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Research Article

Cyclin H is targeted to the nucleus by C-terminal nuclear localization sequences

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Abstract. Cdk-activating kinase (CAK) is a trimeric complex consisting of cdk7, cyclin H, and MAT1, which activates the cell-cycle-regulating cdks through T loop phosphorylation. In addition, other substrates of the CAK complex have been identified when CAK is assembled with the TFIIH core proteins, thereby regulating transcription and nucleotide excision repair. Little is known about the regulation of the CAK complex through cyclin H. In this study we further analyzed cyclin H regulation and identified two basic clusters in the C terminus of the protein as putative nuclear localization sequences (NLSs). Fusion constructs of full-length and truncated cyclin H sequences demonstrated the functionality of the NLSs. A peptide-binding assay revealed that at least one NLS interacts with the nuclear import receptors importin α/β. Phosphorylation in the vicinity of the NLSs by cyclin C/cdk8 or protein kinase CK2, however, does not influence the nuclear translocation of cyclin H.

Key words. CAK; nuclear localization signal; cyclin/cdk; protein kinase CK2; cell cycle; importin.

The concerted activation of cyclin-dependent kinases (cdks) is a prerequisite for accurate progression through the cell cycle. The exact regulation of these kinases is therefore essential for proper replication and division of the genome. Consequently, imbalance by either overexpression or deletion of single components is usually detrimental to the cell and the basis for malignant transformation. Several control mechanisms have evolved to ensure the correct activation or deactivation of cdks. One is the activation of cdk molecules by dephosphorylation and phosphorylation of defined residues in the cdk polypeptide chain [1]. Notably, a cyclin/cdk complex itself is the core component of the cdk-activating kinase (CAK), [2–4]. Cyclin H/cdk7 together with the assembly factor MAT1 activates cell-cycle-regulating cdks by phosphorylation of a conserved threonine residue in their T loop region [5–7].

CAK can be isolated from cells as a trimeric enzyme, but it is also found as part of the multiprotein complex TFIIH, a general transcription factor complex involved in the regulation of RNA polymerase II (RNA Pol II) – dependent transcription. TFIIH is also an essential part of the nucleotide excision repair (NER) complex [8–11]. Whether CAK is part of TFIIH in the repair complex *in vivo* and performs any kinase activity towards other constituents of the complex is still elusive, but as the kinase activity of TFIIH declines after UV treatment of cells, CAK has at least a regulatory influence upon RNA Pol II and transcription during NER [12]. CAK is also known to phosphorylate several gene-specific transcription factors in a MAT1-dependent manner, among them p53, Oct-1, as well as steroid receptors [13–15]. From these diverse functions of CAK, one can conclude that this complex

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plays a central role in the coordination of events that are necessary to separate gene transcription from genome replication as well as chromosome separation and cell division from genome repair [16, 17].

Little, however, is known about the regulatory mechanisms that enable CAK to perform these different functions. Like other cdks, cdk7 needs to be activated by T loop phosphorylation. Surprisingly, the only CAK-activating kinases identified so far are themselves substrates of CAK, cdk1 and cdk2, raising the possibility of regulatory feedback loops during the different phases of the cell cycle. Phosphorylation in the T loop of cdk7 stabilizes the interaction with cyclin H and renders activity of CAK independent from MAT1, thereby also regulating substrate specificity [6, 15, 18–20]. As MAT1 seems to serve as the linker molecule between core TFIIH and CAK, assembly of cyclin H/cdk7 with MAT1 may also direct CAK towards TFIIH and its associated activities [21].

Although commonly known as 'the regulatory subunit' of the CAK complex, little is known about the regulation of cyclin H. Human cyclin H is a polypeptide of 323 amino acids, with a molecular weight of 38 kDa. p53 was recently demonstrated to interact with cyclin H *in vivo* and *in vitro*. The interaction of cyclin H with wild-type p53, but not with a mutated form of the tumor suppressor, downregulates CAK kinase activity towards cdk2 and the C-terminal domain (CTD) of RNA Pol II. This downregulation is independent of functional p21 and therefore a direct mechanism by which p53 can influence cell cycle progression and transcription upon genomic stress [22]. Furthermore, cyclin H has been shown to be a phosphoprotein. Two kinases were identified that phosphorylate cyclin H and thereby regulate its activity. Cyclin C/cdk8 phosphorylates cyclin H at two positions, one in the N terminus and one in the C terminus. Phosphorylation of cyclin H in the TFIIH complex results in inhibition of its transcription and kinase activity [23]. The second kinase that phosphorylates cyclin H at position 315 is protein kinase CK2. Using point mutants of cyclin H, phosphorylation of Thr315 by the CK2 holoenzyme was shown to be necessary to fully activate the phosphorylation activity of CAK [24].

