REVIEW ARTICLE

The regulation of protein transport to the nucleus by phosphorylation

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INTRODUCTION

Since the identification over 10 years ago of the sequence responsible for the nuclear localization of the simian virus 40 (SV40) large tumour antigen (T-ag) and the demonstration of its ability to target heterologous, normally non-nuclear, proteins to the nucleus, research in the field of nuclear transport has largely revolved around the idea of nuclear localization signals (NLSs) being exclusively responsible for protein targeting to the nucleus. NLSs have been defined for a variety of nuclear proteins, and specific NLS receptors or NLS binding proteins (NLSBPs) have been identified and purported to play an essential role in the transport process. It has become increasingly clear over the last few years, however, that NLS function can be regulated, with phosphorylation as the main mechanism controlling NLS-dependent nuclear localization of a number of proteins, including transcription factors (TFs) such as nuclear factor (NF) κB, c-rel, *dorsal*, the α-interferon-stimulated gene factor 3 (ISGF-3), the γ-interferon activated factor (GAF), the nuclear factor of activated T-cells (NF-AT) and the yeast TF SWI5. Nuclear translocation of various TFs and oncogene products accompanies changes in the differentiation or metabolic state of eukaryotic cells precisely, indicating that nuclear protein import is a key control point in the regulation of gene expression.

This review seeks to expound the considerable evidence presently available for the regulated nuclear localization of proteins. This will include analysis showing that even the nuclear localization of the archetypal NLS-containing T-ag is subject to regulation, whereby phosphorylation sites for casein kinase II (CKII) and the cyclin-dependent kinase (cdk) cdc2 in the vicinity of the NLS determine both the rate and the absolute amount of T-ag that is maximally accumulated in the nucleus in an NLS-dependent fashion. The regulatory module for regulated T-ag transport, i.e. the CcN motif, comprising the phosphorylation sites together with the NLS, has been identified in a variety of other nuclear-localized proteins. It appears to be a special type of phosphorylation-regulated NLS (prNLS), where kinases can specifically regulate the nuclear import of particular proteins through phosphorylation at site(s) close to their respective NLSs. The review will also describe the mechanisms by which phosphorylation regulates nuclear protein import, including cytoplasmic retention factors, intra- and inter-molecular NLS masking and direct NLS masking by phosphorylation.

NUCLEAR–CYTOPLASMIC PROCESSES IN THE EUKARYOTIC CELL

The nuclear pore complex (NPC) as a molecular sieve

The fact that eukaryotic cells possess a nucleus has important implications for cellular function. The existence of a nuclear compartment means that the genetic material, the DNA, is in a different subcellular compartment from the site of protein synthesis, the cytoplasm. The two compartments are separated by a double membrane structure, the nuclear envelope [1]. Gene transcription and translation thus occur in different subcellular compartments, meaning that specific transport events between the two are necessary for a eukaryotic cell to be functional: mRNA must make its way from the nucleus to the cytoplasm in order to be translated into protein; and proteins which are needed in the nucleus (structural nuclear proteins such as histones and lamins; the enzymes required for DNA replication, gene transcription, activation of gene expression, etc.) have to be transported from their site of synthesis in the cytoplasm into the nucleus. All transport goes through the nuclear-envelope-localized NPCs, which are very large (about 10^5 kDa), almost organelle-like, structures composed of at least 30 distinct protein components [1–3]. Between 100 and 5×10^7 NPCs are present per nucleus depending on the metabolic and differentiation state of the cell, with more complexes present in active or differentiating cells, underlining the importance of nuclear transport in cellular processes. As its name suggests, the NPC has a pore or molecular sieve function whereby molecules smaller than 40–45 kDa can diffuse freely between cytoplasm and nucleus [1–3].

Nuclear localization signals

Although there are exceptions, proteins larger than 45 kDa require an NLS in order to be targeted to the nucleus. Through mutation/deletion analysis and/or their ability to target a normally cytoplasmically localized protein to the nucleus, NLSs have been defined as the sequences necessary and sufficient for nuclear localization. They function through recognition/ligand–receptor-like interactions rather than through conferring binding capabilities to chromatin or nuclear structures, etc. ([2–5]; see however [6]); consistent with this, *in vivo* lateral mobility measurements indicate that nuclear accumulated NLS-carrying proteins are not immobilized [4,5]. Proteins of > 45 kDa microinjected into the nucleus remain nuclear whether they possess an NLS or not [2–4], suggesting that NLSs function as entry signals rather than as signals for retention in the nucleus.

The archetypal NLS is that of T-ag (Pro-Lys-Lys-Lys-Arg-Lys-Val^{132}). It is functional in targeting normally cytoplasmic carrier proteins to the nucleus, whether present within the coding sequence of the carrier protein or as a peptide covalently coupled to the carrier [7–9]. The mutation of a single amino acid residue within this sequence (Lys^{128} to either Thr or Asn) abolishes its nuclear targeting function. The T-ag NLS, with its concentration

Abbreviations used: SV40, simian virus 40; T-ag, SV40 large tumour antigen; NPC, nuclear pore complex; NLS, nuclear localization signal; prNLS, phosphorylation-regulated NLS; NLSBP, NLS binding protein; CLSM, confocal laser scanning microscopy; CKII, casein kinase II; cdk, cyclin-dependent kinase; TF, transcription factor; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; C- and R-subunits, catalytic and regulatory subunits respectively; PKC, Ca^{2+}/phospholipid-dependent protein kinase; MAP-K, mitogen-activated protein kinase; M-CAMPK, multifunctional calmodulin-dependent protein kinase; NF-κB, nuclear factor κB; GlucR, glucocorticoid receptor; GlucH, glucocorticoid hormone; ISGF-3, interferon-stimulated gene factor; GAF, γ-interferon-activated factor; NF-AT, nuclear factor of activated T-cells; IL, interleukin; EGF, epidermal growth factor; rNFIL-6, rat nuclear factor induced by IL-6.

of basic amino acids preceded by a β-turn, has served as the basis of the search via sequence homology for NLSs in other nuclear proteins [2,10]. Another class of NLS, i.e. the 'bipartite NLS' with two clusters of basic amino acid residues separated by an intervening 10–12-amino-acid spacer, has more recently been established as a variant of the T-ag NLS archetype [1,11,12]; examples are those for the *Saccharomyces cerevisiae* TF SWI5 and the *Xenopus laevis* nuclear phosphoproteins nucleoplasmin and N1N2. A further class of NLSs is based on similarity to the yeast MAT α2 NLS (Asn-Lys-Ile-Pro-Ile-Lys-Asp[8]; see [2,10]).

NLSs have been shown to be recognized by binding proteins (NLSBPs; e.g. [13–18]) hypothesized to have a receptor/carrier role in the active transport of NLS-carrying proteins to the nucleus. Their exact NLS binding specificity and precise subcellular location remain controversial, however. Direct evidence has been provided that the molecular chaperonin HSC70/HSP70 is both necessary for NLS-dependent nuclear transport in reconstituted systems (see, however, [19]) and capable of specifically binding NLSs [20,21].

METHODOLOGICAL CONSIDERATIONS

A drawback of many studies to date relating to the identification of NLSs is the experimental approach used. Generally, eukaryotic cells are transfected with a protein-expressing plasmid construct, and then fixed 1–2 days later to be scored qualitatively for subcellular localization of the expressed protein using indirect immunofluorescence. Apart from the fact that fixation procedures may artifactually affect the results, such an approach provides no information with respect to the rate of nuclear import, since analysis is performed at steady state. In addition, the high levels of protein expression in the cells analysed are often not physiologically relevant, especially in the case of TFs which are normally present in very low amounts. Some of these problems can be avoided by examining the rate of transport of carrier proteins containing multiple covalently attached NLS-peptides microinjected into living cells, although the protein constructs generally have very high NLS/protein molecular mass ratios, a factor which has been shown to be important in several studies (e.g. [22,23]). Alternative methods such as subcellular fractionation suffer from the caveat that protein redistribution may occur during the fractionation process.

