Phosphorylation of the Human Ubiquitin-conjugating Enzyme, CDC34, by Casein Kinase 2

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The ubiquitin-conjugating enzyme, CDC34, has been implicated in the ubiquitination of a number of vertebrate substrates, including p27\textsuperscript{Kip1}, IκBα, Wee1, and MyoD. We show that mammalian CDC34 is a phosphoprotein that is phosphorylated in proliferating cells. By yeast two-hybrid screening, we identified the regulatory (β) subunit of human casein kinase 2 (CK2) as a CDC34-interacting protein and show that human CDC34 interacts in vivo with CK2β in transfected cells. CDC34 is specifically phosphorylated in vitro by recombinant CK2 and Hela nuclear extract at five sites within the carboxyl-terminal 36 amino acids of CDC34. Importantly, this phosphorylation is inhibited by heparin, a substrate-specific inhibitor of CK2. We have also identified a kinase activity associated with CDC34 in proliferating cells, and we show that this kinase is sensitive to heparin and can utilize GTP, strongly suggesting it is CK2. Phosphorylation of CDC34 by the associated kinase maps predominantly to residues 203 and 222. Mutation of CDC34 at CK2-targeted residues, Ser-205, Ser-222, Ser-231, Thr-233, and Ser-236, abolishes the phosphorylation of CDC34 observed in vivo and markedly shifts nuclearly localized CDC34 to the cytoplasm. These results suggest a potential role for CK2-mediated phosphorylation in the regulation of CDC34 cell localization and function.

The CDC34 gene was first identified as a cell division cycle gene in Saccharomyces cerevisiae required for the G\textsubscript{1} to S phase transition and was later shown to encode a ubiquitin-conjugating enzyme (UBC) or E2 (1, 2). The human homolog of CDC34 functionally complements S. cerevisiae temperature-sensitive strains (3) and has been proposed to participate in the ubiquitination of various substrates during diverse cellular processes in vertebrates (4, 5). The most detailed studies of CDC34 have focused on its function during the onset of DNA replication. In budding yeast, Cdc34p is required to degrade the Cdc28-Clb5,6 kinase inhibitor, p40\textsuperscript{SCC}, to traverse the G\textsubscript{1} to S phase transition and initiate DNA replication (6). Studies in interphase egg extracts of Xenopus laevis show that CDC34 is also required for the onset of DNA replication in vertebrates, implying a protein degradation requirement for the progression into S phase (7).

Functionally, vertebrate CDC34 in association with different ubiquitin protein ligase or E3 complexes has been shown to target many different substrates for ubiquitination and degradation during cell division, signal transduction, and development (reviewed in Refs. 8 and 9). The vertebrate CDC34 substrates that have been characterized to date include IκBα, B-Myb, Wee1, MyoD, ICERIγ, ATF5, p27\textsuperscript{xic1}, and p27\textsuperscript{Kip1} (7, 10–12; reviewed in Refs. 8, 9). Additionally, substrates such as β-catenin, p21\textsuperscript{Cip1}, E2F, cyclin E, and cyclin D are putative substrates of CDC34 by virtue of their SCF requirement for proteolysis (reviewed in Refs. 5, 8). SCF is a multiprotein E3 complex that functions in association with CDC34 and is composed of the F-box binding protein p19\textsuperscript{Skp1}, a cullin protein, an F-box protein, and the ring finger protein Roc1/Rbx1/Htr1 (reviewed in Refs. 4, 13). In mammals, CDC34 in association with SCFp145\textsuperscript{Skp2} participates in the ubiquitination of the cyclin-dependent kinase inhibitor, p27\textsuperscript{Kip1} (14, 15).

The regulation of CDC34-SCF-dependent ubiquitination of substrates is found at many different levels. To date, most of the characterized SCF substrates are targeted to the ubiquitination machinery only upon phosphorylation and subsequent binding to the F-box protein (reviewed in Refs. 13, 16). This suggests two levels of regulation, one at the level of the kinase responsible for substrate phosphorylation and the other at the level of F-box protein expression.

