol support this, as does the presence of CKII in HTC cytosol as shown by Western blots (not shown). A further hypothesis to explain the role of the CKII site; that transport substrate proteins are phosphorylated in the cytoplasm, migrate rapidly to the nucleus, and are then dephosphorylated in the nucleus in order to prevent transport out of the nucleus is inconsistent with the data presented here, whereby the Asp¹¹²-fusion protein (unable to be dephosphorylated) is strongly and rapidly accumulated in the nucleus. A direct role for phosphatases in the nuclear transport process can reasonably be discounted (see also Jans *et al.*, 1991a), consistent with the low rate of turnover of phosphate at Ser¹¹² in SV40 infected cells (Scheidtmann, 1986; Scheidtmann *et al.*, 1991a,b).

The exact basis of CKII-phosphorylation mediated regulation of NLS-dependent nuclear transport has yet to be determined, but it is intriguing to speculate that the combination of groups of negatively (phosphorylated or Asp-substituted CKII site) and positively (NLS) charged amino acids has a mechanistic role. This mechanism may be analogous to that proposed for eukaryotic proteasome component proteins, which possess putative NLS and 'cNLS' (sequence of Asp and Glu residues complementary to the NLS) sequences, whereby a masking interaction between the two regions is proposed (Grziwa *et al.*, 1992; Tanaka *et al.*, 1992). We are currently examining the sequences of nuclear proteins which lack a CKII site for regions of negative charge in the vicinity of the NLS, which may conceivably represent, in conjunction with the NLS, a signal for constitutively rapid nuclear transport similar to the Asp¹¹²-substituted fusion proteins described here.

We have previously defined the CcN motif (Jans *et al.*, 1991a) as the regulatory module for nuclear transport of T-ag which can be found in a number of other nuclear proteins (e.g. Stifani *et al.*, 1992). The CKII site ('C') enhances the rate of NLS ('N')-dependent nuclear transport, whilst the *cdc2*-K site ('c') inhibits nuclear transport and thereby determines the maximal level of nuclear accumulation. A number of examples of cell-cycle regulated nuclear protein transport have now been reported (e.g. Nasmyth *et al.*,

1990; Jans *et al.*, 1991a; Moll *et al.*, 1991; Ofir *et al.*, 1990), consistent with a wider role for *cdc2*-kinase in regulating nuclear protein transport. CKII is activated by proliferative signals such as epidermal growth factor and insulin (Sommercorn & Krebs, 1987; Sommercorn *et al.*, 1987; Klarlund & Czech, 1988; Ackerman & Osherooff, 1989; Manak & Prywes, 1991), implying that one of its general roles may be to regulate rapid nuclear protein transport in response to hormonal signals. A role for CKII in the case of T-ag has been demonstrated here, but one might envisage that other hormonally regulated kinases such as PK-A (Shirakawa & Mizel, 1989; Ofir *et al.*, 1990; Mosialos *et al.*, 1991; Grziwa *et al.*, 1992) may be able to substitute functionally for CKII in regulating nuclear import kinetics, presumably through the appropriate kinase sites in the near vicinity of the NLS. The combination of signal transduction regulated positive signal and cell cycle dependent negative signal for nuclear localisation would enable the very precise regulation of nuclear levels of transcription factors and other proteins required during growth and differentiation.

In summary, this study confirms our previous hypothesis (Rihs *et al.*, 1991) that the rate of NLS-dependent nuclear import of SV40 T-ag proteins is controlled by the CKII site; and shows for the first time that negative charge at the site is the effector enhancing the nuclear import rate. The protein factor(s) which recognise(s) the signal represented by the CKII-site-phosphorylated T-ag CcN motif, and thereby serve to accelerate T-ag nuclear import, are the focus of future work in this laboratory. The Asp¹¹²-substituted fusion protein derived in this study may conceivably have great application in the initial purification of such specific binding factors.

Materials and methods

Chemicals and reagents

Isopropyl- β -thiogalactoside (IPTG), β -galactosidase (EC 3.2.1.23.37), poly-ethyleneglycol (PEG 1500), anti-casein kinase II (CKII) monoclonal antibody 1AD9 and recombinant human CKII α -subunit (EC 2.7.1.3) were from Boehringer-Mannheim. 5-iodoacetamido-fluorescein (IAF) was from Molecular Probes (USA). Other reagents were from the sources previously described (Jans *et al.*, 1991a; Rihs *et al.*, 1991).

Cell lines and cell culture

HTC rat hepatoma tissue culture (a derivative of Morris hepatoma 7288C) and Vero (African green monkey kidney) cell lines were cultured in DMEM supplemented with 10% FCS (Jans *et al.*, 1991a; Rihs *et al.*, 1991).