All identified substrates of CAK are predominantly nuclear and immunofluorescence studies have demonstrated that the CAK complex is localized to the nucleus [25, 26]. However, it is not clear wether CAK is already pre-assembled in the cytoplasm and then transported as a complex or if the subunits translocate separately. So far, a nuclear localization sequence (NLS) has only been identified in cdk7 [27]. In this study we identified two NLSs in cyclin H located in the vicinity of the described phosphorylation sites. We show that one NLS sequence is a binding site for importin α/β but that each of the basic sequences can direct heterologous proteins to the nucleus, albeit with different efficiency. Moreover, by using phosphorylation mutants we could show that nuclear import of full-length cyclin H is independent from phosphorylation at the CK2 and cyclin C/cdk8 sites.

Materials and methods

Construction of expression plasmids and site-directed mutagenesis

Full-length and truncated cyclin H cDNA fragments were amplified by PCR from a full-length cDNA clone [24] and cloned into the *Bam*HI/*Hin*dIII site of the pRSET-C vector (Invitrogen). The GST cDNA sequence with an additional N-terminal *Xho*I site was amplified by PCR from the pAcG2T vector (Pharmingen) and cloned in frame into the *Nhe*I site of pRSET-C-cycH constructs. The GST-cycH fragments were then released by *Xho*I/*Hin*dIII digest and inserted into the *Xho*I/*Hin*dIII site of pEGFP-C3 (Clontech). T315A and T315D mutants were amplified from cyclin H-cDNA-containing pBacPAK-His1 clones [24]. S304A and S304E mutants were generated by an overlap extension PCR procedure using primers with the following base substitutions: TCA to GCA in the forward and TGA to TGC in the reverse primer. The resulting fragments were cloned into pRSET-C. Addition of the GST sequence and cloning into the pEGFP-C3 vector was as described above. Correct insertion of the fragments and base substitutions was verified by sequencing.

Cell culture, plasmid transfection, and immunofluorescence

COS-1 cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS). For transient transfections, 3×10^5 cells were seeded on coverslips in 5-cm petri dishes and transfected the following day by DEAE dextran standard procedure. Cells were transfected with 10 µg of EGFP-GST-cycH expression vector and then cultured for 24 h. Coverslips were rinsed in PBS pH 7.4, fixed in 3.7% formaldehyde in PBS for 10 min, and washed three times for 10 min in PBS. Coverslips were mounted on a drop of mounting medium containing DAPI, and transfected cells were analyzed under a fluorescence microscope.

In vitro translation assay and SDS polyacrylamide gel electrophoresis

In vitro translation was performed with the TNT Coupled Reticulocyte Lysate System (Promega) in a 25-µl reaction volume using 500 ng plasmid template and ^{35}S-methionine. Protein products were analyzed by SDS gel electrophoresis on a 10% polyacrylamide gel according to the procedure of Laemmli [28].

Peptide filters and importin-α/β binding assay

Fourteen-amino-acid-long peptides representing the wild-type or mutated sequences of NLS1 or NLS2 in cyclin H were synthesized and immobilized on a cellulose membrane according to the Spot method with a partially automated synthesizer (Abimed Auto-Spot Robot ASP 222) as recommended by the manufacturer (Genosys).

Mouse importin α(58)-GST and mouse importin β(97)-GST in the pGEX-2T vector were kindly provided by D. Jans [29, 30]. *Escherichia coli* strain M15 (pREP4) was transformed with the plasmids and expression of the proteins was induced by the addition of 1 mM IPTG for 3 h. The pellet was resuspended in 50 ml TE buffer [50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 2 mM dithiothreitol (DTT)] containing 500 mM NaCl and 1 mM phenylmethylsulfonylfluoride (PMSF) and frozen/thawed three times. Bacteria were sonicated on ice for 3 × 30 s. The lysate was clarified by centrifugation (4 °C, 30 min, 15,000 g). The supernatant was incubated with glutathione agarose beads (Amersham Biosciences; pre-equilibrated in TE buffer with 500 mM NaCl) at 4°C overnight. The beads were washed with TE buffer containing 500 mM NaCl until $OD_{280\,nm} < 0.1$. The GST fusion protein was eluted in TE buffer containing 100 mM NaCl and 5 mM imidazole and dialyzed overnight against dialysis buffer (20 mM HEPES, pH 7.3, 100 mM potassium acetate, 2 mM DTT, 1 µg/ml leupeptin, 1 µg/ml pepstatin).

Pre-complexation of importin α/β–GST in a molar ratio of 1:1 was carried out in IB buffer (110 mM KCl, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $CaCl_2$, 20 mM HEPES, 1 mM DTT, 5 µg/ml leupeptin, pH 7.4) for 15 min at 22 °C. The peptide membrane was blocked with IB buffer containing 5% dry milk for 1 h at room temperature and then incubated with the pre-complexed importin α/β–GST heterodimer in IB buffer at 4 °C overnight. After washing the membrane with PBS-Tween-20 three times, detection of importin was performed with an anti-GST-specific antibody (Amersham Biosciences), a peroxidase-coupled secondary antibody and the Lumilight detection system (Roche). The filter was then exposed on an autoradiographic film (AGFA).

