A Consensus cAMP-dependent Protein Kinase (PK-A) Site in Place of the CcN Motif Casein Kinase II Site of Simian Virus 40 Large T-antigen Confers PK-A-mediated Regulation of Nuclear Import*

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The regulation of nuclear protein transport by phosphorylation plays a central role in gene expression in eukaryotic cells. We previously showed that nuclear import of SV40 large tumor antigen (T-ag) fusion proteins is regulated by the CcN motif, comprising phosphorylation sites for casein kinase II and the cyclin-dependent kinase cdc2, together with the nuclear localization signal. Regulation of nuclear uptake by CcN motif kinase sites also holds true for the yeast transcription factors SW15 and the Xenopus nuclear phosphoprotein nucleoplasm. To test directly whether a kinase site other than those of the CcN motif could regulate nuclear import of T-ag, the CcN motif casein kinase II site, which markedly increases the rate of T-ag nuclear import, was replaced by a consensus site for the cAMP-dependent protein kinase (PK-A) using site-directed mutagenesis. The resultant fusion protein could be specifically phosphorylated by PK-A in vitro and in cell extracts. Nuclear import of the fluorescently labeled protein was analyzed in the HTC rat hepatoma cell line both in vivo (microinjected cells) and in vitro (mechanically perforated cells) in the presence and the absence of cAMP and/or PK-A-prephosphorylated protein was also tested. All results indicated that the rate of nuclear import was increased by phosphorylation at the PK-A site (2-5-fold), demonstrating that kinases other than those of the CcN motif can regulate nuclear import in response to stimulatory signals. The phosphorylation-regulated nuclear localization signal derived here represents an important first step toward developing a signal conferring inducible nuclear targeting of molecules of interest.

Although proteins such as histones appear to be constitutively targeted to the nucleus, others are only translocated to the nucleus under specific conditions, otherwise being predominantly cytoplasmic (Nigg et al., 1991; Jans, 1995). The advantages of a conditionally cytoplasmic location for a transcription factor (TF) include the potential to control its activity by regulating its nuclear uptake and its direct accessibility to cytoplasmic signal-transducing systems (Schmitz et al., 1991; Jans, 1995). TFs able to undergo inducible nuclear import include the glucocorticoid receptor (Picard and Yamamoto, 1987), the α-interferon-regulated factor interferon stimulated gene factor 3 (ISGF-3) (Schindler et al., 1992), γ-interferon-activated factor (GAF) (Shuai et al., 1992), the nuclear vjjun oncogenic counterpart of the AP-1 transcription complex member c-jun (Chida and Vogt, 1992; Takagawa et al., 1995), the Saccharomyces cerevisiae TF SW15 (Moll et al., 1991; Jans et al., 1995), the Drosophila melanogaster morphogen dorsal (Govind and Steward, 1991), and the nuclear factor NB-XB (Schmitz et al., 1991; Shirakawa and Mizes, 1989; Lenardo and Baltimore, 1989). The fact that the nuclear translocation of various TFs, developmental morphogens, and oncogene products accompanies changes in the differentiation or metabolic state of eukaryotic cells indicates that nuclear protein import is a key control point in the regulation of gene expression and signal transduction.

Proteins larger than 45 kDa require a nuclear localization signal (NLS) (see Jans, 1995; Jans and Hübner, 1996) in order to be targeted to the nucleus. In addition to the NLS, specific signals carried by the transported proteins function in a regulatory fashion, whereby covalent modifications such as phosphorylation play a central role (Jans, 1995; Jans and Hübner, 1996). We have demonstrated that nuclear import of SV40 large tumor antigen (T-ag) fusion proteins is regulated by the CcN motif (Jans et al., 1991), comprising phosphorylation sites for casein kinase II (CKII) and the cyclin-dependent kinase cdc2 together with the NLS. Although nuclear localization is entirely NLS-dependent (Rihs and Peters, 1989; Rihs et al., 1991), the rate of nuclear import is regulated by the CKII phosphorylation site (Ser111/112) (Jans et al., 1991; Rihs et al., 1991; Jans and Jans, 1994), and phosphorylation at the cdc2 site (Thr124) adjacent to the NLS (amino acids 126–132) determines the maximal extent of nuclear accumulation (Jans et al., 1991). Regulation of nuclear transport by CcN motif kinase sites also holds true for SW15 (Moll et al., 1991; Jans et al., 1995) and the Xenopus nuclear phosphoprotein nucleoplasm (Vancurova et al., 1995). It is likely, however, that other kinases/kinase sites function in analogous fashion to regulate nuclear protein import specifically (Jans and Jans, 1994; Jans, 1995; Jans and Hübner, 1996). The cAMP-dependent protein kinase (PK-A), for example, has been implicated in enhancing
nuclear import of members of the red/dorsal class of TFs, although kinetic analyses have not been performed (Mosialos et al., 1991; Norris and Manley, 1992).

