Multiple Mechanisms Regulate Subcellular Localization of Human CDC6*

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CDC6 is a protein essential for DNA replication, the expression and abundance of which are cell cycle-regulated in Saccharomyces cerevisiae. We have demonstrated previously that the subcellular localization of the human CDC6 homolog, HsCDC6, is cell cycle-dependent: nuclear during G1 phase and cytoplasmic during S phase. Here we demonstrate that endogenous HsCDC6 is phosphorylated during the G1/S transition. The N-terminal region contains putative cyclin-dependent kinase phosphorylation sites adjoining nuclear localization sequences (NLSs) and a cyclin-docking motif, whereas the C-terminal region contains a nuclear export signal (NES). In addition, we show that the observed regulated subcellular localization depends on phosphorylation status, NLS, and NES. When the four putative substrate sites (serines 45, 54, 74, and 106) for cyclin-dependent kinases are mutated to alanines, the resulting HsCDC6A4 protein is localized predominantly to the nucleus. This localization depends upon two functional NLSs, because expression of HsCDC6 containing mutations in the two putative NLSs results in predominantly cytoplasmic distribution. Furthermore, mutation of the four serines to phosphate-mimicking aspartates (HsCDC6D4) results in strictly cytoplasmic localization. This cytoplasmic localization depends upon the C-terminal NES. Together these results demonstrate that HsCDC6 is phosphorylated at the G1/S phase of the cell cycle and that the phosphorylation status determines the subcellular localization.

Little is known regarding the regulation of DNA replication initiation in mammalian cells. In yeast, the origin recognition complex, required for replication initiation, consists of six subunits and is associated with specific DNA sequences (replicators) (1–3). In addition, other factors are required for DNA replication initiation including CDC6, CDC45, and MCM (mini-chromosome maintenance) family proteins (4). Several lines of evidence suggest that ORC, CDC6, and MCM may function together as the replication initiator complex (reviewed in Refs. 5 and 6). Recently several human proteins have been identified that seem to be structural homologs of proteins known to be directly involved in DNA replication in yeast (7–14).

CDC6 in Saccharomyces cerevisiae, Cdc18 in Schizosaccharomyces pombe, and XCDC6 in Xenopus are homologs and have been shown to be essential for DNA replication. For example, in S. cerevisiae the assembly of a prereplication initiation complex at origins of replication requires CDC6 (15). In both S. cerevisiae and S. pombe, the CDC6/Cdc18 protein is degraded at the G1/S transition after phosphorylation by cdk (16, 17). In S. pombe, overexpression of Cdc18 results in re-replication without mitosis (16), whereas specific mutations in S. cerevisiae CDC6 cause over-replication of DNA (18). Together these results demonstrate the importance of CDC6 in DNA replication and suggest that stringent regulation of CDC6 protein levels, such that it is active and/or available in G1, but destroyed soon after initiation, is crucial to ensure that DNA replication occurs precisely once per cell cycle.

The exact function(s) of CDC6/Cdc18 in mammalian cells is not known, but it is likely that it plays a role in the process of assembly of prereplication complexes and/or origin firing. The human homolog of CDC6/Cdc18, HsCDC6 binds cyclin and ORC1 similar to studies previously done with yeast, implicating HsCDC6 in DNA replication (19). However, in contrast to yeast in which CDC6 protein levels are tightly regulated during the cell cycle, levels of HsCDC6 do not decline at the onset of S phase (19–21), suggesting an alternative mechanism of regulation of HsCDC6 at the G1/S transition in human cells. This idea is supported by studies from our laboratory and others that demonstrate that epitope-tagged HA-HsCDC6 is found to be nuclear in G1 cells and cytoplasmic in S phase cells (19, 21, 22). We show that HsCDC6 contains two putative N-terminal NLSs and a leucine-rich C-terminal nuclear export signal (NES), LXXLX(L)(reviewed in Ref. 23). Previous reports of other proteins containing both nuclear import and export motifs describe protein shuttling from one compartment to the other until one of the motifs is masked, whether by phosphorylation, association with other proteins, or other modification (24, 25). Our working hypothesis is that HsCDC6 is synthesized and imported into the nucleus in an NLS-dependent manner, whereby it binds ORC and begins assembly of the prereplicative complex. Unbound (excess) HsCDC6 continues to shuttle. Upon phosphorylation of HsCDC6, possibly by cyclin A/cdk, HsCDC6 is released from the prereplicative complex, whereby it becomes available for export from the nucleus via its NES.

