An engineered site for protein kinase C flanking the SV40 large T-antigen NLS confers phorbol ester-inducible nuclear import

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Abstract Nuclear import of simian virus SV40 large tumour antigen (T-ag) is enhanced by the protein kinase CK2 (CK2) site flanking the nuclear localisation sequence (NLS). We report here that replacement of this site with a consensus site for protein kinase C (PK-C) can alter the regulation of T-ag nuclear import and render it inducible by phorbol ester. Measurement of nuclear import kinetics using fluorescently labelled proteins and confocal laser scanning microscopy show that the introduced PK-C site is functional in enhancing T-ag nuclear import compared to a protein lacking the CK2 site. Treatment with the PK-C activator phorbol 12-myristate 13-acetate (PMA) further increases the level of maximal nuclear accumulation and the initial nuclear import rate. This engineered PMA-responsive NLS may have application in targeting of molecules of interest to the nucleus in response to agents stimulating PK-C.

Key words: Ca^{2+}/phospholipid-dependent protein kinase C; Nuclear import kinetics; Confocal laser scanning microscopy; Microinjection; Phosphorylation-regulated nuclear localization sequence

1. Introduction

Proteins larger than 45 kDa conventionally require a nuclear localisation sequence (NLS) in order to enter the nucleus [1,2]. NLS-dependent nuclear import of the simian virus SV40 large tumour antigen (T-ag), however, is regulated through action of the modular CcN motif [3,4], comprising phosphorylation sites for protein kinase CK2 (CK2) and cyclin-dependent kinase (cdk) together with the NLS. The rate of T-ag nuclear import is enhanced 50-fold by the CK2 phosphorylation site ($Ser^{111/112}$) [3,5–7], whilst phosphorylation at the cdk site (Thr^{124}) adjacent to the NLS (amino acids 126–132) determines the maximal extent of nuclear accumulation [3]. CcN motifs also appear to mediate regulated nuclear import of the yeast transcription factor SWI5 [8,9] and the *Xenopus* nuclear phosphoprotein nucleoplasmin [10].

In analogous fashion to T-ag and SWI5, many proteins contain phosphorylation sites close to their NLSs which regulate NLS activity, the sites together with the NLSs thus constituting modular phosphorylation-regulated NLSs (prNLSs) [1,2] able to confer regulated nuclear import on heterologous proteins [3,9]. prNLSs can be divided into classes where phosphorylation either enhances or inhibits NLS function [1,2], with the CcN motif as a specialised type of prNLS containing both enhancing and inhibitory phosphorylation sites. CK2 and cdk are not the only kinases regulating protein nuclear import [1,2,7]; the cAMP-dependent protein kinase (PK-A), for example, has been shown to enhance nuclear import of the *Drosophila melanogaster* morphogen Dorsal [11,12], whilst phosphorylation by Ca^{2+}/phospholipid-dependent protein kinase C (PK-C) inhibits nuclear import of lamin B_2 [13]. A variety of hormonal or other signals can thus affect the inhibition or enhancement of nuclear localisation of proteins carrying prNLSs [1], where the nature of the kinase site(s) within the prNLS determines which specific signals can modulate nuclear import.

To test whether substituting a kinase site by another could alter the signals regulating nuclear import and as a first step toward developing a prNLS capable of conferring inducible nuclear translocation on other molecules of interest, we recently substituted the T-ag CK2 site by a consensus phosphorylation site for PK-A [14]. The resultant protein exhibited PK-A-mediated enhancement of nuclear import [14], which could have been attributable to the fact that PK-A appears to play an exclusively enhancing role on nuclear transport in the case of a number of other proteins [1,11,15]. We decided to investigate this further by substituting the T-ag CK2 site with a consensus site for PK-C, reported to regulate nuclear import negatively [1,13]. We show that the resultant fusion protein can be specifically phosphorylated by purified PK-C in vitro, and determine its nuclear import kinetics in HTC rat hepatoma tissue cells in the absence and presence of phorbol 12-myristate 13-acetate (PMA), an activator of PK-C [16–18], using confocal laser scanning microscopy (CLSM). Somewhat surprisingly, all results indicate that the PK-C site enhances T-ag nuclear import, implying that it is the position of the phosphorylation site relative to the NLS, rather than the nature of the kinase phosphorylating it, that determines whether the site can enhance or inhibit nuclear import. The PMA-responsive prNLS derived and characterised here may have application in targeting molecules of interest to the nucleus in a clinical or research context.