In order to test directly whether a kinase site other than those of the CcN motif could regulate T-ag nuclear import, and as a first step toward developing a phosphorylation regulated NLS (prNLS) (Jans, 1995) capable of conferring inducible nuclear import was increased by 6-10 separate measurements for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence respectively, with autofluorescence subtracted. Curves are fitted for the function Fn/c = Fn/Fnmax (1 - e^-kt) (Jans et al., 1991, 1995; Jans and Jans, 1994), where t is time in min.

Cell Culture—Cells of the HTC rat hepatoma tissue culture (a derivative of Morris hepatoma 7288C) cell line were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum as described previously (Rihs et al., 1991; Jans et al., 1992).

SV40 T-ag β-Galactosidase Fusion Proteins—T-ag fusion proteins, containing T-ag amino acids 111-135, including the CcN motif (comprising CcN and cyclin-dependent kinase sites) and NLS) fused N-terminally to E. coli β-galactosidase (amino acids 9-1023). The single-letter amino acid code is used, whereby the NLS is double underlined and the phosphorylation sites in bold, with that for CKII underlined and that for PK-A underlined with a dotted line. Capital letters indicate T-ag sequence. The C-, Cc-, and CcN-β-Gal fusion proteins have been previously described (Jans and Jans, 1994, Rihs et al., 1991). Nuclear transport was measured in microinjected (in vivo) or mechanically perforated (in vitro) HTC cells using CLSM as described under “Experimental Procedures” (Jans et al., 1991, 1995; Jans and Jans, 1994). The in vivo measurements 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. The in vitro measurements are from a single typical experiment (see also Table I), each point representing the average of at least 10 separate measurements for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence respectively, with autofluorescence subtracted. Curves are fitted for the function Fn/c = Fn/Fnmax (1 - e^-kt) (Jans et al., 1991, 1995; Jans and Jans, 1994), where t is time in min.

**EXPERIMENTAL PROCEDURES**

Chemicals and Reagents—Isopropyl-β-thiogalactoside, recombinant human CKII (EC 2.7.1.3), the CKII-specific peptide substrate Arg-Arg-Arg-Asp-Asp-Ser-Asp-Asp-Asp, β-galactosidase (EC 3.2.1.23.37), and polyethylene glycol were from Boehringer Mannheim, 5-iodacetamido-fluorescein was from Molecular Probes, and kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and PK-A (EC 2.7.1.37) C-subunit (bovine heart) were from Sigma. Other reagents were from the sources previously described (Rihs et al., 1991; Jans et al., 1991, 1995; Jans and Jans, 1994).

**FIG. 1.** Sequence of the SV40 T-ag fusion proteins used in this study (A) and nuclear import kinetics in vivo and in vitro (B). A. All fusion proteins contain SV40 T-ag sequences fused N-terminal to E. coli β-galactosidase (amino acids 9-1023). The single-letter amino acid code is used, whereby the NLS is double underlined and the phosphorylation sites in bold, with that for CKII underlined and that for PK-A underlined with a dotted line. Capital letters indicate T-ag sequence. The C-, Cc-, and CcN-β-Gal fusion proteins have been previously described (Jans and Jans, 1994, Rihs et al., 1991). Nuclear transport was measured in microinjected (in vivo) or mechanically perforated (in vitro) HTC cells using CLSM as described under “Experimental Procedures” (Jans et al., 1991, 1995; Jans and Jans, 1994). The in vivo measurements 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. The in vitro measurements are from a single typical experiment (see also Table I), each point representing the average of at least 10 separate measurements for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence respectively, with autofluorescence subtracted. Curves are fitted for the function Fn/c = Fn/Fnmax (1 - e^-kt) (Jans et al., 1991, 1995; Jans and Jans, 1994), where t is time in min.
prephosphorylated by PK-A were microinjected and used in vitro as described previously forcdc2-prephosphorylated proteins (Jans et al., 1991). Quantitation of fluorescence using CLSM has been described previously in detail (see) and Jans (1992), and Jans and Pavo (1995) for other applications. Image analysis of CLSM files using the NIH Image public domain software and curve fitting was performed as described (Jans et al., 1995). To examine longer term nuclear import kinetics, histonoechemical staining of fixed cells in situ for β-galactosidase activity after microinjection using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was performed as described (Rihs et al., 1991; Jans et al., 1991, 1995; Jans and Jans, 1994).