Confocal laser scanning microscopy (CLSM) has more recently been applied to quantify fluorescence, enabling the estimation of relative protein concentrations in different subcellular compartments in intact cells [24]. We and others have used microinjection and CLSM techniques to analyse nuclear import kinetics in single living cells and have thus succeeded in resolving the transport process temporally *in vivo* [4,5,25–27]. The establishment of *in vitro*/reconstituted nuclear import assay systems has further contributed to understanding nuclear protein import, elucidating the mechanistic role of NLSBPs in particular [16,20]. In conjunction with CLSM, *in vitro* nuclear transport assays can be used quantitatively to define the individual kinetic steps of nuclear transport. We have established an *in vitro* system based on mechanically perforated rat hepatoma cells [26]. Consistent with findings from other *in vitro* systems [16,20,28–30], NLS-dependent nuclear transport in mechanically perforated hepatoma cells can be shown to involve at least two steps [26], both of which are dependent on cytosolic factors (D. A. Jans, unpublished work; [26]): an ATP-independent 'docking' or binding stage at the nuclear envelope/NPC, and an ATP-dependent active-transport step (see [30]). Roles have recently been established for a 97 kDa protein together with an NLSBP in the docking step [29], and for the monomeric GTP-binding protein Ran/TC4 in the active import stage [28,31]. Analysis of the rates and maximal extent of nuclear transport ([4,5,25–27], and see below) has shown that nuclear transport is not exclusively determined by the NLS; rather, other sequences, and in particular phosphorylation sites, act to modulate NLS function.

REGULATED (CONDITIONAL) NUCLEAR ENTRY

Whereas some proteins such as histones appear to be constitutively targeted to the nucleus, others are only targeted to the nucleus under specific conditions, often being present mostly in the cytoplasm (see [2,32]). TFs regulating nuclear gene expression are no different from other proteins in terms of their being synthesized in the cytoplasm and thereby subject to specific mechanisms regulating nuclear protein import. The advantages to the cell of a conditionally cytoplasmic location for a TF include the potential to control its activity by regulating its nuclear uptake, and its direct accessibility to cytoplasmic signal-transducing systems. The nuclear translocation of various TFs [32–36] and proto-oncogene/oncogene products [37–42] has been shown to accompany changes in the differentiation or metabolic state of eukaryotic cells precisely, indicating that nuclear protein import can be a key control point in the regulation of gene expression and signal transduction.

TFs able to undergo inducible nuclear import include the glucocorticoid receptor (GlucR) [43], the interferon-regulated factors ISGF-3 [44] and GAF [45], the yeast mating switch/HO endonuclease promoter regulator SWI5 [35], the *Drosophila melanogaster* morphogen *dorsal* [36], and the nuclear factors NF-κB [32–34] and NF-AT [46]. A number of examples of regulated transport of proteins to the nucleus are listed in Table 1.

Examples of signal transduction pathways from extracellular hormonal signal to the nucleus ultimately leading to changes in the regulation of gene expression include that of the GlucR, where, upon glucocorticoid hormone (GlucH) binding, the cytoplasmic receptor translocates to the nucleus to modulate gene transcription by direct binding to specific DNA sequences called GlucH-response elements [43]; and that of hormone-stimulated response to elevated cyclic AMP (cAMP) levels. The latter results in translocation of the cAMP-dependent protein kinase (PKA) catalytic (C-) subunit from the cytoplasm to the nucleus [73–75], where it phosphorylates and thereby modulates the activities of nuclear TFs such as the cAMP response element binding protein, CREB. In similar fashion to PKA, the mitogen-activated protein kinases (MAP-Ks) $p42^{mapk}$ and $p44^{erk1}$ [76–79] and members of the *rsk*-encoded kinases also enter the nucleus upon mitogenic stimulation and activation [76], while the 82 kDa α isoform of the Ca^{2+}/phospholipid-dependent protein kinase (PKC) re-localizes to the nuclear envelope upon activation [80]. Hormonally induced nuclear transport of activated kinases is a commonly exploited means of communicating signals to the nucleus in many signal transduction and developmental pathways (see Table 1).

T-ag AND THE CcN MOTIF

As mentioned above, nuclear localization of T-ag is dependent on amino acids 126–132. Mutations within the T-ag NLS abolish nuclear targeting [7–9]. Using quantitative CLSM to measure nuclear transport kinetics at the single-cell level both *in vivo* and *in vitro*, we have demonstrated that NLS-dependent nuclear transport of T-ag is regulated by phosphorylation. Sites for the physiologically important CKII and the cdk cdc2, present close to the T-ag NLS, regulate the kinetics of nuclear transport of T-ag, probably by modulating specific protein–protein binding

Table 1 Examples of regulated nuclear protein transport

The single-letter amino acid code is used. NLSs are underlined, and phosphorylated residues are numbered according to their residue number in the respective protein. Abbreviations: a.a., amino acids; IL-1α, interleukin 1α; TNFα, tumour necrosis factor α; PP-2A, protein phosphatase type 2A; Tyk2, JAK1/JAK2, non-receptor tyrosine kinases; GrH, growth hormone; RB-1, p110^{Rb}, the product of the 'retinoblastoma-susceptibility factor' tumour-suppressor gene; PTF1, pancreas-specific TF1; HMG, high-mobility-group nuclear protein; MAPK-K, MAP-K kinase.