Results

We recently demonstrated that phosphorylation of Thr315 by protein kinase CK2 in the cyclin H protein is necessary to fully activate CAK [24]. In an attempt to further investigate the regulation of cyclin H, we computer-scanned the cyclin H protein sequence in the vicinity of the phosphorylation site for functional domains. In proximity to the defined CK2 phosphorylation site, a PROSITE scan revealed two basic clusters (KKRK and KKSK) between amino acids 292 and 308 of the cyclin H polypeptide chain with high similarity to the consensus

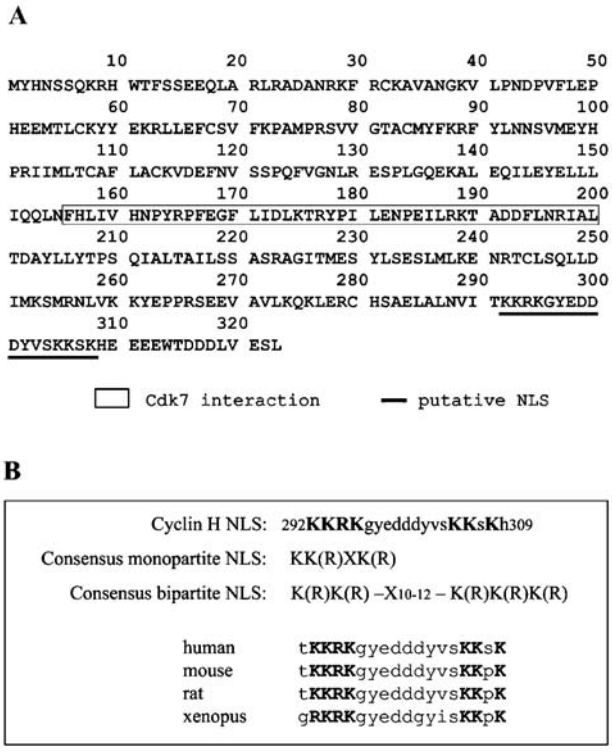

Figure 1. Putative NLS of cyclin H. (*A*) Amino acid sequence of cyclin H. The predicted cdk7-binding region [31] is shown by a box, the solid line depicts the putative NLS region of cyclin H. (*B*) Sequence comparison of the human cyclin H NLS with the consensus sequences for monopartite and bipartite NLSs. Sequence comparison among the different species reveals a high degree of conservation within the putative NLS region of cyclin H.

sequences for classical monopartite and bipartite NLSs (fig. 1A, B). Comparison of available C-terminal cyclin H sequences from different higher eukaryotes showed a high degree of conservation within the basic clusters and in the spacer region separating them (fig. 1B). As cyclin H is predominantly nuclear, we reasoned that cyclin H contains functional NLSs in the C-terminal part of the protein.

To further analyze the putative NLSs in cyclin H, we first cloned the full-length cDNA sequence and several deletion mutants of human cyclin H into pRSET bacterial expression vectors. To eliminate any passive diffusion through the nuclear pore complex by proteins smaller than 40 kDa, a GST sequence was inserted N-terminal to the cyclin H sequences. Correct expression of the fusion proteins was verified by *in vitro* translation from the T7 promoter in the pRSET constructs (fig. 2A) and by bacterial expression of the fusion proteins (data not shown). The GST-cyclin H DNA fragments were then excised and cloned in frame with green fluorescent protein into mammalian expression vectors (fig. 2B).

COS-1 cells were transfected with the resulting EGFP-GST-cyclin H constructs. A control construct harboring EGFP-GST alone localized exclusively to the cytoplasm,