In Vitro Phosphorylation—In vitro phosphorylation of fusion proteins by CKII and PK-A C-subunit was analyzed qualitatively by SDS gel electrophoresis and quantitatively by determination of the stoichiometry of phosphorylation as previously (Botterell et al., 1987; Jans et al., 1987, 1993).

PK-A Activity—PK-A activity was measured in extracts from intact HTC cells subsequent to stimulation with or without the adenylate cyclase activator forskolin (100 μM; Calbiochem) and the phosphodiesterease inhibitor isobutylmethylxanthine (0.5 M; Sigma), as well as in reticulocyte lysate, using kemptide as a specific PK-A substrate, as described previously (Ackerman and Osheroff, 1989; Jans and Jans, 1987; Jans et al., 1987).

Phosphorylation in Cytosolic Extracts—HTC extracts were prepared as described previously (Ackerman and Osheroff, 1989; Jans and Jans, 1994). Phosphorylation in cytosolic extract from HTC cells and reticulocyte lysate (the cytosol routinely used in the in vitro nuclear transport assay) was performed as described (Jans and Jans, 1994), except that where indicated, cAMP (25 μM) and/or PK-I 5–24 peptide (2.5 μM) was included in the incubation (1 h, 30°C). Prior to SDS gel electrophoresis, phosphorylated fusion proteins were separated from components of the cytosolic extracts using affinity chromatography performed in batch. In brief, extracts were incubated with agarose (p-aminobenzyl-1-thio-β-D-galactopyranoside-agarose; Sigma) to which a nonhydrolyzable β-galactosidase substrate is covalently attached, subsequent to the phosphorylation incubation. Conditions for the binding of β-galactosidase to the gel were identical to those routinely used for fusion protein purification (Rihs et al., 1991). Subsequent to washing and centrifugation, the agarose was resuspended in sample loading buffer (Laemmli, 1970) and loaded directly onto the SDS gel (7.5% 30:1 acrylamide-bis-acrylamide). Quantitation of the stoichiometry of phosphorylation in cell extracts was performed using a Molecular Dynamics Phosphorimage, where exposure values for fusion protein bands were converted to absolute values through identical analysis of in vitro phosphorylated samples of predetermined stoichiometry of phosphorylation (Botterell et al., 1987; Jans and Jans, 1994).

**RESULTS**

An Engineered PK-A Site in Place of the Ccn Motif CKII Site in T-ag—In order to test directly the possibility that kinases other than those of the Ccn motif may regulate nuclear protein import and as a first step toward developing a phosphorylation-regulated NLS capable of conferring inducible nuclear translocation on carrier molecules of interest, we set out to replace the T-ag CKII site (Ser111-Ser-Asp-Asp-Glu) by a consensus site for PK-A (Arg-Arg-Ala-Ser111) using site-directed mutagenesis (see “Experimental Procedures” for details). We simultaneously replaced the acidic recognition site with Asn-Asn-Gln115, a substitution that we have previously shown to render the CKII site nonfunctional in terms of both enhancement of nuclear import and phosphorylation at the site (Jans and Jans, 1994). Integrity of the construct was confirmed by DNA sequencing. The relevant amino acid sequence of the resultant AcN-β-Gal T-ag fusion protein is presented in Fig. 1A.