Protein	Stimulus/ kinase/phosphatase	Effect on nuclear transport (sequences involved)
(a) Signal-transduction-related		
T-ag[a]	CKII	CKII site increases the rate of nuclear transport by approx. 40-fold [25,27] ($S^{111}S^{112}$DDE–10 a.a. spacer–PKKKRKV)
c-rel	PKA	PKA site enhances nuclear localization, whereby mutation of S^{266} to alanine abolishes nuclear localization [47] (chicken c-rel: RRPS^{266}–22 a.a. spacer–KAKRQR)
NF-κB	IL-1α[b]/TNFα PKC/PKA	Phosphorylation (of IκB and/or NF-κB subunits) results in unmasking of the PKC/PKA p50/p65 NLSs and NF-κB nuclear localization [33,34,48–51] (human p50/p105: RRKS^{335}DLETSE–16 a.a. spacer–QRKRQK; human p65: RRPS^{258}DRELSE–16 a.a. spacer–EEKRKR)
Lamin B_2	PKC	PKC-mediated phosphorylation inhibits nuclear transport [52] ($RS^{410}S^{411}$RGKRRRIE)
GlucR	GlucH PP-2A?	Hormone binding by GlucR releases it from a cytoplasmic complex with HSP90 and results in its nuclear localization [43]. Nuclear retention of GlucR is impaired during G2, concomitant with a change in GlucR phosphorylation. Inhibition of PP-2A similarly leads to inefficient nuclear retention of GlucH-occupied GlucR, as does transformation by the v-mos oncogenic kinase, implying that site-specific (cell-cycle-dependent) dephosphorylation of GlucR is involved in GlucH-dependent nuclear translocation [53–55] [A constitutive NLS, RKCLQAGMNLEARKTKK (amino acids 479–495 of the rat sequence) is repressed by a second ligand-binding-dependent NLS function, which includes amino acids 600–626 and 696–777] [56]
GrH receptor	GrH	Ligand-dependent nuclear translocation/'anchorage' of the receptor may be accompanied by tyrosine phosphorylation [57] (Receptor residues 294–454 are essential for nuclear anchorage)
c-fos	Serum PKA	Phosphorylation of c-fos reverses its binding to a putative inhibitor protein and retention in the cytoplasm [37,58,59] (KRRIRRIRNKMAAAKCRNRRRL–200 a.a. spacer–RKGS^{362}SS^{364})[c]
Cofilin	Heat shock M-CAMPK	Heat shock induces unmasking of the cofilin NLS via dephosphorylation at a M-CAMPK site, resulting in its nuclear localization [60,61] (RKSS^{24}TPEEKKRKKA) [62]
rNFIL-6	cAMP PKA?	The elevation of intracellular cAMP stimulates phosphorylation of rNFIL-6 and its translocation to the nucleus [63] (KPS^{101}KKPS^{105}??)
ISGF-3	Interferon α/β Tyk2/JAK1(?)	Interferon-induced cytosolic tyrosine phosphorylation of two (p113 and p91/84) of the three ISGF-3 subunits is required to effect their association and translocation to the nucleus [44,64,65][d] (Phosphorylated site is Tyr^{701} in p91/84) [65]
GAF	Interferon γ Allergic reactions/growth factors[e] JAK1/JAK2(?)	Interferon-induced cytosolic tyrosine phosphorylation of the ISGF-3α subunit p91 (also known as STAT-91) is required to effect its nuclear translocation [45,69–71][d,f] (Phosphorylated site is Tyr^{701} in p91) [65]
NF-ATplc	Ca^{2+}/FK506/cyclosporin PKC(?)	Ca^{2+}-dependent activation of calcineurin results in dephosphorylation of cytoplasmic NF-AT and its nuclear localization [46]. Through different mechanisms, the immunosuppressants FK506 and cyclosporin inhibit the phosphatase and thereby induce nuclear translocation of NF-AT[g] (Putative NLS/PKC sites: NF-ATc KPNS^{651}–29 a.a. spacer–GKRKRS^{686}Q)
PKA C-subunit	cAMP	The PKA C-subunit translocates from the cytoplasm to the nucleus upon dissociation from the PKA holoenzyme complex subsequent to cAMP binding by the regulatory (R-) subunit, which plays a cytoplasmic anchor role in the absence of cAMP [73–75]
$p42^{mapk}/p44^{erk1}$	Serum/EGF/GrH $p45^{mapkk}$/PKC/$p56^{lck}$	Mitogenic stimulation results in the phosphorylation, activation and translocation to the nucleus of the p42/p44 MAPKs [76–79] (Phosphorylation sites include Thr^{183} and Tyr^{185} in mouse $p42^{mapk}$; $p44^{erk1}$ has a Tyr^{185} but not a Thr^{183} equivalent; see [79])
$p90^{rsk}$	Growth factors MAPK	Activated MAPK phosphorylates and activates RSK (*rsk*-gene encoded) kinases, inducing their nuclear translocation [76]
PKC-α	Phorbol ester	Phorbol ester binding by the PKC 82 kDa α isoform effects conformational changes which relieve intramolecular NLS masking and result in its localization to the nuclear envelope [80] (Putative NLSs: ^{158}KRGRIYLK and ^{575}KHPGKRLG; see [81])[h]
(b) Cell-cycle-dependent/developmental		
T-ag	cdk/cdc2	cdc2-K-mediated phosphorylation reduces the maximal level of nuclear accumulation by $\approx$ 70% through increasing the affinity of binding to a putative cytoplasmic retention factor [26] (ST^{124}PKKKRKV)
SWI5	cdk/CDC28	Phosphorylation-mediated nuclear exclusion through NLS masking: mutation of the CDC28 serines to alanine results in constitutive nuclear localization [35] (S^{522}PSK–109 a.a. spacer–KKYENVVIKRS^{646}PRKRGRPRKDGTSSVSSS^{664}PIK)
Iodestar	CKII?	Nuclear exclusion until prophase (DESS^{496}DS^{498}DS^{500}EDDKNKKRK) [82]

Table 1 (*cont.*)

Protein	Stimulus/ kinase/phosphatase	Effect on nuclear transport (sequences involved)
v-jun	Serum cdk?/p44^{erk1}?	Cell-cycle-dependent determination of the rate of nuclear localization conferred by S^{248} (faster during G2) (ASKS^{248}RKRKL) [42][i]
dorsal	*toll* PKA?/Ca^{2+}?	Relocalization from the cytoplasm to the nucleus during development in a ventral-to-dorsal gradient, involving components of the *toll* signalling pathway, including the cytoplasmic retention factor *cactus*[j], and 'releasing factors' *pelle* kinase and *tube*.
	pelle kinase	Phosphorylation of *dorsal* by PKA (and/or *pelle*) leads to release from *cactus* and nuclear localization, whereby activated *toll* receptor and increased cytoplasmic Ca^{2+} are required [36,85–88] (RRPS^{312}–22 a.a. spacer–RRKRQR)
Cyclin B1 (B-type cyclins)	cdk??	Nuclear entry at the beginning of mitosis. Cytoplasmic retention during G2 is via cyclin B1 residues 109–154 (numbering according to human sequence; also conserved in cyclin B2)[k]. Phosphorylation within this region is implicated in release from cytoplasmic retention at the onset of mitosis [89,90]
Adenovirus 5 E1a protein	??	Nuclear targeting up to the early neurula stage of *Xenopus* embryonic development through a 'developmentally regulated' NLS (drNLS) [91] (drNLS[l]: amino acids 142–182: FV–20 a.a. spacer–MCSLCYMRTCGMF, distinct from the constitutive C-terminal NLS: ^{285}KRPRP)
xnf7	cdc2(?)	Nuclear entry at the mid-blastula transition stage of embryonic development associated with dephosphorylation at four sites [92–94]. A cytoplasmic retention domain within xnf7 has been defined, which is functional even in the presence of an additional NLS [94]. (KT^{103}PQKRKIEEPEPEPKKAKV–87 a.a. spacer–CT^{209}PVT^{212}PVEKKT^{218}RP; cytoplasmic retention domain: EEYYAHYVGLNRRQNEWVDKSR^{73})
PwA33	??	Nuclear entry at the mid-blastula transition stage of embryonic development [95] (Putative NLS: KRKIEDGDGDQKKRKV^{149}; putative cytoplasmic retention domain: EEFYVHYVGLNRRQNEWVDKSR^{100})
RB-1	cdk/cdc	Cell-cycle-dependent hyperphosphorylation reduces nuclear association ('nuclear tethering') [96,97] (Putative CcN motif: human T^{601}AADMYLS^{608}PVRS^{612}PKKRTSTTR, where T^{601} is a putative CKII site, and S^{608} and S^{612} are cdk sites)[m]
p53	cdk??	Nuclear entry at the G1/S phase transition; nuclear exclusion during G1 [99,100] (CcN motif: human SS^{312}PQPKKKP[n]) [101,102]
c-myc	Fertilization Ca^{2+}?/PKC?	c-myc is stable and cytoplasmic in the mature *Xenopus* oocyte, but is rapidly translocated to the nucleus upon fertilization, concomitant with both an increase in phosphorylation and a reduction in cellular half-life [38,39] (PAAKRVKLD^{326})
PTF1	??	Nuclear entry of the p64 and p48 subunits at early stages of pancreatic development (day 15 in the mouse embryo) through the action of a glycosylated third protein p75 [103,104]
HMG1/2	??	HMG1/2 is predominantly nuclear in undifferentiated cells but largely cytoplasmic (and more highly expressed) in differentiated cells [105]

[a] Regulation of T-ag nuclear localization by CKII is included under signal-transduction-related examples, since CKII is integrally involved in responses to various mitogens ([106–113]; see also the text); there may, however, be sufficient basal CKII activity to make rapid T-ag nuclear transport constitutive in the absence of mitogenic stimulation.