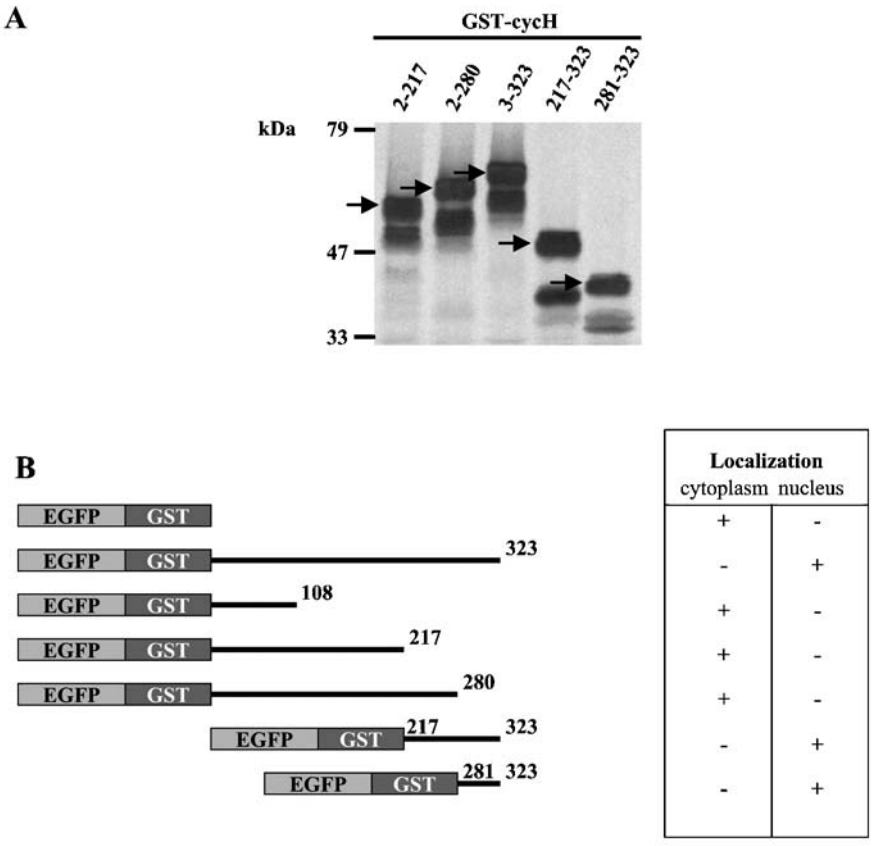

Figure 2. Construction and expression of GST-cyclin H fusion constructs. (*A*) GST fusion constructs of full-length and truncated cyclin H sequences were inserted into the pRSET-C vector and correct expression was verified by *in vitro* translation in the presence of ^{35}S-methionine. Translation products were run on an SDS polyacrylamide gel and exposed on an X-ray film. Correctly sized fusion proteins are depicted by an arrow, smaller bands result from the usage of internal start codons by the T7 polymerase. The numbers above each lane represent the cyclin H amino acid sequence of each fusion clone. (*B*) COS-1 cells were transfected with fusion constructs of full-length/truncated GST-cyclin H sequences and EGFP to evaluate the cytoplasmic or nuclear localization.

demonstrating that the size of the protein excluded passive diffusion and that fusion proteins are not transported to the nucleus by other NLSs within EGFP-GST (figs. 2B, 3A). As expected for correctly targeted cyclin H, EGFP-GST-cyclin H full-length fusion proteins showed nuclear localization in COS-1 cells (figs 2B, 3B). However, when different deletion mutants of cyclin H lacking the putative NLS sequences between amino acids 281–323 were transfected into COS-1 cells, the fusion proteins were retained in the cytoplasm (figs 2B, 3C). In contrast, fusion proteins expressed from constructs carrying only the C-terminal part of the cyclin H protein localized exclusively to the nucleus (figs 2B, 3D). These C-terminal sequences did not code for parts of cyclin H known to interact with the catalytic CAK subunit cdk7 (fig. 1) [31] and, therefore, redistribution of these fragments to the nucleus by cotransport with cdk7 could be excluded. Thus, cyclin H contains functional NLS sequences in the C terminus of the protein that are able to target heterologous, otherwise cytoplasmic, proteins to the nucleus.

Each of the two basic clusters in the cyclin H protein not only matches the consensus sequence for monopartite NLSs but together they may also constitute a bipartite NLS. The prototype of this bipartite NLS-bearing protein is nucleoplasmin, where two basic amino acid clusters are separated by a 10- to 12-amino acid spacer. In cyclin H, the two basic clusters are separated by 9 amino acids (fig. 1, fig. 4). To analyze whether the two basic amino acid clusters of cyclin H represent two separately active NLSs or constitute a bipartite NLS, the sequence between amino acids 281–323 was divided in NLS1 (amino acids 281–300), which is the more N-terminal sequence and NLS2 (amino acids 301–323), the C-terminal basic cluster. Both fragments were fused with EGFP-GST (fig. 4). Expression of the fusion constructs in COS-1 cells revealed that the EGFP-GST-NLS1 protein was exclusively targeted to the nucleus, resembling the localization of full-length cyclin H. Expression of EGFP-GST-NLS2 resulted in a predominantly nuclear localization. However, a fraction of the protein was retained in the cytoplasm of COS-1 cells. These results lead to the conclusion that the