To confirm that the engineered phosphorylation site was functional, phosphorylation was tested in vitro using purified CKII and PK-A C-subunit (Fig. 2). The AcN-β-Gal T-ag fusion protein was not phosphorylated to a significant extent by CKII, in contrast to the wild type Ccn-β-Gal T-ag fusion protein, indicating that as expected, the CKII site was no longer functional. The AcN-β-Gal T-ag fusion protein was, however, spe-
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Table I

| Fusion protein* | Phosphorylation Sites | Treatment | Nuclear import parameter
|-----------------|-----------------------|-----------|-------------------------|
|                 | PK-A                  | CKII      | NLS        | in vivo | in vitro |
| N-β-Gal         | −                    | −         | +          | k(10⁻³) | Fv/cmax (n) | k(10⁻³) | Fv/cmax (n) |
| dN-β-Gal        | −                    | −         | +          | ND     | 0.48 (1)   | ND     | ND |
| Cc-β-Gal        | −                    | −         | +          | ND     | 1.7(1)     | ND     | 1.3(1) |
| Ccn-β-Gal       | −                    | +         | +          | 160 ± 40 | 7.3 ± 1.7 (2) | 36 ± 7 | 6.5 ± 1.6 (3) |
| Acn-β-Gal       | −                    | +         | +          | 130 ± 40 | 3.5 ± 0.2 (3) | 40 ± 4 | 3.6 ± 0.7 (5) |
| Acn-β-Gal       | +                    | −         | +          | 710 ± 26 | 3.1 ± 0.6 (4) | 110 ± 31 | 3.2 ± 0.3 (3) |
| Acn-β-Gal       | +                    | +         | +          | 530     | 2.4 (1)    | 58     | 2.9 (1) |

a T-ag fusion protein sequences are presented in Fig. 1A.

b Raw data (see Figs. 1B and 5, and not shown) were fitted for the function Fv/cmax *(1 − e⁻ᵗ) (Jans et al., 1991, 1995; Jans and Jans, 1994), where t is time in min.

c ND, not determined.
d Stoichiometry of prephosphorylation for AcN-β-Gal in the case of the in vivo and in vitro experiment was 0.53 (phosphorylation for 30 min at 30°C) and 2.7 (phosphorylation for 2 h at 30°C) mol Pi/mol tetramer respectively (see also Table II).

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PK-A specifically phosphorylated by PK-A (Fig. 2), indicating that the introduced PK-A site was functional. The Ccn-β-Gal T-ag fusion protein and β-galactosidase itself were not phosphorylated by PK-A, as expected. The results indicated that we had been successful in introducing a functional PK-A site in place of the CKII site, within the T-ag Ccn motif, in Acn-β-Gal.

Nuclear Import Kinetics—The kinetics of nuclear import of Acm-β-Gal were compared with those of the wild type Ccn-β-Gal T-ag fusion protein, as well as derivatives thereof either lacking a functional CKII site (dN-β-Gal) (Jans and Jans, 1994) or possessing a nonfunctional NLS (Cc-β-Gal T-ag fusion protein) (Rhihs and Peters, 1989; Rhihs et al., 1991) (see Fig. 1A for T-ag sequences) both in vivo and in vitro (Figs. 1B and 3 and Table I). The Acm-β-Gal T-ag fusion protein showed maximal nuclear accumulation about half that of wild type (Ccn-β-Gal) and more than two times higher than that of the Ccn-β-Gal T-ag fusion protein which lacks a functional CKII (or PK-A) site. The rate at which steady state was achieved in terms of nuclear accumulation (k) was comparable between Acm-β-Gal and Ccn-β-Gal (Fig. 1B; Table I). The fact that the Acm-β-Gal T-ag fusion protein was accumulated efficiently in the nucleus of HTC cells both in vivo and in vitro indicated that the PK-A site can function to enhance T-ag nuclear import in the absence of the CKII site. This indicated that a kinase site other than those of the Ccn motif can act to regulate T-ag nuclear import.