2. Materials and methods

2.1. Chemicals

Purified PK-C (rat brain) was from Promega, recombinant human CK2 from Boehringer Mannheim, DL-dithiothreitol (DTT) and PMA from Sigma, and 5-iodoacetamidofluorescein (IAF) from Molecular Probes. Other reagents were from the sources described previously [14,19].

2.2. 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 (DMEM) supplemented with 10% foetal calf serum [3,6].

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2.3. T-ag β-galactosidase fusion proteins

The T-ag β-galactosidase fusion proteins used in this study are shown in Fig. 1A. They contain T-ag amino acid residues 111–135, including the CcN motif (comprising CK2 and cdk phosphorylation sites and NLS), or variants thereof, fused N-terminal to the *Escherichia coli* β-galactosidase sequence (amino acids 9–1023, EC 3.2.1.23.37) (see [3,5–7]). Oligonucleotide site-directed mutagenesis (Clontech Transformer Kit) of plasmid pDAJ2 (see [7]), performed as previously [14], was used to substitute a consensus site for PK-C (Arg-Gly-Ser-Ser^{111}-Ser-Arg) in place of the CK2 site (Ser^{111}-Ser^{112}-Asp-Asp-Glu). Integrity of the constructs was confirmed by DNA sequencing.

2.4. Fusion protein expression, purification and labelling

Isopropyl-β-D-thiogalactopyranoside (1 mM) was used to induce fusion protein expression in *E. coli* strain MC1060 [20]. Proteins were purified by affinity chromatography and labelled with IAF as described [6].

2.5. 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 CLSM (Bio-Rad MRC-600) was as described previously [3,6,7,9]. In the case of in vivo experiments, HTC cells were fused with polyethylene glycol (PEG 1500) at least 1 h prior to microinjection to produce polykaryons [5,6]. Nuclear import of PKC-cN-β-Gal was measured with or without treatment with PMA carried out either by coinjecting with 200 ng/ml PMA in the injection pipette, or preincubating cells with 20 ng/ml PMA for 4 h before injection. cAMP (25 μM in the injection pipette) was also coinjected as a negative control [14]. Image analysis of CLSM files using the NIH Image public domain software and curve fitting were as described [9,14].

2.6. In vitro phosphorylation

In vitro phosphorylation of fusion proteins by CK2 was analysed as previously by SDS gel electrophoresis, and the stoichiometry of phosphorylation determined where appropriate [7,14,21,22]. Phosphorylation by PK-C was performed using $1–1.5\times10^{-3}$ unit/μl PK-C, 1 mg/ml fusion protein or histone H1 in a reaction buffer containing 20 mM HEPES (pH 7.4), 1 mM DTT, 10 mM $MgCl_2$ and 0.2 mM ATP (containing 0.05 μM γ-[^{32}P]ATP) in the presence of 200 μg/ml phosphatidyl serine and 1 mM $CaCl_2$ for 30–60 min at 30°C [23], followed by gel electrophoresis and phosphorimaging [24].

3. Results

3.1. An engineered PK-C site in place of the T-ag CK2 site

A consensus PK-C phosphorylation site (see Fig. 1A) was introduced into the T-ag CcN motif in place of the CK2 site using site-directed mutagenesis. To show functionality of the site, in vitro phosphorylation was performed using purified CK2 and PK-C (Fig. 1B). In contrast to the wild type CcN-β-Gal T-ag fusion protein (stoichiometry of phosphorylation of 1.1 mol Pi/mol tetrameric fusion protein), the engineered PKC-cN-β-Gal fusion protein was not phosphorylated by CK2 (Fig. 1B), indicating that the CK2 site was no longer functional. PKC-cN-β-Gal was, however, able to be specifically phosphorylated by PK-C (Fig. 1B; phosphorylation levels almost 6-fold those of cN-β-Gal, lacking a PK-C site at the CK2 site position – see Fig. 1A), indicating functionality of the introduced PK-C site. The wild type CcN-β-Gal fusion protein was not phosphorylated by PK-C to any significant extent (not shown), while β-galactosidase itself was not phosphorylated by PK-C at all (Fig. 1B). The results indicated that a PK-C site had been successfully introduced in place of the CK2 site in the T-ag CcN motif.