[b] The fact that IL-1α is a physiological stimulus for NF-κB activation/nuclear localization is interesting in the context of NF-κB, rel and *dorsal* homologies, since the *toll* receptor in the *dorsal* pathway shows sequence homology with the IL-1 receptor. Common nuclear signalling mechanisms are clearly implicated.

[c] The PKA site of c-fos is absent from v-fos, which is constitutively nuclear.

[d] Treatments blocking tyrosine phosphorylation prevent nuclear translocation of ISGF-3 and GAF [64,66,67]. Tyrosine phosphorylation clearly precedes subunit association and nuclear translocation [64–68,71], with no non-phosphorylated p91 able to be detected in the nucleus [68]; DNA binding in the case of ISGF-3 appears to be mediated mainly by the p48 subunit (ISGF-3γ), which is found in both nucleus and cytoplasm and binds DNA constitutively (see [44,65]).

[e] Allergic reactions and EGF have both been found to induce GAF activation and nuclear translocation [69–71].

[f] The GrH- and erythropoietin-stimulated factors GHSF and EPOSF respectively, which may have the p91 component in common with ISGF-3 and GAF, are also induced to translocate to the nucleus upon tyrosine phosphorylation [72].

[g] NF-ATp/c (p denotes pre-existing; c, cytoplasmic; which are essentially identical [46]) has a *rel* similarity region related to those of NF-κB and *dorsal* [46], interestingly including the NLS.

[h] Deletion of either the regulatory or kinase domains of PKC-α leads to its constitutive nuclear localization, probably through NLS unmasking [81].

[i] Constitutively nuclear c-jun possesses Cys^{248}, which is implicated in redox-mediated regulation of c-jun's DNA-binding activity [83]. Phosphorylation does not affect v-jun's DNA-binding capabilities [84], implying that Ser^{248} does not enhance nuclear localization through affecting DNA binding. Importantly, the sequence shown is capable of conferring cell-cycle-dependent nuclear localization on an IgG carrier [42].

[j] The C-terminus (LQISNLSIST^{678}) of *dorsal* is implicated in cytoplasmic retention, since its deletion results in nuclear localization of the *dorsal* gene product. Proteolysis of *dorsal* or *cactus* may also be involved.

[k] Cyclin B1 residues 109–160 can induce cytoplasmic location of the normally nuclear human cyclin A; cytoplasmic retention of cyclin B1, however, can be overridden by the nucleoplasmin NLS [90].

[l] Homologous sequences have been identified in the rat (amino acids 594–623), mouse and human GlucH binding domains of GlucR (see [90]), known to include a part of the ligand-binding-dependent NLS [43,56].

[m] Although not sufficient for complete nuclear localization, a 'bipartite' NLS purported to participate in nuclear localization of mouse RB has been identified: KRSAEFFNPPKPLKKLR^{869}–24 a.a. spacer–S^{894}IGE, where S^{894} is a putative CKII site [98].

[n] Two additional NLSs have been identified in the p53 C-terminus [102] (see Table 2).

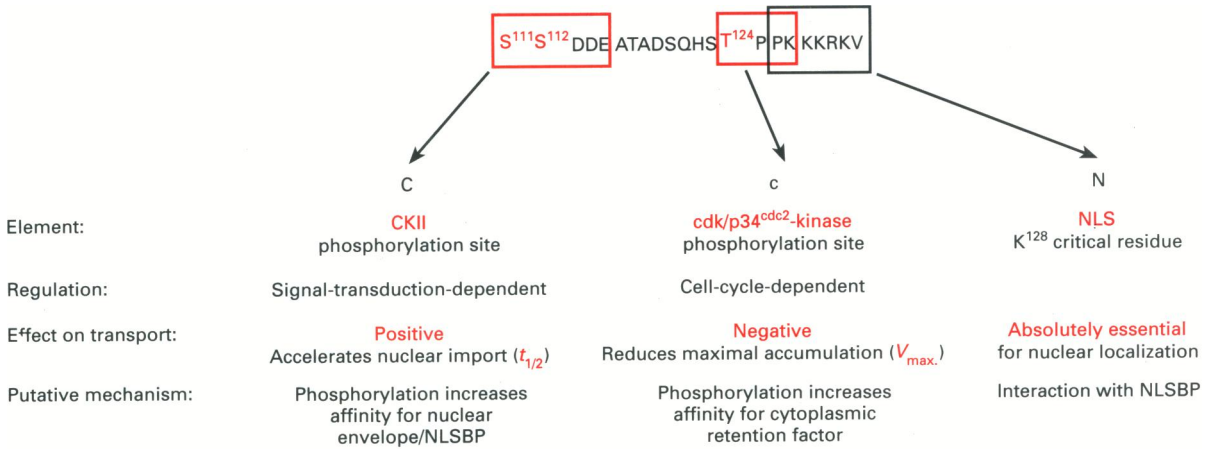

Figure 1 Summary of the regulation of T-ag transport by the CcN motif

The single-letter amino acid code is used, with phosphorylated residues numbered, the CKII and cdk sites boxed in red and the NLS boxed in black. The yeast TF SWI5 shows identical regulation by the cdk-sites of its CcN motif in terms of $V_{max.}$ ([35,150]; D. A. Jans, unpublished work).

affinities and recognition events [4,25–27]. The CKII site (the serine at position 112) increases the rate of NLS-dependent nuclear import, so that maximal accumulation within the nucleus occurs within 15–20 min, which compares with the 10 h taken when phosphorylation by CKII is prevented through deletion or mutation of the CKII site [25,27]. In contrast to the enhancing effect of the CKII site, phosphorylation by cdc2 (at Thr^{124}) inhibits nuclear transport, drastically reducing the maximal level of nuclear accumulation [26]. We have named this regulatory module for T-ag nuclear transport the 'CcN motif', comprising CKII ('C') and cdk ('c') sites, and the NLS ('N') [26] (see Figure 1). This complex regulatory system for SV40 T-ag nuclear localization involving two different kinases demonstrates the existence of specific mechanisms regulating nuclear entry. The NLS is clearly not the sole determinant of nuclear localization; rather, the kinetics of NLS-dependent nuclear import are regulated by phosphorylation in the vicinity of the NLS.

Activation of CKII is known to be integral in mediating cellular responses to mitogens such as insulin and epidermal growth factor (EGF) [106–113]. While the regulation of CKII by such mitogenic signals is generally accepted not to be acute, it is not inconceivable that mitogens may conditionally regulate nuclear transport through the CcN motif CKII site. Activity of the cdk cdc2 is known to be tightly regulated during the cell cycle, increasing sharply before mitosis and falling after M phase [114–116], suggesting that the CcN motif cdc2 site could regulate nuclear localization of T-ag in a cell-cycle-dependent fashion. Hence, through a potentially signal-transduction-responsive CKII site positively regulating the rate of nuclear transport, and a cell-cycle-dependent cdc2 site negatively regulating the maximal extent of accumulation, the level of T-ag present in the nucleus could be precisely regulated, presumably as required with respect to the eukaryotic cell cycle and stages of the viral lytic cycle [27]. It should be stressed, however, that kinetic analyses of T-ag nuclear transport in either the presence or the absence of the above mitogens, or with respect to stages of the cell cycle, have not been performed, so that the possibility that 'basal' CKII and cdc2 activities may constitutively determine the nuclear import of T-ag cannot as yet be formally dismissed.