PK-A Activity in HTC Cells—The fact that Acm-β-Gal was accumulated efficiently in nuclei of HTC cells in the absence of exogenous stimulus of PK-A implied that the basal level of PK-A activity was sufficient to enhance fusion protein nuclear import. In order to quantify the basal level of PK-A activity in untreated and polyethylene glycol-treated HTC cells, kemptide phosphorylation was measured in cell extracts in the absence (basal activity) and the presence (total stimulatable activity) of 10 μM cAMP (Fig. 4). The basal PK-A activity was found to be about 0.5 unit/mg cell extract (activity ratio of about 0.16), which is about 5 times higher than the activity we have found in other cell lines (e.g., Botterell et al., 1987; Jans et al., 1987). This relatively high basal PK-A activity is presumably sufficient to effect rapid nuclear import of the Acm-β-Gal T-ag fusion protein in the absence of hormonal or other stimulation of HTC cells; analogous results have been reported in other systems (e.g., that of the myogenic factor MyoD) (Vanromunde et al., 1994). Upon stimulation for 1 h with the adenylate cyclase activator forskolin in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine, PK-A activity was increased to about 1.4 units/mg (activity ratio, 0.45) (Fig. 4), which compares quite well with results for other cell lines (e.g., Botterell et al., 1987; Jans et al., 1987).

Induction of PK-A Activity Enhances Nuclear Import—Nuclear import of the Acm-β-Gal T-ag fusion protein was assessed in response to treatments inducing phosphorylation at the PK-A site (Figs. 3 and 5; Tables I and II). Control import kinetics were initially compared in response to cAMP in the absence and the presence of the specific PK-A C-subunit peptide inhibitor PK-I 5–24 both in vivo and in vitro. No marked differences were observed in terms of the maximal level of nuclear accumulation (Fv/cmax) in the presence or the absence of cAMP or PK-I 5–24, in contrast to the rate of import that increased 3–5-fold in the presence of cAMP (Fig. 5 and Tables I and II; compare the bottom left and right panels of Fig. 3, A and B). Nuclear accumulation of Acm-β-Gal in the presence of cAMP closely resembled that of the wild type Ccn-β-Gal at early time points, as can be seen in the comparison of the right hand upper and lower panels of Fig. 3 (A and B). PK-I 5–24 eliminated the increase in the rate of nuclear import induced by cAMP (Fig. 5; Tables I and II), strongly implying that the effect of cAMP on nuclear import of Acm-β-Gal was mediated by the PK-A C-subunit.

More detailed examination of the nuclear import kinetics showed that the effect of cAMP appeared to be exerted at the level of the initial rate of nuclear import (Table II). The initial
rate of import of AcN-β-Gal was increased by about 2-fold in response to cAMP, the effect being abrogated by PK-1 5–24 (Table II). As expected, cAMP had no effect on the rate of nuclear import of the CdcN-β-Gal fusion protein that lacks the PK-A site (Table II), indicating that the enhancement of nuclear uptake by cAMP was specific to AcN-β-Gal.

The above results closely paralleled results for the phosphorylation of AcN-β-Gal in cytosolic extracts (Fig. 6, and not shown). As observed previously, the wild type CdcN-β-Gal fusion protein, which contains the CKII site, was strongly phosphorylated due to the presence of cytosolic CKII (Jans and Jans, 1994). The basal level of phosphorylation of AcN-β-Gal in the absence of cAMP was about 20% that of CdcN-β-Gal (Fig. 6). This basal phosphorylation activity is presumably sufficient to support the nuclear transport of AcN-β-Gal in vitro at the rate observed in the absence of exogenous cAMP (Fig. 1B and Table I). The addition of cAMP increased the phosphorylation of AcN-β-Gal over 2-fold above this basal level (Fig. 6). Both basal and cAMP-induced phosphorylation of AcN-β-Gal was inhibited markedly by PK-1 5–24 (Fig. 6), demonstrating that PK-A was indeed the kinase responsible for AcN-β-Gal phosphorylation in reticulocyte lysate. Consistent with this, the PK-A C-subunit was demonstrated to be present in reticulocyte lysate by Western blot analysis, as well as in cytosolic extracts from HTC cells (not shown).