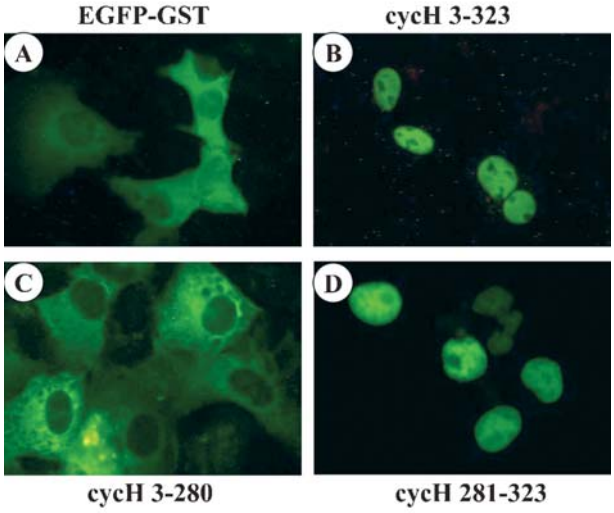

Figure 3. Subcellular localization of EGFP-GST-cyclin H constructs. Fusion constructs were transfected into COS-1 cells by a DEAE dextrane method and expression of fusion proteins was analyzed 24 h after transfection by fluorescence microscopy. (*A*) Control EGFP-GST protein was localized to the cytoplasm, demonstrating the lack of additional NLSs in the fusion constructs. (*B*) Full-length cyclin H fusion constructs were exclusively localized to the nucleus. (*C*) A cylin H fusion construct lacking the NLS-bearing C-terminal sequence was retained in the cytoplasm. (*D*) A fusion construct haboring the putative cyclin H NLS could direct the EGFP-GST protein to the nucleus. The numbers represent the cyclin H amino acid sequence of each clone.

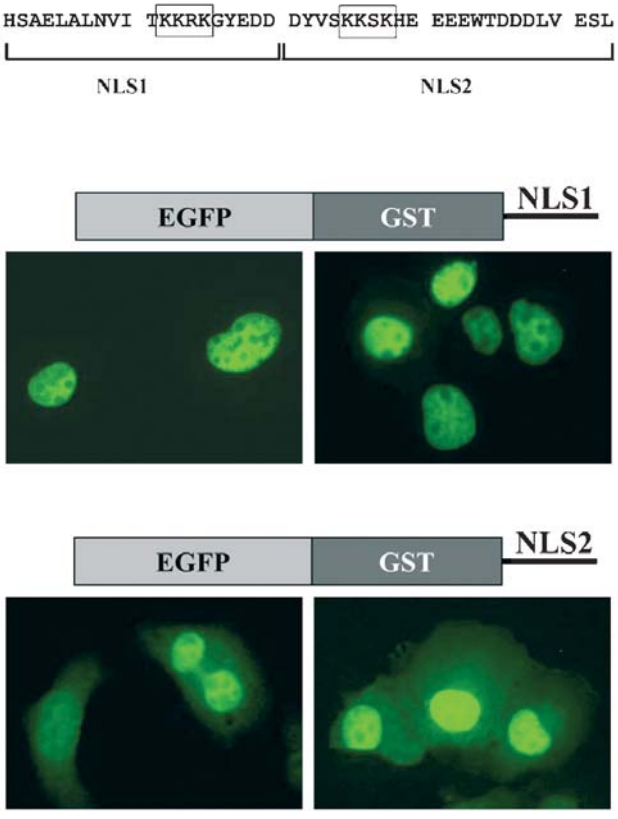

Figure 4. Analysis of the two C-terminal basic clusters in cyclin H. The NLS-bearing sequence was divided between the two clusters (NLS1 and NLS2) and the fragments were cloned separately as EGFP-GST fusion constructs. The plasmids were transfected into COS-1 cells and analyzed 24 h after transfection by fluorescence microscopy. Note that EGFP-GST-NLS1 localized exclusively to the nucleus, whereas EGFP-GST-NLS2 was retained to some degree in the cytoplasm.

two basic clusters in the C-terminus of cyclin H represent two separate NLSs that can operate independently in targeting the proteins to the nucleus. The two NLSs only differ in the effectiveness of the nuclear translocation. NLS1 achieves complete nuclear localization of heterologous proteins and is probably the stronger signal in full-length cyclin H, whereas NLS2 alone could impose some degree of regulation on the localization of cyclin H.

The first step of nuclear protein import involves the recognition of NLSs by members of the importin family. This step is energy independent and translocates proteins to the cytoplasmic side of the nuclear pore complex. Conventional nuclear localization sequences are believed to be recognized specifically by the α/β importin heterodimer, as has been shown for the importins from several species [ref. 32 and refs. therein]. We therefore wanted to assess whether the nuclear import of cyclin H is accomplished by binding to these classical import receptors. Synthetic 14-amino-acid-long peptides encompassing each of the two NLSs with flanking sequences were spotted on a support membrane. The NLS of the SV40 large T-antigen served as a positive control. Filters were incubated with pre-complexed GST-importin α/GST-importin β heterodimer. Specific binding of the import receptors was visualized by binding and detection of a GST-specific antibody. Autoradiography after antibody binding showed that NLS1 of cyclin H interacted with the importin α/importin β dimer (fig. 5). To elucidate the relative importance of a basic residue within the NLS for import receptor binding, the peptide sequence was subsequently modified, substituting one or two basic amino acids of the NLS by alanine residues. Substitution of one or two basic amino acids within the NLS1 sequence influenced the binding affinity of the import receptors. In this assay, the second lysine within the KKRK sequence seemed to have the biggest influence on binding affinity (fig. 5, spot 3). Substitution of two basic amino acids completely abolished the binding of importin α/β (fig. 5, spots 6 and 7). In contrast to NLS1, NLS2 as well as the corresponding alanine mutants were unable to interact with the importin α/importin β heterodimer (fig. 5, spots 8–14). We therefore conclude that cyclin H can be directed to the nucleus by at least two different mechanisms. First, efficient translocation of the protein into the nucleus is achieved through direct interaction of NLS1 with importin α/β. Second, a transport