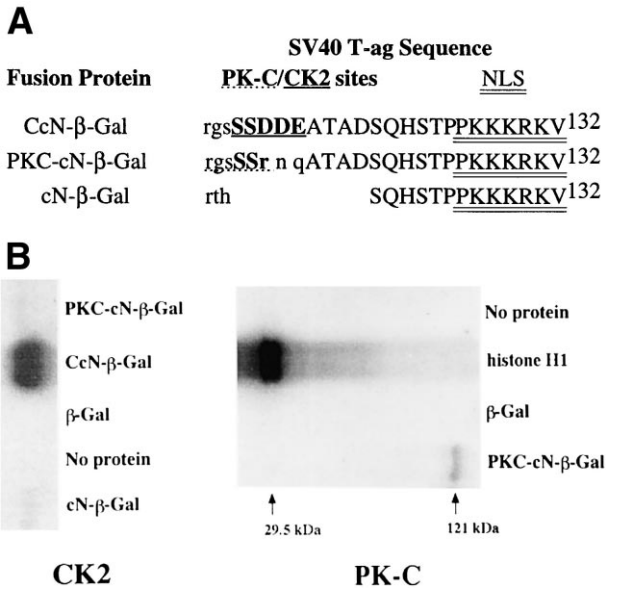

Fig. 1. Sequence of the T-ag fusion proteins used in this study and specific CK2 and PK-C phosphorylation in vitro. A: All proteins contain T-ag sequences, shown in capital letters, fused N-terminal to *E. coli* β-galactosidase (amino acids 9–1023). Linker and mutated sequences are shown in small letters. The single letter amino acid code is used, with the T-ag-NLS double underlined, phosphorylation sites in bold, the CK2 site underlined and the PK-C site underlined with a dotted line. The consensus site for PK-C phosphorylation created in PKC-cN-β-Gal is Arg-X-X-Ser-X-Arg, where X is any amino acid, and Ser is the site of phosphorylation. B: T-ag fusion proteins were phosphorylated using either CK2 or PK-C as indicated at 30°C for 30–60 min prior to analysis by gel electrophoresis and autoradiography. Histone H1 was used as a positive control for PK-C phosphorylation.

Table 1
Nuclear import kinetics of SV40 T-ag fusion proteins in the presence and absence of various treatments

Fusion protein[a]	Kinase sites		Treatment	Nuclear import parameter[b]			
	CK2	PK-C		in vivo		in vitro	
				Fn/c_{max}	Initial rate[c] Fn/c/min	Fn/c_{max}	k (10^{-3})
cN-β-Gal	−	−		1.65 ± 0.05	ND[d]	1.43 ± 0.03 (1)	ND[d]
CcN-β-Gal	+	−		5.87 ± 0.19	1.14 ± 0.27 (4)	6.95 ± 0.60 (3)	35 ± 5
PKC-cN-β-Gal	−	+		2.15 ± 0.07	0.57 ± 0.08 (3)	3.98 ± 0.39 (3)	52 ± 10
PKC-cN-β-Gal	−	+	PMA (in medium)	3.04 ± 0.19	0.63 ± 0.13 (2)		
PKC-cN-β-Gal	−	+	PMA (coinjected)	3.07 ± 0.24	0.72 ± 0.10 (2)		
PKC-cN-β-Gal	−	+	cAMP (coinjected)	1.92 ± 0.12	0.47 ± 0.14 (2)		

[a]T-ag fusion protein sequences are presented in Fig. 1A.
[b]Raw data (see Fig. 2Fig. 3) were fitted as described in the legend to Fig. 2 and represent the mean ± S.E.M. (*n* in parentheses).
[c]The initial rate represents the initial nuclear import rate determined up to 3 min after microinjection.
[d]ND, not determined.