In budding yeast, regulation of substrate ubiquitination has also been demonstrated at the level of the components of the ubiquitination machinery. The ring finger protein Roc1/Rbx1/Htr1 in yeast modulates the level of active Cdc34p that associates with the SCF complex, thus regulating the level of substrate ubiquitination (17, 18). Recent studies have also indicated that the ubiquitination machinery is regulated by post-translational modifications as well. Cullin proteins are found to be post-translationally modified by NEDD8 conjugation, and this modification has been shown to be required for full activation of a Roc1-Cul1 ubiquitin ligase complex (19–21). Roc1/Rbx1/Htr1 and F-box proteins themselves appear to be regulated by ubiquitination and proteolysis as well. In budding yeast, F-box proteins Grr1, Cdc4, and Met30 have been shown to be ubiquitinated by core components of SCF complexes, targeting them for degradation and potentially resulting in changes in the levels of functional F-box proteins (22–25).
mammalian cells, ectopically expressed Roc family proteins are degraded when unassociated with cullin proteins (26). In addition, Cdc34p in S. cerevisiae (ScCdc34p) has been shown to dimerize and to be phosphorylated and ubiquitinated, although these post-translational modifications have not yet been linked to an essential biological function (27–29).

In this study we show that human CDC34 (hCDC34) is a phosphoprotein that is phosphorylated in proliferating cells. We also identify the regulatory subunit (β) of human casein kinase 2 (CK2) as a novel CDC34-interacting protein through yeast two-hybrid screening and characterize the putative phosphorylation of hCDC34 by CK2. CK2 is a constitutive and ubiquitously expressed serine-threonine kinase (reviewed in Refs. 30, 31) that is essential for viability and cell cycle progression in S. cerevisiae (32, 33). CK2 is also required for cell cycle progression in mammals (34), and misregulation of CK2 expression in the lymphocytes of transgenic mice has been shown to result in the development of lymphoma (35).

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Metabolic Labeling, Transfection, and Immunofluorescence**

Cultured Xenopus laevis in Dublecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone). Serum starvation was used to synchronize cells and for the generation of quiescent cells. Cells at 40–50% confluency were used to seed 24-well plates with 25% (v/v) of cell culture medium. Xenopus laevis hCDC34/pcDL-SR were transfected as described above. Cells were metabolically pulse-labeled for 3 h with [3H]thymidine (BrdUrd, Amersham Pharmacia Biotech) for 3 or 10 h as indicated, aspirated and boiled in 1× Laemml sample buffer (39), and the samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A percentage of the input [3H]thymidine in vitro translated protein (2.5%) was included on the gel to quantitate the percentage of co-immunoprecipitated protein. Cells were quantitated by PhosphoImager analysis using ImageQuant software (Molecular Dynamics).

**Immunoprecipitation-Western (IP-Western) assays** were conducted using whole cell lysates generated with RIPA buffer. For immunoprecipitations, the amount of lysate used was normalized to an equal amount of total protein as determined by Bradford analysis (Bio-Rad) or Ponceau staining. Immunoprecipitates were probed with goat anti-rabbit/mouse or protein A coupled to horseradish peroxidase (Bio-Rad) followed by chemiluminescence using ECL reagent (Amersham Pharmacia Biotech).

Double-immunoprecipitation assays were performed on cell lysates following [3H]methionine or [32P]orthophosphate labeling of cells as previously described (40). Labeled cell lysates were precipitated with trichloroacetic acid and normalized by using equivalent amounts of trichloroacetic acid-precipitable counts for each sample. Between 4 × 10^6 and 1 × 10^7 trichloroacetic acid-precipitable counts from transfected cell extracts were immunoprecipitated.

**Kinase Assays**—The in vitro CK2 kinase assays were performed with recombinant human CK2 enzyme, cell extracts, or immunoprecipitates. For in vitro kinase assays utilizing recombinant human CK2, a 20-µl reaction containing approximately 5–50 µg of recombinant 6×His-hCDC34 protein or other recombinant proteins, CK2 buffer (50 mM Hepes, pH 7.2, 150 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol), 0.1 or 0.25 milliunit of human recombinant CK2 (Roche Molecular Biochemicals or New England Bio-Labs, respectively), and 0.08 µCi of [γ-32P]ATP (PerkinElmer Life Sciences) was incubated at 30 °C for 30 min. 32P incorporation was determined by boiling samples in 1× Laemmli sample buffer, followed by SDS-PAGE, Coomassie Blue staining, and autoradiography. Kinase assays using cell lysates were performed by adding 5–50 µg of nuclear or total cell extract to a 30-µl reaction with 0.5 µg of recombinant 6×His-hCDC34 protein. X. laevis S100 and HeLa cell nuclear extracts were generated as previously described (7, 41). Reactions were incubated at
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RESULTS