Significantly, a variety of proteins other than T-ag appear to possess CcN motifs [26], implying a general role for the CcN motif in regulating nuclear protein transport. Some of these, where phosphorylation at the CKII and cdc2 sites has been shown *in vivo* and/or *in vitro*, and the nuclear targeting function of the NLS has also been confirmed, are shown in Table 2. Others which have been identified based on the search for CKII and cdc2 sites in the vicinity of an NLS include the *Drosophila* 'Notch group' of genes and human homologues [133–135], the yeast TF SNF2 [136] and human and *Drosophila* homologues [137], the family of interferon-induced TFs including IFi204 and IFi16 [138,139], the Swi4 family of mismatch repair enzymes [140], bovine poly(A) polymerase [141], yeast DNA topoisomerase II [142], the DNA repair helicase ERCC6 [143], the protein tyrosine phosphatase PEP [Pro-, Glu-, Ser-, Thr-rich (PEST) domain phosphatase] [144], and the $p85^{s6k}$ (s6 kinase [145]). The last-named is interesting because its alternatively spliced variant $p70^{s6k}$ lacks the putative N-terminal NLS ($RRRRRR^7$) and is cytoplasmic [145]. The upstream putative CKII site (S^{40}EDELEE) does not appear to be necessary for nuclear localization, but analysis of transport rates is not available (see [145]).

Putative CcN motifs can also be found in the sequences of proteins localized in the plant cell nucleus [146–148], underlining the apparent conservation of function across the animal/plant kingdoms with respect to the regulation of NLS-dependent transport by phosphorylation. This is supported by the functionality of the T-ag NLS in yeast and plant cells, etc. ([142]; see [148,149]), of the plant-cell NLS of *Agrobacterium tumefaciens* VirD2 protein in yeast cells [147] and of the cdk-regulated yeast NLS of SWI5 in rat and monkey cells ([150]; D. A. Jans, unpublished work). In the latter case, we have been able to show not only that the SWI5 NLS is functional in nuclear targeting, but also that the cdk-site-mediated regulation of its function (see below) operates in mammalian cells. This argues for phosphorylation and the CcN motif having a general role in regulating nuclear protein import. Notably, in the case of the TF SWI5, we have been able to show that the cdk sites regulate the extent of maximal nuclear accumulation [150], exactly as phosphorylation by cdc2 does in the case of T-ag, although the mechanism may be different (see below).

While several examples of cell-cycle-dependent phosphorylation inhibiting nuclear transport are known (see Table 1), the

Table 2 Potential and confirmed CKII and cdk phosphorylation sites in the vicinity of the NLSs of various nuclear proteins

The single-letter amino acid code is used. The CKII consensus site comprises an acidic amino acid three residues C-terminal to the phosphorylatable S/T, with an elevated number of acidic residues in the vicinity of the site increasing its affinity for CKII [51]; cdks phosphorylate S/T residues N-terminal to proline residues with a basic amino acid one or two residues C-terminal to the proline. References apply as indicated to confirmed *in vitro* or *in vivo* (indicated by *) phosphorylation sites for CKII and cdc2, and the NLS. The phosphorylated residues are numbered.

Protein	CKII site(s)	cdk site(s)	NLS
SV40 T-ag	$S^{111}S^{112}$DDE* [117–119]	ST^{124}PPK* [118–120]	$PKKKRKV^{132}$ [4,7–9]
Human p53	GPDS^{389}D* [121,122]	SS^{312}PQP* [122,123]	$PQPKKKP^{319}$ [101,102]
			$LKTKKGQ^{372}$ (NLS II [102])
			$SRHKKTM^{381}$ (NLS III [102])
Human c-myc		PST^{315}RKP	$PAAKRVKLD^{326}$ [124]
	SS^{348}DTEE* [125]	TS^{344}PRS	$RQRRNELKRS^{373}$ [124]
Lamin A/C	S^{458}NED	SPS^{392}PTS* [126]	$SVTKKRKLE^{412}$ [127]
Mouse c-abl IV	ESS^{322}ISDE	$APDT^{566}$PEL* [128]	$SALIKKKKKMAP^{631}$ [42,129]
		$PAVS^{588}$PLL* [128]	
Human B-myb	S^{431}FLD	T^{440}PKST^{444}PVK [130]	$LKRQRKRRV^{418}$ [131]
		TS^{560}PVR [130]	$KRKPGLRRSPKKVRKS^{586}$ [131]
SWI5 (*S. cerevisiae*)		KRS^{646}PRK* [35]	$KKYENVVIKRSPRKRGRPRK^{655}$ [35]
		SSS^{664}PIK* [35]	
CTP:phosphocholine cytidylyltransferase	T^{25}EED* [132]		KVNDRKRRK^{14} [132]†

† Nuclear localization is dependent on amino acids 6–28, i.e. including the CKII site. Both nuclear transport (no kinetic analysis) and phosphorylation are reduced if the site is deleted [132].

demonstration of CKII-mediated effects on transport have thus far been restricted to T-ag. This may in part be attributable to the fact that, of the proteins listed in Table 2 which possess putative CcN motifs, only T-ag and SWI5 have been quantitatively examined in terms of nuclear import kinetics.

MECHANISMS OF REGULATION OF NLS-DEPENDENT NUCLEAR TRANSPORT

There appear to be a number of highly specific cellular mechanisms regulating NLS-dependent nuclear protein transport in a precise fashion. These include cytoplasmic retention factors, intra- and inter-molecular masking of NLSs, and direct NLS masking by phosphorylation.

Cytoplasmic retention factors

One mechanism of regulation of nuclear protein import is that of cytoplasmic retention, whereby a cytoplasmically localized retention factor/'anchor' protein binds an NLS-containing protein and prevents it from migrating to the nucleus [151]. Cytoplasmic retention has been described for c-fos [37] and for the GlucR, where the HSP90 heat-shock protein complexes with and retains the GlucR in the cytoplasm in the absence of GlucH [43]. Hormone binding by the GlucR dissociates the complex with HSP90 to allow NLS-dependent nuclear translocation of the receptor [43]. Cytoplasmic retention has also been described for the B-type cyclins (see Table 1), which translocate to the nucleus as cyclin-B–cdk complexes at the onset of mitosis [89]. Phosphorylation has been implicated in regulating the release of cyclin B1 from cytoplasmic retention (see [90]).

Further examples are the *Xenopus* proteins xnf7 (*Xenopus* nuclear factor 7) and PwA33, both of which are cytoplasmic until dephosphorylation at the mid-blastula transition stage of embryonic development when they become nuclear [92–95]. In conjunction with four phosphorylation sites, a 22-amino-acid cytoplasmic retention domain within xnf7 (also present in PwA33; see Table 1) has been defined as being responsible for overriding the xnf7 NLS [94]. This cytoplasmic retention is dominant even in the presence of an additional NLS [94], indicating that the cytoplasmic retention domain does not function through direct masking of the xnf7 NLS (see below).

As already mentioned, the PKA C-subunit translocates to the nucleus upon dissociation from the PKA holoenzyme complex subsequent to the binding of cAMP by the regulatory (R-) subunit [73–75]. The PKA R-subunit can be regarded as playing a cytoplasmic anchor role similar to that of HSP90 and the *rel/dorsal* family IκB proteins (see below), since it functions to retain the C-subunit in the cytoplasm (in the vicinity of the perinuclear Golgi in the case of the type II R-subunit and type II PKA holoenzyme [75]) in the absence of cAMP-mediated stimulation.