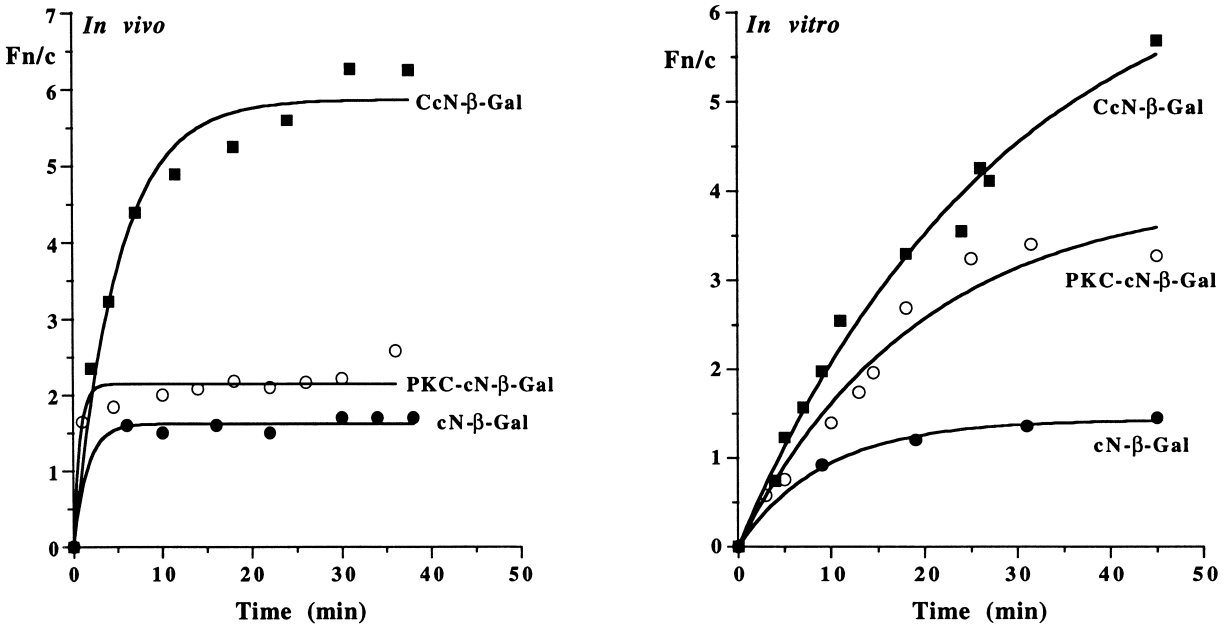

Fig. 2. Nuclear import kinetics in vivo (left) and in vitro (right). Nuclear import was measured in HTC cells using CLSM and image analysis as described in Section 2. Results represent the average of at least two separate experiments, where each point represents the average of 6–10 separate measurements for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence, respectively, with autofluorescence subtracted. Curves were fitted for the function $Fn/c\ (t) = Fn/c_{max}\ (1-e^{-kt})$ [9,14], where Fn/c_{max} is the maximal nuclear accumulation level, k is the nuclear import rate constant, and t is time in minutes [3,6,7].

3.2. The introduced PK-C site enhances T-ag nuclear import

The kinetics of nuclear import of PKC-cN-β-Gal were compared to those of the wild type CcN-β-Gal T-ag fusion protein, as well as a derivative lacking a functional CK2 site (cN-β-Gal) [6] (see Fig. 1A for T-ag sequences), both in vivo and in vitro (Figs. 2 and 3A). PKC-cN-β-Gal showed maximal nuclear accumulation about 30 and 60% that of the wild type CcN-β-Gal in vivo and in vitro respectively, well above that of cN-β-Gal, lacking a functional phosphorylation site at the CK2 site position (Figs. 2 and 3A; Table 1). The fact that PKC-cN-β-Gal accumulated efficiently in the nucleus of HTC cells both in vivo and in vitro indicated that the PK-C site is functional in enhancing T-ag nuclear import in the absence of agents stimulating PK-C activation, presumably attributable to phosphorylation at the site stemming from basal PK-C activity [14].