Here we show that HsCDC6 is phosphorylated by cyclin/ckds in vitro via association with cyclin/ckds through the Cy motif and becomes dephosphorylated in G1 and phosphorylated at the G1/S transition in vivo. A functional Cy motif and intact phosphorylation sites are required for the cytoplasmic displace-
ment of HsCDC6 in S phase. Furthermore, conversion of the cyclin/cdk-targeted serines (SPXK) to phosphate-mimicking aspartic acid resulted in nearly exclusive cytoplasmic localization of the protein during G1 phase when cdk is inactive. Together these results indicate that dephosphorylated HsCDC6 localizes to the nucleus through the action of either of two NLSs, and is phosphorylated at the G2/S border, whereby HsCDC6 relocates to the cytoplasm, possibly through the unmasking of the NES.

EXPERIMENTAL PROCEDURES

Binding and Competition Studies—The conditions for competition of binding reactions with peptides PS100 and PS101 was described previously (26, 27). PS100 (ACRRLFGPVDSE) is derived from the cdk2, and GST (b) peptide PS101.

Plasmids and Mutagenesis—The plasmids pAHpHsCDC6 (hemagglutinin-tagged) and pA3MHsCDC6 (Myc-tagged) were described previously (19). All point mutant plasmid constructs were generated using polymerase chain reaction-based mutagenesis using Pfu polymerase (Stratagene). Plasmids containing mutants were sequenced and subjected to in vitro transcription translation assays (TnT, Promega) to demonstrate full-length and accurate sequences. The following primers were used to generate mutations: Cy1, 5’-acataacaattgagggagagagctgctgacaatt-3’ (RRVL > GGSV); Cy2, 5’-acattagggagagagctgctgacaatt-3’ (RRVL > RVG); Alav, 5’-gctgacaattgagggagagctgctgacaatt-3’; Asp54, 5’-agaattctcttttcgaaggagccttaat-3’; Asp106, 5’-gctgacaattgagggagagctgctgacaatt-3’. These constructs were subjected to sequencing analysis and others have published previously that HsCDC6 is phosphorylated at the G1/S transition, whereby HsCDC6 relocates to the nucleus through the action of either of two NLSs, and is phosphorylated at the G2/S border, whereby HsCDC6 relocates to the cytoplasm, possibly through the unmasking of the NES.
plasm during S phase of the cell cycle (19, 21, 22, 28). Because the activity of many regulatory proteins is modulated by phosphorylation via specific kinases and phosphatases, we examined the phosphorylation level of endogenous HsCDC6 during G1 and S phase in HeLa cells, a human cervical carcinoma cell line. Autoradiography revealed that endogenous HsCDC6 is dephosphorylated in G1-blocked cells (mimosine) and phosphorylated in S phase (hydroxyurea) and asynchronously growing

Fig. 3. Subcellular localization of HsCDC6. A, U2OS cells were transfected with HA-tagged HsCDC6, ΔCy, A4, and D4 plasmid constructs. B, indirect immunofluorescence was performed on transfected cells using an anti-HA antibody (12CA5 mouse monoclonal antibody) followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. For each plasmid construct, >50 transfec-
tants were counted per experiment. All transfections were repeated at least once. 4,6-diamidino-2-phenylindole staining shows all nuclei in the field, whereas anti-HA staining shows HA-CDC6 only in the transfected cells.
cells (Fig. 1A). Western analysis of the nitrocellulose blot reveals similar protein levels of HsCDC6 in asynchronous and mimosine- and hydroxyurea-treated cells, indicating that the low level of phosphorylation observed in mimosine-treated cells is not caused by the absence of endogenous protein levels (Fig. 1B). These results demonstrate that HsCDC6 is phosphorylated as cells enter S phase.