The *rel/dorsal* family

A cytoplasmic anchor function has been described for the NF-κB-binding inhibitor proteins IκBα (also known as MAD-3) and IκBβ in retaining the NLS-carrying NF-κB p65 (relA) subunit in an inactive complex in the cytoplasm. Phorbol ester or other treatment induces release of p65 and its migration to the nucleus [32–34]; *in vitro* experiments imply that this can be effected by phosphorylation either of IκB by PKC or of p65 by PKA ([48]; see also [85]). Roles similar to that of IκB have been proposed for the sequence-related molecules *cactus*, which negatively regulates nuclear localization of the *Drosophila* morphogen *dorsal* [36], and $p40^{rel}$, which may retain the c-rel proto-oncogene in the cytoplasm in analogous fashion [152]. Phosphorylation of *dorsal* has been shown to effect its release from *cactus* and subsequent nuclear translocation [87]; *dorsal* is constitutively nuclear in the absence of *cactus* [153,154]. Overexpression of *dorsal* in the presence of *cactus* can overcome cytoplasmic retention, indicating that it is titratable (see [85]).

Other IκB family members include IκBγ (a discrete gene product identical to the 70 kDa C-terminus of the NF-κB p50 precursor p105 [155]) and the proto-oncogene *bcl*-3 [156], both of which can bind the NF-κB p50 subunit *in vitro* and may function to retain it in the cytoplasm *in vivo*. All of the IκB/*cactus* family members contain 5–7 ankyrin repeats, structural elements that are involved in protein–protein interactions. Some of these appear to be directly involved in cytoplasmic retention, since

deletion of ankyrin repeat 7 together with part of repeat 6 inactivates *bcl*-3 binding of NF-κB p50 [156]. The mechanism of cytoplasmic retention in the case of the IκBs appears to be through NLS masking (see below).

T-ag

Cytoplasmic anchoring may be the basis of the cdc2-mediated inhibition of T-ag nuclear import described above [26]. Maximal inhibition of transport of tetrameric T-ag–β-galactosidase fusion proteins (containing four copies of each of the NLS and the cdc2 site) is effected by a stoichiometry of phosphorylation of only 1 at the cdc2 site [26], which indicates that one phosphorylated cdc2 site is sufficient to retain the protein in the cytoplasm even in the presence of three non-cdc2-phosphorylated CcN motifs. Phosphorylation presumably increases the affinity of the specific interaction between T-ag and a putative cytoplasmic retention factor. The inhibitory effect of cdc2-phosphorylation of Thr^{124} can be overcome by increasing the concentration of cytosolic T-ag fusion protein (D. A. Jans, unpublished work), implying that there may be a finite, titratable cellular level of the factor which retains Thr^{124}-phosphorylated T-ag in the cytoplasm.

NLS masking

A number of proteins possessing apparently functional NLSs are predominantly cytoplasmic due to the inaccessibility or masking of their NLSs. This may be effected through interaction with another protein (e.g. a factor binding to the NLS itself) or conformational effects whereby the NLS is masked by other parts of the molecule. Phosphorylation is an efficient and potentially rapidly responsive means of modulating NLS accessibility.

NF-κB and intra- and inter-molecular masking

The active (nuclear) form of NF-κB is composed of the p50 and p65 protein components, which contact specific DNA sequences as a homo- or hetero-dimer to activate κ light-chain gene transcription [32,34]; the homodimer of p50 is the TF KBF-1. Both subunits are homologous within an approx. 300-amino-acid NLS-containing sequence (the *rel* homology region) required for DNA binding and dimerization, which is shared with members of the *rel* oncogene family and the *D. melanogaster* developmental control gene *dorsal*, all of which are TFs.

As outlined in the previous section, initial models for the regulation of the nuclear localization of NF-κB revolved around the observation that phorbol ester treatment could effect the release of the NF-κB p65 subunit from a cytoplasmic complex with IκB, in order to be transported in an NLS-dependent fashion to the nucleus [32–34,48]. A dual mechanism involving NLS masking of both NF-κB components has more recently been established [49–51], whereby the C-terminus of the p105 precursor of NF-κB p50 (or the IκBγ molecule; see above) appears to retain the NF-κB p50 subunit in the cytoplasm through intramolecular masking of its NLS. Deletion analysis indicates that a short sequence including ankyrin repeat 6 and an adjacent acidic sequence in the C-terminus of p105, absent from p50, is responsible [157]. Antibodies specific to the NLS recognize p50 but not p105, implying that the NLS is inaccessible in the larger precursor [49]. One mechanism of unmasking of the NLS is probably through proteolytic processing of the p105 C-terminus [158–160].

Intermolecular masking of its NLS by IκB appears to be the mechanism of NF-κB p65's cytoplasmic retention, whereby deletion or mutation of the p65 NLS eliminates binding to IκB [50]. NLS unmasking appears to be brought about through IκB degradation [160] induced by PKC phosphorylation (probably by the PKC-ζ isotype, known to be activated by tumour necrosis factor α, one of the physiological stimuli for NF-κB [161]), with IκB degradation probably being proteasome-mediated [162]. PKA-phosphorylation of p65 ([48]; see also [85]) can also effect the dissociation of p65 from IκB.

Nuclear localization of the NF-κB TF is thus dually regulated by specific intra- and inter-molecular masking of the NLSs of the two NF-κB subunits. Signal transduction-triggered phosphorylation regulates the masking events precisely to enable a rapid response in terms of nuclear translocation and gene induction. Figure 2(a) is a schematic representation of the molecular events purported to be involved in the regulation of NF-κB nuclear transport, and may also serve as a framework to understanding nuclear uptake of the related TFs c-rel and *dorsal*.

NLS masking by phosphorylation

NLS masking can also be directly effected by phosphorylation close to or within an NLS which masks or inactivates it through charge or conformational effects. Since dephosphorylation appears to be the key event in triggering the nuclear translocation of a number of other proteins, such as NF-AT and the GlucR (see Table 1 and below), this may be a quite widespread mechanism.

Cell-cycle-dependent nuclear exclusion of the *S. cerevisiae* TF SWI5 involved in mating type switching is effected by phosphorylation by the cdk CDC28 [35], the yeast cdc2 homologue. Three CDC28 sites, one of which is within the spacer of the SWI5 bipartite NLS (see Tables 1 and 2), inhibit nuclear localization by inactivating or masking the function of the SWI5 NLS [35]. At anaphase, CDC28 activity falls and SWI5 is dephosphorylated to effect its nuclear entry and the subsequent activation of transcription of the HO mating switch endonuclease; removal by mutation of the CDC28 sites results in constitutive nuclear localization [35,149].

NLS-dependent nuclear transport of lamin B2 is inhibited by phosphorylation at two PKC sites N-terminally adjacent to the NLS (see Table 1), both *in vivo* in response to phorbol ester stimulation and *in vitro* [52]. Negative charge close to the NLS is presumed to inactivate the NLS in a similar fashion to CDC28-mediated inactivation of the SWI5 NLS. Analogously, nuclear translocation of the actin-binding protein cofilin upon heat-shock treatment is accompanied by dephosphorylation at a consensus multifunctional calmodulin-dependent protein kinase (M-CAMPK) site adjacent to the putative cofilin NLS (see Table 1; [60,61]). Phosphorylation at this site is proposed to mask the function of the NLS [61].