Mammalian CDC34 is a Phosphoprotein That Is Phosphorylated in Proliferating Cells—To determine if CDC34 is phosphorylated in mammalian cells, we metabolically labeled WI-38 human diploid fibroblast cells and NIH3T3 mouse fibroblast cells with \(^{32}P\)orthophosphate or \(^{35}S\)methionine. Cell lysates were immunoprecipitated with CDC34 antibodies and analyzed by SDS-PAGE and autoradiography. The results show that CDC34 is readily phosphorylated in mammalian cells (Fig. 1A, lanes 3 and 7). To further characterize the in vivo phosphorylation status of mammalian CDC34, NIH3T3 cells were synchronized by serum deprivation followed by serum stimulation and metabolic pulse labeling with \(^{35}S\)methionine or \(^{32}P\)orthophosphate. In parallel, synchronized cells were incubated with BrdUrd to measure the incorporation of nucleotides following the addition of serum to determine the time of DNA replication onset. Cell lysates were immunoprecipitated with CDC34 antibodies and analyzed by SDS-PAGE and autoradiography.

The results indicate that the cells entered S phase between 13 to 16 h following serum stimulation, with the majority of cells undergoing DNA replication during the period 16–19 h post-serum stimulation (Fig. 1B). CDC34 immunoprecipitated from \(^{35}S\)methionine-labeled cell extracts revealed that the steady-state level of CDC34 protein varied no more than 2-fold between quiescent cells (Fig. 1B, right upper panel, lane 1) and serum-stimulated cells (Fig. 1B, right upper panel, lanes 2–4). Immunoprecipitation of CDC34 from \(^{32}P\)orthophosphate-labeled extracts indicates that CDC34 is not appreciably phosphorylated in quiescent cells but is readily phosphorylated within 6 h following serum stimulation and remains phosphorylated at all time points during the cell cycle (Fig. 1B, right lower panel). These results indicate that CDC34 is readily phosphorylated in proliferating cells but not highly phosphorylated in quiescent cells.

Isolation of Human Casein Kinase 2 Regulatory Subunit as a CDC34-Interacting Protein by Two-hybrid Screening—To identify potential regulators of CDC34, we performed a yeast two-hybrid screen (37). Full-length hCDC34 was cloned into plasmid pBTM116, resulting in the expression of a fusion protein between the activation domain of Lex A and hCDC34. This chimera was used to screen a normal human adult lung plasmid library expressing GAL4 DNA binding domain fusion proteins. Three strong positives were identified, all encoding the full-length hCK2\(\alpha\) regulatory subunit. CK2 functions as a heterotetramer comprised of \(\alpha\) and \(\alpha'\) forming the catalytic subunit and 2\(\beta\) subunits forming the regulatory subunit, which is primarily responsible for substrate binding (30, 31). This result suggests that CDC34 is a potential substrate of CK2 or that CK2\(\beta\) may be a potential substrate of CDC34.

CDC34 and CK2\(\beta\) Interact in Vitro and in Vivo in Transiently Transfected Human Cells—To determine whether CK2\(\beta\) can interact directly with CDC34, we performed an in vitro co-immunoprecipitation assay using recombinant hCDC34 and in vitro translated hCK2\(\beta\). The results show that hCK2\(\beta\) could be specifically co-immunoprecipitated with hCDC34, suggesting that these proteins interact in vitro (Fig. 2A). A reciprocal co-immunoprecipitation experiment using in vitro translated hCDC34 and recombinant hCK2\(\beta\) also resulted in specific co-immunoprecipitation of the two proteins (data not shown).

Efforts to identify the interacting domains of hCK2\(\beta\) and CDC34 by in vitro co-immunoprecipitation, indicated that the carboxyl terminus of CK2\(\beta\) was dispensable for CDC34 binding while domains within the central region of CDC34 appeared to be required for CK2\(\beta\) binding (data not shown).