**HsCDC6 Associates with Cyclin/cdk5 through the Cy Motif**—The N-terminal one third of HsCDC6 contains one Cy motif (an RRLVF cyclin-docking motif). To determine the importance of the Cy motif in the association of HsCDC6 with cyclin/cdk5, competition studies were performed using a peptide containing a Cy motif (PS100) and another peptide with mutations in the Cy motif (PS101) as a negative control. GST-CDC6 specifically binds in vitro 35S-radiolabeled cyclin A produced by in vitro transcription and translation. The Cy peptide competitively inhibited this association of HsCDC6 with cyclin A as compared with the mutant Cy peptide (Fig. 2a). To determine directly

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**Fig. 4. FACS Analysis.** U2OS cells were co-transfected with plasmid expressing farnesylated GFP and empty vector (A), HsCDC6 (B), A4 (C), D4 (D), ΔCy (E), or p21 (F). After 48 h, the cells were harvested, stained with propidium iodide, and analyzed by FACS to determine the cell cycle distribution of the GFP-positive (transfected) cells. The G1, S, and G2 population estimated by the Modfit software are superimposed on the raw data histogram.
U2OS cells were transfected with HA-tagged HsCDC6 and mutant HsCDC6 constructs. After 48 h, the cells were fixed and permeabilized, and indirect immunofluorescence was performed. Transfected cells were scored for nuclear (% NUC) and cytoplasmic (% CYTO) localization of the HA-CDC6 protein. WT, wild type.

**Fig. 5. CDC6 has two NLSs.** U2OS cells were transfected with HA-tagged HsCDC6 and mutant HsCDC6 constructs. After 48 h, the cells were fixed and permeabilized, and indirect immunofluorescence was performed. Transfected cells were scored for nuclear (% NUC) and cytoplasmic (% CYTO) localization of the HA-CDC6 protein. WT, wild type.

**Fig. 6. Phosphorylation-mimicking mutations of HsCDC6.** U2OS cells were transfected with HA-tagged HsCDC6 and mutant HsCDC6 plasmid constructs. After 48 h, the cells were fixed and permeabilized, and indirect immunofluorescence was performed. Transfected cells were scored for nuclear (% NUC) and cytoplasmic (% CYTO) localization of the HA-CDC6 protein. WT, wild type.
significant number of cells transfected with A4Δ80–81 showed a redistribution from the nucleus to the cytoplasm (58% nuclear) as compared with the exclusively nuclear staining of A4 (Fig. 5). The second NLS candidate, similar to the putative c-Myc NLS, was mutated (Arg66Lys67Arg68 to alanines A4Δ66–68), and the CDC6 protein was examined for subcellular distribution. This mutation resulted in 61% nuclear location. Mutation of both of these putative NLSs on the A4 CDC6 plasmid (A4Δ66–68, 80–81) resulted in a 90% cytoplasmic localization (Fig. 5). Therefore, nuclear localization depends on either of the two NLSs on HsCDC6.

To determine whether there was residual nuclear localization activity, LMB, an inhibitor of nuclear export, was added to transfected cells (Fig. 5). In the transfections with mutations in the individual NLSs, A4Δ66–68 and A4Δ80–81, an increased nuclear localization was seen, suggesting residual nuclear localization activity when either NLS was mutated alone. However, in cells transfected with the combined mutation A4Δ66–68, 80–81, there was less than 25% nuclear localization in response to LMB, suggesting that NLS activity was confined to these two signals.