The CcN motif: positive and negative regulation by dual phosphorylation

We have established that the CKII and cdc2 sites function independently of one another in terms of both regulating T-ag nuclear transport and influencing phosphorylation at the other site [26]; i.e. CKII-dependent phosphorylation does not enhance nuclear import by reducing phosphorylation at the inhibitory cdc2 site; nor does Thr^{124} phosphorylation inhibit nuclear transport by impairing Ser^{112} phosphorylation [26]. Both phosphorylations almost certainly occur in the cytoplasm [26,27,118], implying that the regulatory events determining nuclear import kinetics are cytoplasmic, consistent with the cytosolic requirement for signal-dependent nuclear transport *in vitro* [26,28–30]. Nuclear phosphorylation at either kinase site to effect retention in the nucleus is clearly not the mechanism of

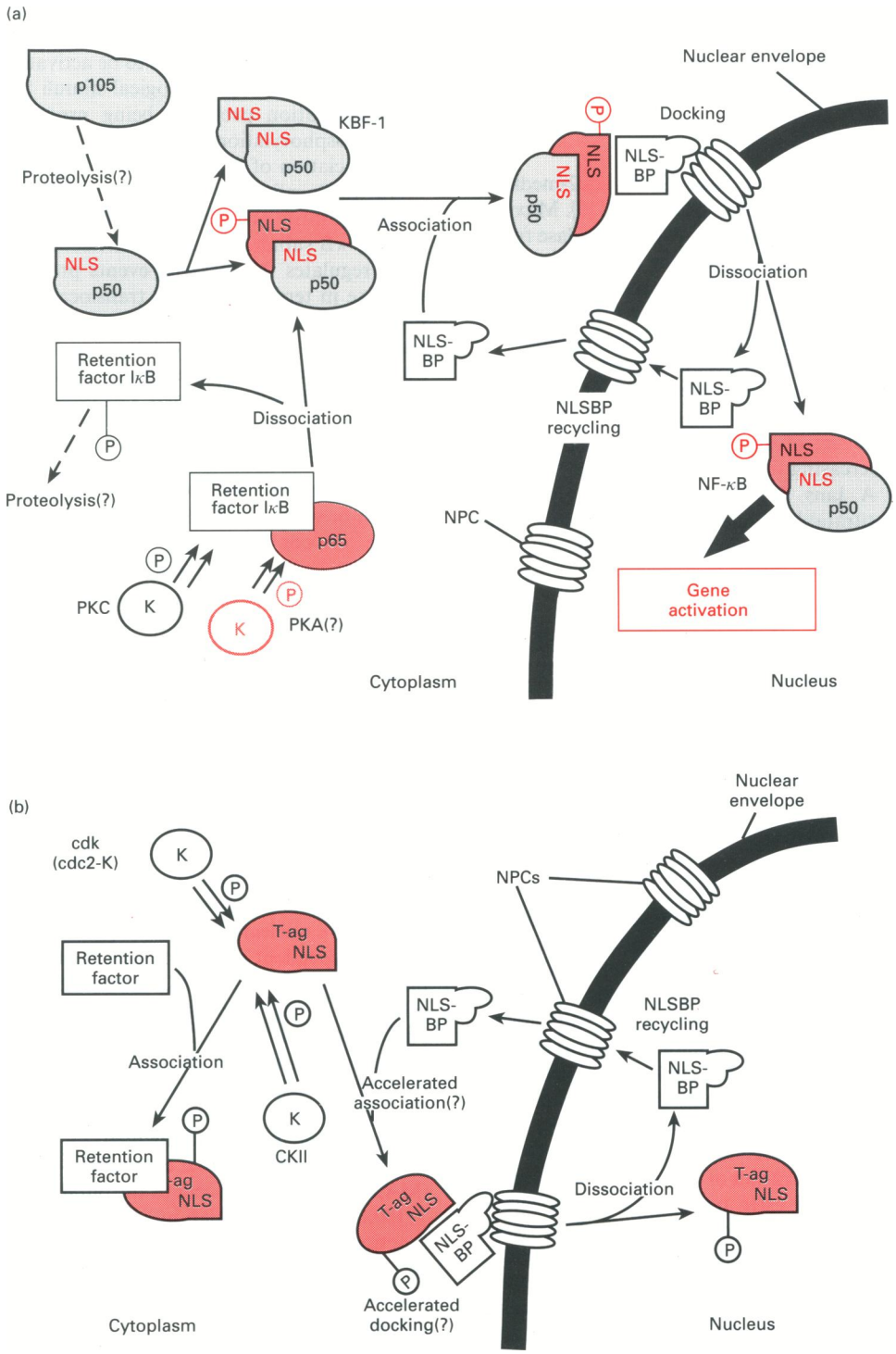

Figure 2 Regulation of nuclear protein transport by phosphorylation

A simplistic representation of some of the steps involved in the regulation of the nuclear transport of NF-κB (**a**) and SV40 T-ag (**b**). The NLSBP-mediated interactions, docking at the nuclear envelope, etc., are speculative. (**a**) NF-κB nuclear transport is regulated by phosphorylation and intra-/inter-molecular masking. NF-κB p65 is retained in the cytoplasm by IκB, which binds directly to the p65 NLS [51], until phosphorylation of IκB by PKC (inducing IκB degradation [160,161]) and/or of p65 by PKA (see [85]) dissociates the complex and p65 is free to translocate to the nucleus. Proteolysis of the NF-κB p105 subunit to the mature NF-κB p50 form is probably the means by which the NLS, masked by the C-terminal portion of the molecule in p105 [50], is unmasked in p50 [157–159]. It is unclear where dimerization of p50 with itself (TF KBF-1) or with p65 (TF NF-κB) occurs, or whether it is necessary for nuclear transport. (Overexpression of IκB can also lead to its nuclear localization, although the mechanism and significance of this are unclear [50].) It should be emphasized that the exact role in NF-κB nuclear transport of the activation of DNA binding by phosphorylation remains unclear. (**b**) SV40 T-ag nuclear transport is regulated by phosphorylation and the CcN motif. Phosphorylation by cdc2 results in cytoplasmic retention of T-ag [26], probably through increasing the affinity for a cytoplasmic anchor protein. Phosphorylation at the CKII site increases the nuclear import rate [25,27], probably by increasing the affinity and kinetics of docking at the nuclear envelope/NPC, perhaps through regulating the NLSBP interaction directly. The two CcN motif kinase sites are completely independent in terms of both phosphorylation at the respective sites and their effect on nuclear import [26]. Dephosphorylation at either site is not directly involved in the transport process [26,27].