3.3. Induction of PK-C activity enhances nuclear import

Nuclear import of PKC-cN-β-Gal was assessed in vivo in response to treatment with the PK-C activator PMA (Fig. 3 and Table 1), administered either by coinjection, or preincubation of the cells for 4 h prior to microinjection. In contrast to coinjection with cAMP as a negative control, PMA treatment increased the maximal level of nuclear accumulation of PKC-cN-β-Gal by about 40%, and the initial import rate by 20% (Fig. 3A,B; Table 1). PMA had a negligible effect on nuclear import of CcN-β-Gal containing the wild type CK2 site (not shown), and this, together with the lack of an effect of cAMP on PKC-cN-β-Gal nuclear import (Fig. 3B and Table 1), underlined the specificity of the PMA effect on nuclear import of PKC-cN-β-Gal. The results thus indicate that nuclear import of PKC-cN-β-Gal can be enhanced by PMA treatment in terms of both maximal nuclear accumulation and initial nuclear import rate, through increased PK-C-mediated phosphorylation at the engineered site.

4. Discussion

We have previously shown that a PK-A site in place of the T-ag CK2 site can enhance nuclear import of a heterologous protein in response to PK-A activation [14]. Here we show that an engineered PK-C site flanking the T-ag NLS can also enhance nuclear import, despite the fact that PK-C has been shown to inhibit nuclear import of proteins such as lamin B2 [1,13]. Nuclear accumulation of PKC-cN-β-Gal in vivo and in vitro was 30 and 60% respectively that of the wild type CK2 site-containing protein, significantly higher than that of a protein lacking a functional phosphorylation site at the CK2 site position, indicating that the engineered PK-C site is functional in enhancing T-ag nuclear import. Treatment with the PK-C activator PMA further increased the initial nuclear import rate and the maximal nuclear accumulation, consistent with PK-C-mediated enhancement of nuclear import. Clearly, a functional PMA-responsive PK-C site can enhance T-ag fusion protein nuclear import in similar fashion to the wild type CK2 phosphorylation site. That the enhancing effect of the PK-C site on nuclear import was not absolutely dependent on PMA treatment (see Fig. 2) is attributable to phosphorylation at the PK-C site through basal cytosolic PK-C activity (see [14]). This means that, at least in HTC cells, the engineered prNLS does not appear to be completely dependent on exogenous stimulation for nuclear targeting activity (see also [14]).

As mentioned above, all results indicate that the engineered PK-C site enhances T-ag nuclear import, even though PK-C has previously been reported to inhibit rather than enhance nuclear import [1,13]. The PK-C site in PKC-cN-β-Gal was