NLS1

1 LNVITKKRKGYEDDD
2 LNVITAKRKGYEDDD
3 LNVITKARKGYEDDD
4 LNVITKKAKGYEDDD
5 LNVITKKRAGYEDDD
6 LNVITAARKGYEDDD
7 LNVITKKAAGYEDDD

NLS2

8 DDYVSKKSKHEEEEW
9 DDYVSAKSKHEEEEW
10 DDYVSKASKHEEEEW
11 DDYVSKKAKHEEEEW
12 DDYVSKKSAHEEEEW
13 DDYVSAASKHEEEEW
14 DDYVSKKAAHEEEEW

15 AAADPKKKRKVRTAA (NLS-T-Ag)

Figure 5. Peptide filter binding assay to assess importin α/β interactions with the cyclin H NLS. Fourteen-amino-acid-long peptides encompassing the wild-type or mutated sequences of both cyclin H NLSs were synthesized on a membrane and incubated with pre-complexed GST-importin α/β heterodimers. The specific interaction of the import receptors with the peptides was visualized by anti-GST antibody (upper panel). Whereas importin α/β readily bound the wild-type NLS1 sequence (box 1), substitution of one basic amino acid to alanine reduced binding activity (boxes 2–5) and binding was completely absent in double-substituted peptides (boxes 6 and 7). The NLS2 peptide sequence did not interact with importin α/β in any form (boxes 8–14). Box 15 represents the peptide sequence of the importin-α/β-interacting NLS from the SV40 large T-antigen. The exact peptide sequences are denoted in the lower panel. Alanine substitutions are underlined.

mechanism that acts through NLS2 but has yet to be identified directs the majority of the protein to the nucleus but may also result in cytoplasmic retention of cyclin H.

Nuclear translocation has been shown to be regulated by phosphorylation at or near the NLS. Phosphorylation next to the NLS in the SV40 T-antigen by cdc2 has a negative effect on nuclear transport, whereas phosphorylation of a serine residue near the NLS by CK2 results in nuclear accumulation of the protein [33, 34]. A similar mechanism has been demonstrated for a variety of proteins, e.g. cdc25C [35]. We have shown earlier that cyclin H is phosphorylated by protein kinase CK2 at threonine 315 [24]. This phosphorylation is necessary for full activation of the CAK kinase activity. Thr315 is in close proximity to the described NLSs of cyclin H. Furthermore, a second phosphorylation at serine 304 by cyclin C/cdk8 was described [23]. This phosphorylation site is just next to the first basic residue of NLS2. We therefore wanted to investigate whether phosphorylation at these two sites has any influence on the nuclear translocation of cyclin H, thereby regulating CAK activity. To test this hypothesis, we mutated the phosphorylation sites at positions 304 and 315 within the cyclin H sequence to either alanine (which cannot be phosphorylated) or to aspartic acid (Thr315) or glutamic acid (Ser304), both mimicking phosphorylation through their negative charges (fig. 6A). The mutations were introduced into the full-length cyclin H sequence as well as into the NLS-bearing C-terminal truncations. COS-1 cells were then transfected with plasmids that contained the fragments fused to EGFP-GST. Mutation of Thr315 to either alanine or aspartic acid had no influence on the nuclear localization of cyclin H or the NLS-bearing sequence (fig. 6B). Both mutations resulted in nuclear accumulation of the fusion proteins to a similar extent as was observed for wild-type cyclin H constructs. As seen for the Thr315 phosphorylation site, mutation of the cyclin C/cdk8 phosphorylation site at Ser304 to either alanine or glutamic acid had no influence on the subcellular distribution of the fusion proteins, resulting in complete nuclear transfer of the full-length and the truncated NLS-bearing proteins. We therefore have to conclude that the phosphorylation status at Ser304 and Thr315 within the NLS region of cyclin H has no influence on the subcellular localization of the protein.