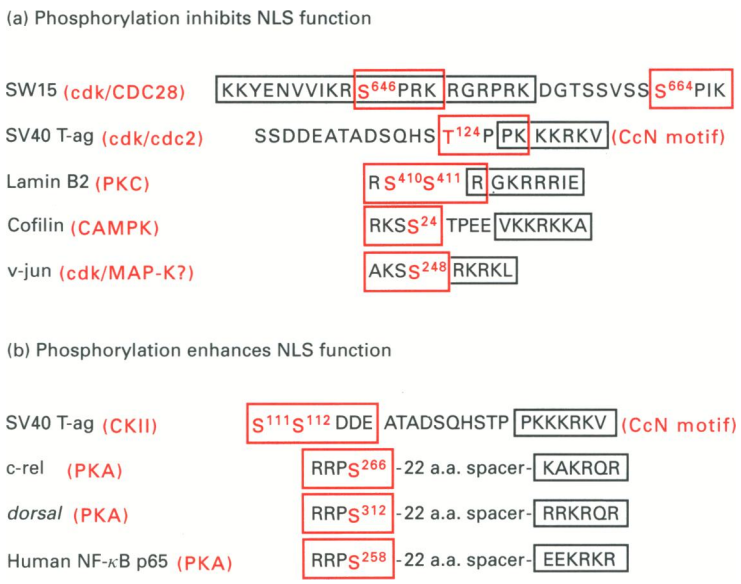

Figure 3 prNLSs where phosphorylation sites close to the NLS regulate the latter's function

The prNLSs are grouped into those where phosphorylation inhibits (**a**) or enhances (**b**) nuclear transport. The single-letter amino acid code is used, with phosphorylated residues numbered, the phosphorylation sites (kinases named in parentheses in red) boxed in red and the NLSs boxed in black. In the case of the prNLSs, where tested [those of T-ag (the CcN motif), SWI5 and v-jun], the prNLSs are completely sufficient to confer phosphorylation-regulated nuclear localization on a heterologous protein ([25–27,35,42,150]; D. A. Jans, unpublished work). Abbreviation: a.a., amino acids.

nuclear accumulation, as also demonstrated by mutational analyses [25–27]. Aspartic acid can substitute functionally for phosphoserine/threonine at both phosphorylation sites of the T-ag CcN motif [26,27], indicating that negative charge, normally provided by specific phosphorylation, is what is functionally important in terms of regulating nuclear import. This implies that the phosphorylated sites may represent signals in themselves which are recognized by components of the cellular nuclear transport machinery. The results with Asp-substituted proteins also indicate that phosphatase activity at either of the CcN motif kinase sites has no direct role in the nuclear transport process; i.e. nuclear dephosphorylation at either the CKII or the cdc2 site is not necessary for T-ag accumulation in the nucleus [26,27].

Although cytoplasmic retention appears to be the basis of the inhibition of T-ag nuclear transport via the cell-cycle-regulated cdc2 as discussed above, the mechanism of CKII-phosphorylation-mediated enhancement of the rate of nuclear transport is not clear. Preliminary evidence from our *in vitro* system (D. A. Jans, unpublished work) implies that phosphorylation at the CKII site increases the rate of docking at the nuclear envelope/NPC, which is almost certainly mediated by NLSBPs. The CKII site may enhance T-ag nuclear import through facilitating/accelerating the interaction with NLSBPs, whereby phosphorylation may directly modulate the affinity of binding. Figure 2(b) represents some of the events thought to be involved in the regulation of T-ag nuclear import by the CcN motif, involving cdc2 regulation of cytoplasmic retention and CKII regulation of the kinetics of docking at the NPC.

Phosphorylation-regulated NLSs (prNLSs)

The CcN motif can be regarded as one example of a phosphorylation-mediated regulatory module for nuclear protein localization (a prNLS), where nuclear protein import is regulated through phosphorylation site(s) close to the NLS of a particular karyophilic protein. These can be grouped into those in which phosphorylation either enhances or inhibits NLS-dependent nuclear transport, and a selection of these is shown in Figure 3.

PKA is directly implicated in enhancing nuclear localization of the c-rel proto-oncogene product ([47,163,164]; see Table 1) in comparable fashion to CKII and T-ag. Furthering the analogy, alanine at the PKA site in c-rel (Ser^{266}) abolishes c-rel nuclear localization, while aspartic acid at the site simulates PKA phosphorylation in inducing nuclear translocation [47]. Linker insertion analysis indicates that spacing between the PKA site and NLS (22 amino acids) is of prime importance in regulating c-rel nuclear localization [47], implying that the two sequence elements may be recognized in concert, as we have speculated for the T-ag CcN motif (see above; [27]). Significantly, this PKA site, together with the NLS, is conserved in all members of the *rel/dorsal* family within the *rel* homology region (see [32]), and has been shown to be the domain of *dorsal* to which *cactus* binds [165]. Experiments in which the cDNAs encoding *dorsal* and the PKA C-subunit have been cotransfected into Schneider cells clearly implicate PKA and this conserved site (Ser^{312} in *dorsal*) in regulating *dorsal* nuclear localization in response to activation of *toll* [85]. Co-expression of PKA enhances nuclear localization of *dorsal*, which can be blocked by specific PKA-peptide inhibitors [85]. Mutation of Ser^{312} to glutamine significantly reduces nuclear localization of *dorsal* in response to *toll*, while aspartic acid in place of Ser^{312} can partially induce nuclear transport, indicating that negative charge at the site is mechanistically important [85]. PKA-phosphorylation of c-rel, *dorsal* and perhaps also of the NF-κB subunits thus appears to modulate their specific interactions with cytoplasmic retention factors, thereby controlling nuclear translocation in a similar fashion to phosphorylation of the cytoplasmic retention factors themselves (see above).

A further example of putative PKA regulation of nuclear localization is that of rNFIL-6 (rat nuclear factor induced by interleukin-6), which translocates to the nucleus upon the elev-

ation of intracellular cAMP levels in rat PC12 pheochromocytoma cells ([63]; see Table 1). PKC and the M-CAMPK also appear to be signal-transduction-responsive kinases able to regulate nuclear protein import. NLS-dependent nuclear transport of lamin B2 is inhibited by PKC phosphorylation at sites N-terminally adjacent to the NLS (see Table 1) [52], while nuclear localization of the actin-binding protein cofilin is similarly negatively regulated by an M-CAMPK site N-terminal to the NLS (see Table 1) [61,62].

CONCLUSION

NLS function appears to be conserved across all eukaryotes, with particular NLSs active in terms of subcellular targeting in a variety of different organisms and cell types. While NLSs are clearly essential for nuclear protein localization, it is clear that phosphorylation can regulate NLS-dependent nuclear import of a number of TFs and other proteins, through cellular mechanisms such as cytoplasmic retention factors or through prNLSs, where phosphorylation at sites close to the NLS specifically modulates its function. Even the archetypal NLS-containing T-ag is subject to regulated nuclear localization through a particular type of prNLS, the CcN motif, where phosphorylation determines both the rate and the end-point of NLS-dependent nuclear import. Putative CcN motifs are present in a number of proteins from diverse sources, implying a wider role in regulating protein transport to the nucleus. The cellular mechanisms controlling nuclear protein import, such as cytoplasmic retention factors, intra- and inter-molecular NLS masking, NLS masking by phosphorylation, etc., also appear to be common to many eukaryotes, a striking example being the conservation of the structure and function of the related IκB, pp40^{rel} and *cactus* cytoplasmic retention factor proteins, together with the prNLSs of their specific TF partners. The fact that the nuclear targeting activity, as well as its regulation by cdk-phosphorylation conferred by the cell-cycle-regulated prNLS of the yeast TF SWI5, are active in mammalian cells [150] indicates that the signals regulating NLS function can also be functional in widely diverse organisms and cell types. It is possible that the signals regulating nuclear protein transport may well be as universal as the NLSs themselves.

FUTURE PROSPECTS

Knowledge concerning the mechanisms regulating nuclear protein transport can be applied to target molecules of interest to the nucleus. prNLSs such as those shown in Figure 3 conferring tightly regulated nuclear localization according to hormonal stimuli or the stage of the cell cycle may enable 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 [166,167]). Alternatively, toxic molecules might be efficiently targeted to sensitive subcellular sites such as the nucleus in order to effect tumour cell killing [168,169]. The universality of NLS function, and also perhaps of the mechanisms of regulation of nuclear protein transport, would imply that these approaches are likely to succeed through their potential widespread applicability in terms of cell type and protein/gene.

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