Similar results to those above for nuclear transport were obtained in in vitro experiments in which the PK-A C-subunit was included together with AcN-β-Gal, the initial rate of nuclear transport being increased over 2-fold (Table II, in vitro). PK-1 5–24 abrogated the effect of the inclusion of C-subunit, which did not affect nuclear import of the control CdcN-β-Gal fusion protein (Table II, in vitro). Finally, the nuclear import kinetics of AcN-β-Gal prephosphorylated by PK-A in vitro were also measured both in vivo and in vitro (Tables I and II). Prephosphorylated AcN-β-Gal was accumulated at a 2–3-fold higher rate (Table I), which was attributable to a 2–3-fold higher initial rate of transport (Table II). As a control, the CN-β-Gal T-agar fusion protein lacking the PK-A site was incubated with PK-A, and the nuclear import kinetics was subsequently measured (Tables I and II). Preincubation with PK-A had no significant effect on the rate of import, meaning that the enhanced import rate of prephosphorylated AcN-β-Gal could be directly attributed to phosphorylation at the PK-A site.

**DISCUSSION**

This study constitutes the first report of a kinase site being replaced by a consensus site for another kinase in order to alter the regulation of the physiological effects of phosphorylation at the site in question. It shows that the introduction of a consensus site for PK-A in place of the CKII site of the T-ag CcN motif can confer PK-A-mediated regulation of the kinetics of nuclear import of T-ag fusion proteins. The addition of cAMP or PK-A C-subunit or prephosphorylation by PK-A at the site increases the rate of nuclear import 2–5-fold, largely through increasing the initial rate of nuclear import. The PK-A-specific inhibitor peptide PK-1 5–24 inhibits the cAMP-induced and PK-A C-subunit-induced enhancement of nuclear import, indicating that the effects are mediated by PK-A phosphorylation at the PK-A site. That PK-A may regulate nuclear protein import in the case of TFS such as those of the rel/dorsal family has been established by others (Mosialos et al., 1991; Norris and Manley, 1992); this, however, is the first time that a PK-A site has been engineered in place of a kinase site in a heterologous protein and shown to be capable of regulating nuclear import. The results here clearly demonstrate that kinases other than those of the Cdkn motif can regulate nuclear import of T-ag if the appropriate phosphorylation site is present.

The fact that PK-A regulates the rate rather than the maximal extent of nuclear import of AcN-β-Gal is consistent with the fact that the CcN site, replaced by the consensus PK-A site in AcN-β-Gal, regulates the rate of nuclear import of the wild type T-ag CdcN-β-Gal fusion protein (Jans and Jans, 1994; Rihs et al., 1991). Phosphorylation at the PK-A/CCKII site thus modulates the same parameter of nuclear protein import, but because the kinases phosphorylating at the respective sites exhibit distinct regulation, this results in differences in the stimuli enhancing nuclear import of the respective fusion proteins. Although treatments activating PK-A enhance nuclear import of AcN-β-Gal, CKII-mediated enhancement of that of CdcN-β-Gal appears to be largely constitutive (see Jans, 1995; Jans and Jans, 1994), due to the fact that CKII activity appears to be largely constitutive in most cell types (Allende and Allende, 1995). The engineered AcN signal is thus a prNLS conferring inducible nuclear import, hormonal, or other stimuli that activate PK-A able to directly modulate the rate of nuclear entry of a carrier protein to which it is attached.
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Table II

| Fusion protein | Treatment | Initial nuclear import rate |
|----------------|-----------|-----------------------------|
|                |           | Fn/min (n)* | Relative untreated |
| In vivo        |           |              |                   |
| AcN-β-Gal      | None      | 0.30 (2)     | 100               |
| AcN-β-Gal      | cAMP      | 0.57 ± 0.04 (3) | 190               |
| AcN-β-Gal      | cAMP/PK-I 5–24 | 0.29 (1) | 97                |
| AcN-β-Gal      | PK-A prephosphorylationa | 0.52 (1) | 173               |
| CN-β-Gal       | None      | 1.20 (1)     | 100               |
| CN-β-Gal       | PK-A preincubationa | 1.20 (1) | 100               |
| CN-β-Gal       | PK-A preincubationb | 1.10 (1) | 92                |
| In vitro       |           |              |                   |
| Experiment 1 (n = 2) |          |              |                   |
| AcN-β-Gal      | None      | 0.19 ± 0.01 (2) | 100               |
| AcN-β-Gal      | cAMP      | 0.39 ± 0.10 (2) | 119               |
| AcN-β-Gal      | cAMP/PK-I 5–24 | 0.22 (1) | 97                |
| AcN-β-Gal      | PK-I 5–24 | 0.18 (1)     |                   |
| Experiment 2 (n = 2) |          |              |                   |
| AcN-β-Gal      | None      | 0.23 ± 0.04 (2) | 100               |
| AcN-β-Gal      | +PK-A C-subunit | 0.61 ± 0.10 (2) | 265               |
| AcN-β-Gal      | +PK-A C-subunit/PK-I 5–24 | 0.28 ± 0.04 (2) | 122               |
| CN-β-Gal       | None      | 0.50 (1)     | 100               |
| CN-β-Gal       | +PK-A C-subunit | 0.40 (1) | 80                |
| Experiment 3 (n = 1) |          |              |                   |
| CN-β-Gal       | None      | 0.52         | 100               |
| CN-β-Gal       | PK-A preincubationb | 0.35 | 67                |
| AcN-β-Gal      | None      | 0.08         | 100               |
| AcN-β-Gal      | cAMP      | 0.21         | 263               |
| AcN-β-Gal      | PK-A prephosphorylationb | 0.25 | 313               |