**Cyclin/cdk Phosphorylation of HsCDC6 Is Responsible for Cytoplasmic Localization**—The loss of the cyclin binding motif restricts HsCDC6 localization to the nucleus. Because the absence of a Cy motif is predicted to diminish the efficiency with which cyclin/cdkks dock on HsCDC6 and phosphorylate target serines, we proposed that the phosphorylation of HsCDC6 is important for cytoplasmic localization. To test this hypothesis, U2OS cells were transfected with a plasmid encoding HA-tagged HsCDC6 with mutations of serines 45, 54, 74, and 106 to aspartates (D4) to mimic the negative charge of a phosphate. Immunofluorescence studies of transfected cells demonstrated cytoplasmic staining in >95% cells (Fig. 3). FACS analysis of cells co-transfected with D4 and a GFP marker plasmid showed no significant change in cell cycle distribution when compared with mock transfected cells (Fig. 4D), indicating that cytoplasmic localization of D4 is not a result of S phase arrest. To precisely define the regulatory serine(s) involved in cytoplasmic localization, we individually mutated serines 54 and 74 to aspartates (Asp54 and Asp74). To prevent the phosphorylation of other cyclin/cdk targeted serines in HsCDC6, which may contribute to the cytoplasmic displacement of Asp54, serines 45, 74, and 106 were converted to alamines (ADAA) (Fig. 6), and similar mutations were added to the mutant Asp74 (AADAA). These plasmids were transfected into U2OS cells and cells were scored for cytoplasmic localization. Mutation of either serine 54 or 74 alone to aspartates resulted in nearly exclusive cytoplasmic localization, thus defining that phosphorylation of either of these serines is sufficient for nuclear exclusion.

**Phosphorylation Does Not Inactivate the CDC6 NLS**—Because serine 54 and 74 are located near the two NLSs, it is possible that the negative charge acquired by the phosphorylation of these residues (mutation to aspartates) causes cytoplasmic localization by inactivating the NLS. Addition of LMB to cells transfected with D4 CDC6 (Fig. 5), however, increased nuclear localization to ~50% of cells. This nuclear localization suggests that the NLS is still active in the phosphorylated HsCDC6 (or in HsCDC6 with phospho-mimicking mutations).

**The Nuclear Export Signal of HsCDC6 Is Located in the C Terminus**—Another model to explain the phosphorylation-dependent subcellular distribution of HsCDC6 is that the phosphorylation of key serines results in a conformational change that unMASKS a NES. To identify the location of a potential NES(s), truncations of the constitutively cytoplasmic HsCDC6D4 were transfected into U2OS cells and scored for localization. Truncations at amino acids 364 (D4–364T) and 462 (D4–462T) resulted in predominantly nuclear localization as compared with full-length D4, which is cytoplasmic (Fig. 7). However, D4–488T demonstrated cytoplasmic localization in >90% cells, suggesting the possibility that an NES exists in residues 462–488. Sequence analysis of HsCDC6 predicts a highly hydrophobic and classical NES in this region, ILVCSLMLLIRQLKI.

Although this study clearly demonstrates a role for phospho-
rylation in cytoplasmic localization, the exact mechanism of nuclear export could require either 1) phosphorylation alone of HsCDC6 or 2) phosphorylation and continued association between cyclin/cdk. To distinguish between these two possibilities, U2OS cells were transfected with HA-tagged HsCDC6D4 also containing a Cy1−2 mutation that does not disrupt the NLS (Fig. 6). Transfected cells show predominantly cytoplasmic staining similar to HsCDC6D4, indicating that phosphorylation alone without continued association with cyclin/cdk is sufficient for cytoplasmic localization.

HsCDC6-ORC1 Interaction—We have previously described HsCDC6-ORC1 interactions (19). To determine the role of ORC1 binding to nonphosphorylated and phosphorylation-mimicking HsCDC6 mutants, A4 and D4, respectively, T7-tagged ORC1 was co-transfected with Myc-tagged HsCDC6-A4 or -D4 into 293T cells. Cell lysates were immunoprecipitated using an anti-T7 antibody and analyzed for co-immunoprecipitation of Myc-A4 and Myc-D4 by Western blot (Fig. 8) using an anti-Myc antibody. ORC1 binds similar levels of A4 and D4, suggesting that the binding of ORC1 to HsCDC6 is not regulated by the phosphorylation status of HsCDC6.