SV40 T-ag/ β -galactoside fusion proteins

Plasmids expressing SV40 T-ag/ β -galactosidase fusion proteins, in which SV40 T-ag amino acids 111–135 are fused to the N-terminus of *E. coli* β -galactosidase (amino acids 9–1023) have been described (Rihs & Peters, 1989; Jans *et al.*, 1991a; Rihs *et al.*, 1991). The plasmid encoding fusion protein derivative T-ag(Asn-Asn-Gln^{113/115}) was derived as previously (Jans *et al.*, 1991a; Rihs *et al.*, 1991) using oligonucleotide site-directed mutagenesis (Taylor *et al.*, 1985). The mutated 108 bp EcoRI fragment encoding T-ag amino acids 111–135 was inserted into

EcoRI partially restricted *lacZ*-plasmid vector pPR2 (Rihs & Peters, 1989) to yield plasmid pDAJ2.

For construction of the plasmids encoding the T-ag(Ala-Gly^{111/112}) and Asp¹¹² fusion proteins, the 159 bp EcoRI fragment from plasmid pPR11 (Rihs, 1989), encoding T-ag amino acids 87–135 was subcloned into M13mp19, yielding M13DAJ2, which was then used for site-directed mutagenesis. In the case of plasmid pPJR1, encoding T-ag(Ala-Gly^{111/112}), a Sau3A restriction site was inserted at amino acids 108/109, to enable excision of the mutated 108 bp Sau3A fragment, and subsequent ligation into BamHI-partially digested pPR2 vector N-terminal to the *lacZ* sequence. In the case of the Asp¹¹²-substituted fusion protein-encoding plasmids, a Sau3A restriction site was inserted at amino acids 110/111, enabling isolation of the mutated 101 bp Sau3A fragment. The 5'-overhanging ends were digested with Mung Bean nuclease, followed by the addition of 10mer SmaI linkers, restriction and ligation into the SmaI site of pPR2, yielding plasmid pDAJ10, encoding the fusion protein T-ag(Gly¹¹¹Asp¹¹²). Construct integrity was routinely confirmed by DNA sequencing (Sanger *et al.*, 1977).

Fusion protein expression, purification and labelling

1 mM IPTG was used in media to induce T-ag fusion protein expression in *E. coli* strain MC 1060 (Shapira *et al.*, 1983), after which proteins were purified by affinity chromatography and labelled with IAF as described (Rihs & Peters, 1989).

Microinjection and laser microscopy

HTC cells were fused with PEG about 1 h prior to microinjection to produce polykaryons (Rihs & Peters, 1989). Microinjection and single cell microscopic fluorescence measurements on HTC and Vero cells were performed as previously (Rihs & Peters, 1989; Jans *et al.*, 1991a; Rihs *et al.*, 1991). Quantitation of fluorescence using confocal microscopy and microphotolysis has been described previously in detail (Jans *et al.*, 1989, 1990, 1991a,b; Rihs & Peters, 1989; Pearson *et al.*, 1991; Rihs *et al.*, 1991; Jans, 1992, for applications). Histochemical staining of fixed cells for β -galactosidase activity after microinjection was performed as described previously (Rihs & Peters, 1989; Rihs *et al.*, 1991).

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Preparation of cell extracts

Cytosolic and nuclear extracts from HTC cells for measurement of CKII activity and Western blotting were prepared as described by Ackerman & Osheroff (1989). Briefly, cells were homogenised with 10 strokes from a 22 gauge needle and 2 ml syringe in homogenising buffer (15 mM Tris, pH 7.9, 5 mM KCl, 0.5 mM MgCl₂, 0.05 mM EDTA, 0.5 mM DTT, 0.05% Triton X-100, 350 mM sucrose, 1 mM PMSF, 10 mM Na-metabisulfite), followed by separation into cytosolic and nuclear fractions by centrifugation (Ackerman & Osheroff, 1989).

Phosphorylation

Phosphorylation using casein kinase II α -subunit was carried out in 50 mM Tris, pH 8.2, 50 mM NaCl, 25 mM KCl, 10 mM MgCl₂, 10 μ M Na-orthovanadate (Ackerman & Osheroff, 1989) with 0.2 mM ATP (2 Ci mmol⁻¹, New England Nuclear) at concentrations of 2 mg ml⁻¹ T-ag fusion protein (Jans *et al.*, 1991a) for 3 h at 30°C unless otherwise indicated. The stoichiometry of phosphorylation was determined as described previously (Botterell *et al.*, 1987; Jans *et al.*, 1991a) by spotting on to phosphocellulose paper (P-81, Whatman) and washing with orthophosphoric acid (Glass *et al.*, 1978). SDS-gel electrophoresis (according to Laemmli, 1970), gel drying and autoradiography was performed as described previously (Jans *et al.*, 1991a).

Western blotting

Proteins after gel electrophoresis were transferred to nitrocellulose and visualized by staining with Ponceau S. Nitrocellulose was then blocked with Tween and subsequently incubated with the anti-CKII monoclonal antibody 1AD9 according to the specifications of the manufacturer, using alkaline phosphatase and NBT/BCIP (nitro blue tetrazolium/bromochloroindolyl phosphate) (Sigma Chem. Co.).

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