To examine whether CDC34 and CK2\(\beta\) can interact in vivo, 293 cells were transfected with HA-tagged hCDC34 and FLAG-tagged CK2\(\beta\) followed by immunoprecipitation with FLAG antibodies and immunoblot analysis with CDC34 antibodies. The result shows that both HA-hCDC34 and endogenous CDC34 co-precipitate with FLAG-hCK2\(\beta\) (Fig. 2B). Western analysis of the transfected cell lysate showed two CDC34-immunoreactive bands representing HA-tagged and endogenous CDC34 (Fig. 2B). The reciprocal experiment was not possible, because the electrophoretic migration of FLAG-hCK2\(\beta\) is coincident with...
mass markers (\(M\)) are as indicated in kilodaltons.

Malian expression plasmids encoding FLAG-tagged human CK2/\(\beta\) was in vitro translated with \(^{[\text{35}S]}\)methionine and incubated with bacterially expressed human CDC34. The samples were then immunoprecipitated with normal rabbit serum (NRS) or CDC34 antibody (α-CDC34). Lane 3 shows 2.5% of the input CK2/\(\beta\) protein used in the reaction (2.5% INPUT). Quantitation was performed by PhosphorImager analysis and is shown as the percentage of the total input in vitro translated CK2/\(\beta\) immunoprecipitated (% INPUT IP). Molecular mass markers (M) are as indicated in kilodaltons. B, human 293 cells were co-transfected with mammalian expression plasmids encoding FLAG-tagged human CK2/\(\beta\) (FLAG–CK2/\(\beta\)) and HA-tagged human CDC34. Equivalent amounts of total protein were immunoprecipitated from transfected cell lysates (IP: FLAG–CK2/\(\beta\)) with anti-FLAG (α-FLAG Ig) or nonspecific mouse immunoglobulin followed by Western blot analysis (WESTERN) with CDC34 antibody (α-CDC34) (lanes 2 and 3). Lane 1 shows the total cell lysate analyzed by Western blotting alone. The arrows indicate the protein bands corresponding to endogenous CDC34 (dashed arrow) and HA-tagged human CDC34 (solid arrow). Molecular mass markers (M) are as indicated in kilodaltons.

that of the immunoglobulin light chain. Transiently expressed hCK2/\(\beta\) was observed to associate with endogenous CDC34 in the presence or absence of transiently co-expressed human CK2α (data not shown). These results demonstrate that CDC34 and CK2/\(\beta\) interact both in vitro and in vivo in transiently transfected cells.

**Human CDC34 Is Specifically Phosphorylated in Vitro by Recombinant Human CK2**—To test whether CDC34 may be a substrate of CK2, recombinant human CK2 was tested for its ability to phosphorylate recombinant CDC34 in vitro. To determine whether the phosphorylation of CDC34 was specific, several other proteins not previously shown to be CK2 substrates were tested in parallel for CK2 phosphorylation. Among those proteins tested for CK2 phosphorylation, including bovine serum albumin (BSA), lysozyme, chicken serum albumin, rabbit immunoglobulin, histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, and catalase, only CDC34 was specifically phosphorylated by CK2 (Fig. 3A, upper panel, lane 1). Importantly, human RAD6, a ubiquitin-conjugating enzyme closely related to CDC34 by amino acid sequence, is not phosphorylated by CK2 (Fig. 3B, left panel, lane 2). Protein bands corresponding to the α- and β-subunits of CK2 were also observed to be phosphorylated in every sample due to the autophosphorylation activity exhibited by CK2 (43). Coomassie Blue staining of the gels showed that the proteins tested were present at quantities similar to CDC34 (Fig. 3A, lower panel, and Fig. 3B, left panel). These results show that CDC34 is phosphorylated by CK2 in vitro and suggests that CDC34 may be a bona fide substrate of CK2.

These results do not exclude the possibility that the interaction between CDC34 and CK2/\(\beta\) also may indicate that CK2/\(\beta\) is a substrate of CDC34, although CDC34 is not generally believed to directly bind to its substrates. To address whether CK2/\(\beta\) may be polyubiquitinated and proteolyzed in a CDC34-dependent manner, we measured the stability of \(^{[\text{35}S]}\)methionine in vitro translated Xenopus CK2/\(\beta\) in control- or CDC34-immunodepleted Xenopus interphase egg extract. Removal of CDC34 from egg extract was evaluated by immunoblotting with CDC34 antibody and by confirming the loss of DNA replication activity in extracts after CDC34 immunodepletion as described previously (7) (data not shown). We observed little turnover of CK2/\(\beta\) and no difference in the stability of CK2/\(\beta\) in control- or CDC34-depleted extract suggesting CK2/\(\beta\) is not targeted for CDC34-dependent proteolysis in Xenopus interphase extract (data not shown). However, this result does not eliminate the possibility that CK2/\(\beta\) may be a substrate of CDC34 in a different context or that putative CDC34-dependent ubiquitination of CK2/\(\beta\) may not result in proteolysis.