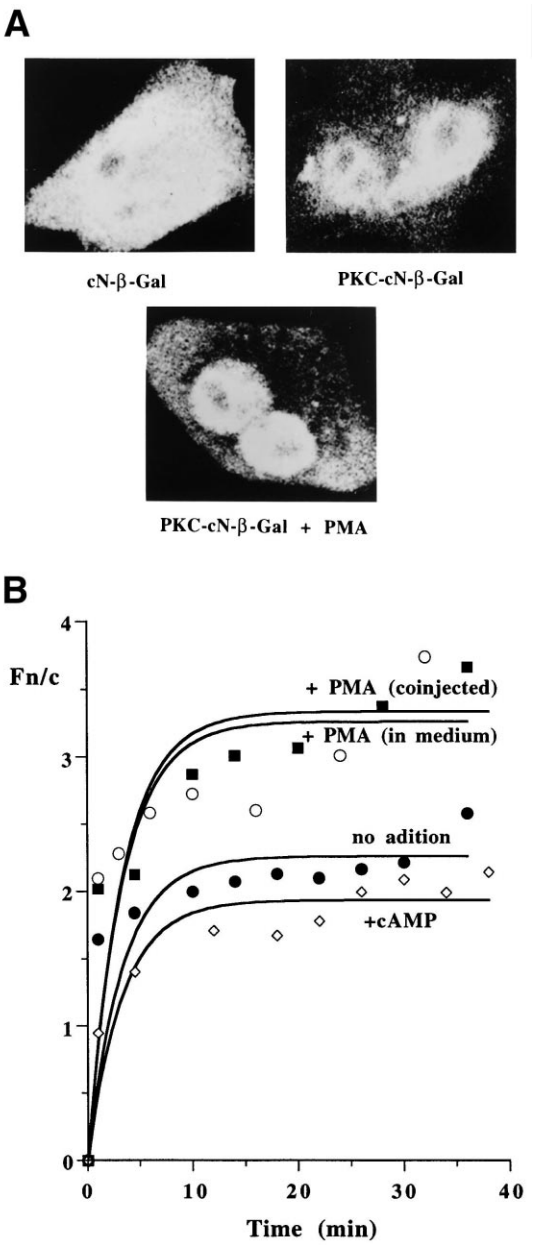

Fig. 3. Nuclear uptake of PKC-cN-β-Gal in the absence or presence of exogenous treatments in vivo. A: Cells were preincubated for 4 h without or with 20 ng/ml PMA as indicated, prior to microinjection with the proteins indicated and examination using CLSM after 10 (top two panels) or 6 min (bottom panel). B: Nuclear import kinetics of PKC-cN-β-Gal either in cells preincubated in the absence or presence of PMA as in A above, or in cells coinjected with PKC-cN-β-Gal together with either PMA or cAMP (negative control). Results represent the average of at least two separate experiments (see also Table 1), where each point represents the average of at least 8 separate measurements for each of Fn and Fc respectively, with autofluorescence subtracted. Curves were fitted as described in the legend to Fig. 2.

placed in the position of the nuclear import enhancing T-ag CK2 site, implying that rather than the exact nature per se of the kinase phosphorylating within a prNLS being important, it is the position of its site relative to the NLS that determines whether it enhances or inhibits NLS function. This is completely consistent with our recent findings [19,24] with respect to the mechanism of CK2 enhancement of T-ag nuclear import, which appears to be through direct involvement of the residues of the CK2 site in mediating interaction with the NLS-binding importin α subunit; negative charge through phosphorylation at the CK2 site increases the affinity of the interaction with importin α, whilst altering the distance between the CK2 site and NLS reduces the binding affinity [19]. By analogy, it seems reasonable to postulate that negative charge at the PK-C site through PMA-induced or basal PK-C activity in the case of PKC-cN-β-gal is the mechanism by which the site enhances nuclear import; altering the sequence, but not the position, of the kinase site simply alters the nature of the cellular signals that can enhance nuclear import. Whether the introduction of a consensus site for another kinase (e.g. for the nuclear import enhancing PK-A) in place of the T-ag CcN motif cdk site, which inhibits T-ag nuclear import, can alter the signals which inhibit T-ag nuclear import (eg. make them cAMP-dependent in the case of a PK-A site) is currently under investigation.

Engineered prNLSs such as that described here where PK-C or PK-A sites enhance nuclear import are of particular interest since they potentially confer tightly regulated nuclear localisation according to hormonal or other stimuli, thus enabling precise cuing of the nuclear entry of relevant proteins and other molecules according to need [14]. This may have application in facilitating the directed transport of DNA molecules encoding a gene of interest to the nucleus of relevant cells to increase transfection and/or homologous recombination efficiencies, or to correct inborn errors of metabolism in gene therapy [25,26]. Alternatively, engineered prNLSs can be used to deliver drugs to the nucleus; agents that might be used in this context include anti-cancer drugs or photosensitiser molecules for which the nucleus is a hypersensitive site. Conjugates to which variants of the T-ag CcN motif were linked in order to target the photosensitiser chlorin e6 to the nucleus [27], for example, have been shown to increase photosensitising activity up to 2000-fold. The prNLSs derived and characterised in this and previous [14] studies thus represent an important step towards developing a signal conferring inducible nuclear targeting of molecules of interest.

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