Discussion

CAK plays a central role in cellular mechanisms that regulate DNA repair, transcription, and cell cycle progression. Therefore, tight control of CAK activity itself is necessary to ensure coordinated cellular activity. The sub-cellular distribution and the transport to specific substrates constitutes a major regulatory mechanism but is not well understood for CAK and its subunits. Through expression of fusion constructs in COS-1 cells, we demonstrated here that the cyclin H subunit of CAK con-

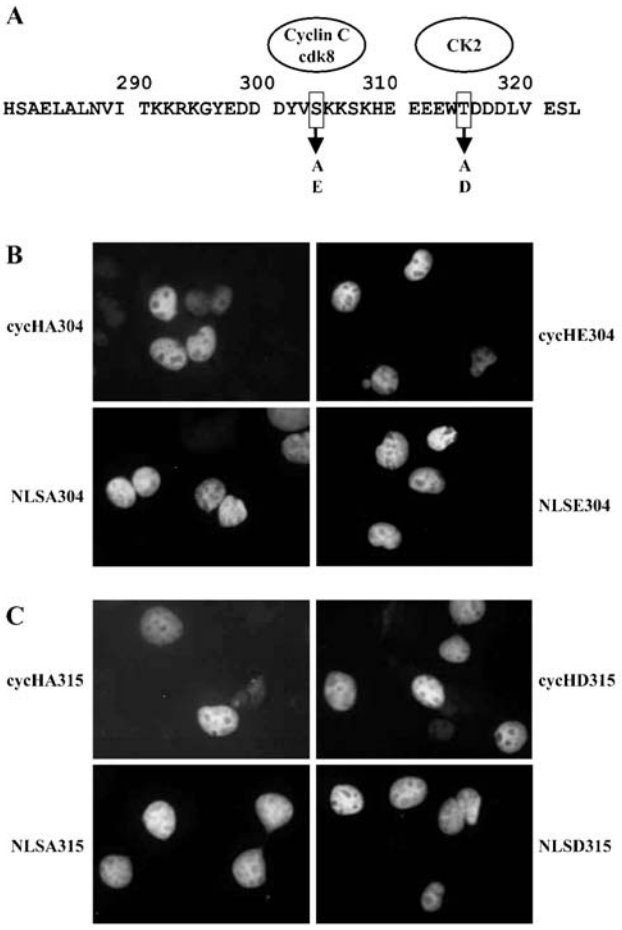

Figure 6. Analysis of the cyclin C/cdk8 and CK2 phosphorylation sites in the cyclin H NLS. (*A*) C-terminal amino acid sequence of cyclin H showing the cyclin C/cdk8 (Ser304) and CK2 (Thr315) phosphorylation sites in the vicinity of the NLS. The sites were either both mutated to alanine, or to glutamic acid or aspartic acid. (*B*, *C*) EGFP-GST fusion constructs of the mutated full-length (cycH) or the truncated (NLS) cyclin H proteins were transfected into COS-1 cells and analyzed 24 h after transfection by immunofluorescence. Mutations of the cyclin C/cdk8 or the CK2 phosphorylation sites did not influence the nuclear import of full-length cyclin H or its NLS-bearing sequences.

tains two basic clusters of amino acids in its C-terminal sequence that serve as NLSs. Nuclear targeting of proteins bearing both NLSs is independent of phosphorylation at two distinct sites near the NLSs. We have also shown that at least one NLS of cyclin H displays significant specific binding to the importin α/β dimer and is therefore translocated by the classical pathway of nuclear import. In this respect, cyclin H behaves like cyclin E, which binds to the importin α adaptor subunit through a basic NLS in the N-terminal region [36]. Despite their structural and functional relationship, this is, however, not a general mode of nuclear translocation among cyclin family members. The cyclin A/cdk2 complex is structurally related to cyclin H/cdk7 but transported by a pathway that does not require exogenous importin α or β. Moreover, cyclin A/cdk2 does not require additional NLS-containing carrier proteins to get into the nucleus [37]. Cyclin B1 as well as the cyclin B/cdk1 regulator cdc25C are imported into the nucleus through pathways independent of importin α [35, 38, 39]. Interestingly, protein kinase CK2 has been demonstrated to regulate nuclear import of both proteins [35, 40]. Cyclin D1 is imported into the nucleus possibly by piggybacking on the $p21^{Cip1}/p27^{Kip1}$ cdk inhibitors [41, 42].

In accordance with our results on the nuclear transport of cyclin H, cyclin H has been reported to be predominantly nuclear during the whole cell cycle. However, cyclin H is usually isolated from the cell as either a trimeric CAK complex consisting of cyclin H, cdk7, and MAT1, or as part of the multi-protein complex TFIIH [5, 6]. Not clear to date is where the assembly of the complex takes place. For some cyclin/cdk complexes, cytoplasmic assembly is a prerequisite for entering the nucleus, and the cytoplasmic-nuclear redistribution is mostly conferred by the cyclin subunit of the complexes [37]. Cdk7 itself bears an NLS in its C-terminal region and can, therefore, enter the nucleus by itself [27]. The third component, MAT1, does not contain an obvious NLS and probably has to be co-translocated with one of the other constituents. MAT1 has been reported to interact directly and specifically with cdk7, independently of the presence or absence of cyclin H [31]. Most of the CAK and TFIIH substrates like the C-terminal domain of RNA Pol II, the cyclin/cdk complexes, or gene-specific transcription factors are located and active in the nucleus. The cell may therefore benefit by ensuring efficient translocation of the individual components. Individual transfer may also provide a regulatory mechanism to block premature activation of the CAK complex outside the nucleus. *In vitro* experiments have shown that assembly of cyclin H with cdk7 and MAT1 can activate the complex without a requirement for phosphorylation in the activating T loop of cdk7 [6].