**The in Vitro Phosphorylation of CDC34 by Recombinant CK2 and by Cell Extracts Is Inhibited by Heparin, a Substrate-specific Inhibitor of CK2—**CK2 is characterized by its sensitivity to low concentrations of the glycosaminoglycan, heparin, although the critical inhibitory concentration of heparin can vary depending on the acidic nature of the specific substrate (30, 44). We tested the phosphorylation of CDC34 by recombinant CK2 in the presence of heparin and observed that 50% inhibition of CDC34 phosphorylation is achieved at a concentration of 2.5–3 nM heparin (Fig. 4A). Coomassie Blue staining of the gel showed that hCDC34 was equivalent in each reaction (data not shown). This result indicates that heparin is an inhibitor of CDC34 phosphorylation by CK2.

To determine whether CK2 is a predominant CDC34 kinase in vertebrate cells, we performed in vitro kinase assays using cell extracts and recombinant CDC34 with and without the addition of heparin. Previous studies have shown that CK2 is an abundant kinase in mammalian extracts, particularly nuclear extracts (30, 45). Following the kinase reaction, the CDC34 protein was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The results show that CDC34 is phosphorylated by whole cell extracts from 10T/12 cells, nuclear extracts from HeLa cells, and *X. laevis* interphase egg extract (Fig. 4B). Between 40 and 70% of the observed phosphorylation of cell extracts was inhibited by heparin at a concentration of \(2 \mu M\). Taylor et al. (46) observed an inhibition of IκBα phosphorylation by CK2 in U937 cell extracts at a concentration of 10 μM heparin, while Lin et al. (45) observed a 76% inhibition of c-Jun phosphorylation at a concentration of 0.1 μM heparin using nuclear extracts from metallothionein-v-Sis-transformed NIH3T3 cells. Coomassie Blue staining of the gel indicated that equivalent amounts of CDC34 were evaluated for each cell extract sample (data not shown). These results indicate that CDC34 is phosphorylated in cell extracts predominantly by a heparin-sensitive kinase.

**The in Vitro CK2 Phosphorylation Sites of CDC34 Are Located Within the Carboxyl-terminal 36 Amino Acids of CDC34**—Human CDC34 contains a highly acidic tail domain within amino acids 200–236, including several potential CK2 phosphorylation sites (Fig. 5A). CK2 is a unique kinase in that it preferentially phosphorylates substrates containing the acidic amino acid residues glutamate and aspartate immediately downstream (+1 to +3) from the phosphoacceptor site (47, 48). The classic CK2 consensus site has been described as Ser*/Thr*/Asp*/Glu*/Val*/Sp*/P*/Y*P*/X*_{2–3}*. Where the asterisk indicates the phosphoacceptor serine or threonine, X represents any non-basic

![Figure 2](http://www.jbc.org/)
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A. A top panel (KINASE ASSAY): An in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons. B, left panel (KINASE ASSAY): An in vitro kinase assay was performed using recombinant CK2 and hCDC34 (CDC34), or human RAD6 (hRAD6). The arrows indicate the phosphorylated hCDC34 protein band, and the asterisks indicate autophosphorylated CK2 α and β bands. Right panel (COOMASSIE): Coomassie Blue staining of the kinase assay gel.

B. FIG. 3. CDC34 is a specific in vitro casein kinase 2 substrate. A, top panel (KINASE ASSAY): An in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons.

C. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant CK2 and CDC34 in the absence (lane 1) or the presence (lanes 2 and 3) of increasing concentrations of heparin. The arrow indicates the phosphorylated hCDC34 protein band, whereas the asterisks indicate the autophosphorylated CK2 α and β protein bands. The percentage phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the 0 mM heparin sample. Molecular mass markers (M) are as indicated in kilodaltons. B, cell lysates from mouse 10T-1/2 cells (10T-1/2), HeLa cell nuclear extract (HELA NE), or X. laevis S100 (XL-S100) were subjected to kinase assays with recombinant hCDC34 either without (lanes 1, 3, 5) or with 2 µM heparin (lanes 2, 4, 6). The arrow indicates the phosphorylated hCDC34 protein bands. The percent phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the phosphorylation without heparin for each sample. Molecular mass markers (M) are as indicated in kilodaltons.

D. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons.

E. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons. B, cell lysates from mouse 10T-1/2 cells (10T-1/2), HeLa cell nuclear extract (HELA NE), or X. laevis S100 (XL-S100) were subjected to kinase assays with recombinant hCDC34 either without (lanes 1, 3, 5) or with 2 µM heparin (lanes 2, 4, 6). The arrow indicates the phosphorylated hCDC34 protein bands. The percent phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the phosphorylation without heparin for each sample. Molecular mass markers (M) are as indicated in kilodaltons.

F. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons. B, cell lysates from mouse 10T-1/2 cells (10T-1/2), HeLa cell nuclear extract (HELA NE), or X. laevis S100 (XL-S100) were subjected to kinase assays with recombinant hCDC34 either without (lanes 1, 3, 5) or with 2 µM heparin (lanes 2, 4, 6). The arrow indicates the phosphorylated hCDC34 protein bands. The percent phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the phosphorylation without heparin for each sample. Molecular mass markers (M) are as indicated in kilodaltons.

G. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons.

H. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons.

I. FIG. 4. CDC34 in vitro phosphorylation by CK2 and cell extracts is inhibited by the CK2-specific inhibitor, heparin. A, in vitro kinase assay was performed using recombinant human CK2 and human CDC34 (hCDC34), bovine serum albumin (BSA), lysozyme, chicken serum albumin (CSA), rabbit immunoglobulin (RABBIT IG), histone H1, glutathione S-transferase (GST), ubiquitin, aldolase, or catalase. The arrows indicate the phosphorylated CK2 α, β, and hCDC34 protein bands. Bottom panel (COOMASSIE): The kinase assay gel was stained with Coomassie Blue to visualize the relative amounts of substrate proteins assayed. Molecular mass markers (M) are as indicated in kilodaltons.
Fig. 5. The residues of CDC34 phosphorylated in vitro by recombinant CK2 and HeLa cell-derived kinases are located within the CDC34 tail domain at residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236. A, the carboxyl-terminal amino acid sequence of human CDC34 from residues 190 to 236 indicating the location of putative CK2 phosphorylation sites in boldface. B, schematic representation of the phosphorylation sites in the CDC34 wild-type protein (WT), the point mutations of the CDC34 3 point mutant (3 PT MUT), the point mutations of the CDC34 5 point mutant (5 PT MUT), and the truncation mutation of the CDC34-(1–200) mutant (1–200). C, upper panel: An in vitro kinase assay was performed using recombinant CK2 (R2) and recombinant WT CDC34 (WT), a 1–200 truncation mutant of hCDC34-(1–200), a triple point mutant of CDC34 (S231A,T233A,S236A) (3 PT MUT), and a quintuple point mutant of CDC34 (S203A,S222A,S231A,T233A,S236A) (5 PT MUT). The arrow and asterisk show the phosphorylated hCDC34 and CK2β protein bands, respectively. Bottom panel: Coomassie Blue-stained gel of kinase assay. The solid and dashed arrows show full-length and a proteolytic fragment of CDC34-(1–200) protein, respectively. The percent phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the phosphorylation of the WT CDC34 protein (lane 1). D, an in vitro kinase assay was performed using HeLa nuclear extract (HELA NE) and recombinant CDC34 wild-type (WT), a 1–200 truncation mutant of hCDC34-(1–200), CDC34 3 PT MUT, and CDC34 5 PT MUT. The percent phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the phosphorylation of the WT CDC34 protein (lane 1).

and 4). Coomassie Blue staining of the proteins in Fig. 5D indicated that the CDC34 mutants were assayed at comparable levels (data not shown). These results demonstrate that kinases present in HeLa nuclear extract target only five carboxyl-terminal residues of CDC34 for phosphorylation.