DISCUSSION

Our laboratory and others (19, 21, 22) demonstrated previously that subcellular localization of HsCDC6 is cell cycle-dependent, whereby HsCDC6 is nuclear in G1 phase and cytoplasmic in S phase. We also showed cell cycle-specific association of cyclin A/cdk2 with HsCDC6 (19). We show here that endogenous HsCDC6 is phosphorylated at the G1/S border, a finding temporally consistent with cyclin A expression, origin firing, and the redistribution of HsCDC6 to the cytoplasm. In addition, we investigate the role of this phosphorylation in HsCDC6 localization through mutational analysis of 1) the four putative cyclin/cdk phosphorylation sites, 2) the cyclin-docking motif, Cy, 3) N-terminal nuclear localization signals, and 4) a C-terminal nuclear export signal. It was reported previously that HsCDC6 is phosphorylated on serines 54, 74, and 106 by cyclin/cdk (21, 22). We have similar results2 and extended the analysis to show that the Cy motif of HsCDC6 improves the $K_m$ of a CDC6-derived peptide substrate of cdk5 by 100-fold (30).

The results described here indicate a role for phosphorylation and NLS in the subcellular localization of HsCDC6. Both A4 and ΔCy mutants are limited to the nuclei only when the NLS are intact. However, disruption of each individual putative NLS results in a significant shift toward cytoplasm, 42% and 39% as compared with <10%. Mutation of both sites results in nearly complete inhibition of nuclear import. The addition of LMB, a CrmI nuclear export inhibitor, further demonstrates an incomplete block to nuclear import in individual NLS mutations. Because LMB did not significantly increase nuclear localization of A4Δ66−68, 80−81, we can eliminate the possibility that these mutations merely enhance nuclear export and thereby increase cytoplasmic localization. The results presented here support the idea that HsCDC6 utilizes either of two NLSs to gain access to the nucleus.

We show here that mutation of either serine 54 or 74 to phosphate-mimicking aspartates results in cytoplasmic localization of HsCDC6. This is not an effect of blocking nuclear import, because we see nuclear localization of the D4 mutant when cells are treated with LMB, suggesting D4 cycles in and out of the nucleus. In addition, we see nuclear staining in cells transfected with truncated D4 mutants (D-364T and D-462T), suggesting that the NLS sequences are functional despite the localized increase in negative charges mimicking phosphorylation. Also, we show that phosphorylation and export of HsCDC6 does not depend upon a continued association with cyclin A/cdk, because the elimination of the cyclin-interacting Cy motif on the ΔD4 mutant does not abolish cytoplasmic localization.

In addition, we identify a leucine-rich C-terminal NLS. On the basis of reports of other proteins containing both NLS and NES motifs, shuttling from one compartment to the other continues to occur until one of the motifs is masked whether by phosphorylation, association with other proteins, or other modification. Our working hypothesis is that HsCDC6 is synthesized and imported into the nucleus in an NLS-dependent manner, whereby it binds ORC and begins assembly of the prereplicative complex. Upon phosphorylation, possibly by cyclin A/cdk, HsCDC6 is released from the prereplicative complex and is exported from the nucleus via its NES. It is possible that conformational changes induced by phosphorylation of HsCDC6 allow the unmasking of the C-terminal NES required for nuclear export.

Phosphorylation of HsCDC6 was expected to be linked with origin firing. Therefore, the A4 mutant might have acted as a dominant negative protein, which causes a block to origin firing. It is surprising that A4 does not cause a block in G1/S. Although this result is consistent with the results of Petersen, et al. (21), it is possible that levels of exogenously expressed A4 is lower than endogenous HsCDC6, thus allowing origin firing by endogenous HsCDC6. Further, as in yeast, very few origins may need to fire per chromosome for complete and rapid replication of the genome (31). Therefore, we cannot yet state that the failure to phosphorylate HsCDC6 is without any effect on the replication machinery, particularly in view of the effects on replication seen in other experimental systems with similar mutants (22, 32).

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