Phosphorylation modifies protein function through direct influence on the enzymatic properties or alteration of the binding characteristics for specific partners. Phosphorylation of cyclin H by protein kinase CK2 and cyclin C/cdk8 has been shown to change the kinase activity of the CAK complex towards its substrates cdk2 and the CTD of RNA Pol II. Whereas CK2 phosphorylation is necessary for full activation of CAK, phosphorylation by cyclin C/cdk8 on two sites in the cyclin H molecule downregulates TFIIH activity towards the RNA Pol II CTD. Although the consequences of this phosphorylation in terms of CAK activity were investigated, the exact molecular mechanisms for this regulation are not clear. The CK2 and one of the cyclin C/cdk8 phosphorylation sites are found in close proximity to each other in the C terminus of cyclin H. We have now demonstrated that these phosphorylation sites reside within or close to

two nuclear localization signals that are able to translocate cyclin H very efficiently to the nucleus. However, the phosphorylation status does not seem to have a large influence on the subcellular distribution of cyclin H, as demonstrated by mutation analysis. This is very intriguing, as in some proteins with a similar arrangement of NLSs and phosphorylation sites, the phosphorylation status clearly has an influence on the efficiency of nuclear translocation. The so-called CcN motif usually consists of an NLS with phosphorylation sites for protein kinase CK2 and a cyclin-dependent kinase in the vicinity of the nuclear-targeting sequence. The CcN motif was characterized initially as being responsible for phosphorylation-regulated nuclear localization of the simian virus 40 large tumor antigen (T-ag) [33]. Subsequently, this motif was identified in a diversity of proteins, including nucleoplasmin [43], the yeast transcription factor SW15 [44], phosphatase inhibitor-2 [45], and, most recently, cdc25C [35]. In all cases investigated, phosphorylation at a short distance from the NLS modulates the nuclear import rate and the phosphorylation next to the NLS regulates the maximal level of nuclear accumulation [33, 46, 47]. In cyclin H, the protein kinase CK2 phosphorylation site lies seven amino acids downstream from the last basic residue of the second NLS and the phosphorylation site for cyclin C/cdk8 is just next to the second NLS in cyclin H. From these data one could conclude that the second NLS of cyclin H comprises a phosphorylation-regulated NLS. However, in our experiments, mutation of both sites to unphosphorylatable alanine had no influence on the efficiency of nuclear transport. This effect could be explained in two ways. First, phosphorylation by protein kinases CK2 and cyclin C/cdk8 solely regulates the activity of CAK, but does not account for nuclear import of cyclin H. This is supported by the fact that both CAK-regulating kinases are predominantly nuclear and probably activate/deactivate the complex only when localized to the nucleus. A second explanation would be that the importin-α/β-dependent, stronger NLS1 of cyclin H directs the protein to the nucleus constitutively, independent of the phosphorylation status in NLS2. Only if NLS1 is not accessible by the import receptors does NLS2 serve as the targeting signal and allow some degree of regulation of subcellular distribution, as has been seen in fusion constructs that carry NLS2 alone. The accessibility of a domain can be modified by conformational changes or by interaction with other proteins. In this context, structural information about cyclin H reveals that the unique C-terminal helix bearing the identified NLS may have some degree of flexibility [31]. In addition, binding partners of cyclin H have been identified recently. As the regulatory subunit, cyclin H interacts with cdk7 in a domain that is very close to the identified NLS in the linear sequence. However, structural comparison with the very closely related cyclin A/cdk2 complex does not suggest an influence of cdk7 binding on the C-terminal helix [31]. The binding sites for two other cyclin-H-binding factors, protein kinase CK2 and the tumor suppressor p53, have not been identified so far. However, noteworthy in this context is that protein kinase CK2, although predominantly nuclear, is found in several cytoplasmic compartments, including the Golgi apparatus [48], and substrates of CAK like cyclin E/cdk2, cyclin B/cdk1, and cdk5-p35 are not only active in the nucleus, but also present in the cytoplasm [37, 49–52]. Under certain conditions, therefore, e.g. cell cycle phase specific or under stress, there is conceivably a requirement for functional CAK in the cytoplasm. The identification of additional binding partners for cyclin H is underway and will provide valuable information on the regulation of cyclin H, additional substrates of CAK, and the distribution of the subunits.

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