*a The Fn/c for the initial 5 min of accumulation is expressed per minute.

*b Stoichiometry of phosphorylation for AcN-β-Gal of 0.53 mol P_i/mol tetramer (phosphorylation for 30 min at 30 °C). The CN-β-Gal protein (Rihs et al., 1991) lacks the PK-A site and was preincubated with PK-A C-subunit for 30 min at 30 °C as a negative control.

*c Stoichiometry of phosphorylation for AcN-β-Gal of 2.7 mol P_i/mol tetramer (phosphorylation for 2 h at 30 °C). The CN-β-Gal protein was preincubated with PK-A C-subunit for 2 h at 30 °C as a negative control.

Fig. 6. Phosphorylation of T-ag fusion proteins in the absence or the presence of cAMP and PK-I 5–24 in cytosolic extract. Subsequent to the incubation of fusion proteins in reticulocyte lysate (60 min at 30 °C), affinity chromatography, and SDS gel electrophoresis (7.5% 30:1 acrylamide-bis-acrylamide), the stoichiometry of phosphorylation was determined as described under “Experimental Procedures” using phosphor imaging of the dried gel. The standard used was the CN-β-Gal protein phosphorylated by purified CKII to a stoichiometry of 0.54 mol P_i/mol tetramer.

prNLSs have an important potential application in targeting molecules of interest to the nucleus (Jans, 1994, 1995). Those such as the engineered prNLS described here where the PK-A site modulates the rate of nuclear import are of particular interest, because they potentially confer tightly regulated nuclear localization according to hormonal or other stimuli, thus enabling precise cueing of the nuclear localization of relevant proteins and other molecules according to need. This may have application in gene therapy through facilitating the directed transport of DNA molecules to the nucleus of mammalian or plant cells to increase transfection and/or homologous recombination efficiencies (see Jans, 1994; Rosenkranz et al., 1992). Alternatively, toxic molecules might be efficiently targeted to sensitive subcellular sites such as the nucleus in order to effect tumor cell killing (Akhylnina et al., 1993, 1995). The prNLS derived and characterized in this study represents an important first step toward developing a signal conferring inducible nuclear targeting of molecules of interest. Although the PK-A-T-ag-NLS prNLS did not show absolute dependence on induc- tion of PK-A activity for nuclear translocation in HTC cells, presumably due to the relatively high basal PK-A activity (see Figs. 4, 5; Vandromme et al., 1994), it may, however, be useful for conditional inducible nuclear targeting of molecules of interest in other cell lines where PK-A activity is more tightly regulated. Apart from the derivation of further inducible vari- ants of the T-ag CcN motif, future work in this laboratory will include investigation of the efficacy of this prNLS in various cell lines, including somatic cell PK-A mutants (Borterell et al., 1987), as well as the investigation of its use in conjunction with plasmid constructs encoding the cDNAs for the PK-A C-subunit and/or PK-A inhibitor PK-I (see Norris and Manley, 1992) expressed from inducible promoters. Our ultimate aim is to achieve fully inducible/conditional nuclear targeting of mole- cules of interest for use in a variety of cell types with widespread clinical and research applications.

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