Taken together, these results indicate that CDC34 is phosphorylated in vitro by both recombinant CK2 and HeLa nuclear extract-derived kinases at residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236 within the acidic carboxyl-terminal tail domain of CDC34. Furthermore, mutation of these sites results in the elimination of CDC34 phosphorylation.

CDC34 Immunopurified from Proliferating Mammalian Cells Is Associated with a Heparin-sensitive Kinase That Can Utilize GTP—To characterize the in vivo CDC34 kinase, CDC34 from mammalian cells was immunopurified and found to be associated with a kinase that could phosphorylate CDC34. Affinity-purified CDC34 antibody was used to immunoprecipitate endogenous CDC34 from cells. Specific anti-CDC34 immunoprecipitates or non-specific rabbit immunoglobulin immunoprecipitates were then subjected to in vitro kinase assays followed by re-immunoprecipitation with CDC34 antibody. Results show that CDC34 from proliferating cells, but not quiescent cells, is associated with a kinase activity that phosphorylates CDC34 (Fig. 6A, left panel, lanes 1 and 2). Western analysis of the cell extracts indicated that the steady-state level of CDC34 in cycling and quiescent cells was similar (Fig. 6A, right panel, lanes 5 and 6).

To determine whether the CDC34-associated kinase exhibits attributes that are hallmarks of CK2, cell lysates from 10T-1/2 cells were immunoprecipitated and kinase assays were performed in the presence of heparin (30, 44). The results show that the CDC34-associated kinase is sensitive to low concentrations of heparin with 50% inhibition achieved at 0.2 μg heparin (Fig. 6B, lane 3). Other kinase inhibitors were also tested for their ability to inhibit the CDC34-associated kinase. Staurosporine, an inhibitor of protein kinase C and cyclin-dependent kinases (49), and wortmannin, an inhibitor of phosphatidylinositol-3-kinases (50) did not inhibit the phosphorylation of CDC34 by the associated kinase compared with heparin (Fig. 6C). A further distinction of CK2 is that it can utilize both ATP and GTP as a phosphate donor (30). Immunoprecipitation and kinase assays were performed, and the results indicate that the CDC34-associated kinase can phosphorylate CDC34 using either ATP or GTP (data not shown). Taken together, these results suggest that, in vivo, CDC34 is tightly associated with a kinase in proliferating mammalian cells that bears all the characteristics of CK2. This is consistent with previous findings that demonstrate an increased level of CK2 activity in actively proliferating cells versus quiescent cells (31, 51).

To identify the specific residues of CDC34 phosphorylated by the CDC34-associated kinase, CDC34 mutants were expressed in cells, immunopurified, and subjected to in vitro kinase assays. Versions of CDC34 WT, 1–200, 3 PT MUT (S231A,T233A, S236A), 4 PT MUT (S203A,S231A,T233A,S236A), and 5 PT MUT (S203A,S222A,S231A,T233A,S236A) tagged with the
influenza hemagglutinin (HA) epitope were transiently expressed in 10T-1/2 cells. Cell lysates were immunoprecipitated with antibody (CDC34 Ig) or rabbit immunoglobulin (Rig) from cell lysates derived from proliferating (Cyc) or serum starved (Quiesc) NIH3T3 cells, and kinase assays were performed on the immunoprecipitates. Right panel (WESTERN). Cell lysates were analyzed in parallel by Western blotting using CDC34 antibody. B, cell lysates from exponentially growing 10T-1/2 cells were immunoprecipitated with CDC34 antibody (CDC34 Ig) or rabbit immunoglobulin (Rig) and subjected to kinase assay without (lanes 1 and 2) or with 0.2 μM (lane 3) or 2.0 μM heparin (lane 4). The percent phosphorylation (% REL PHOS) was determined by Phosphorimagery analysis and was normalized relative to the phosphorylation of CDC34 in the absence of heparin (lane 2). Molecular mass markers (M) are as indicated in kilodaltons. C, cell lysates from exponentially growing 10T-1/2 cells were immunoprecipitated with CDC34 antibody, and kinase assays were performed on the immunoprecipitates with buffer (None) or kinase inhibitors heparin, staurosporine (StauRo), and wortmannin (WortM) at the indicated micromolar concentrations (CONC). D, left panel (IP-KINASE): HA-tagged CDC34 wild-type (WT) and mutations were transiently expressed in 10T-1/2 cells. The CDC34 mutants analyzed were the 3 PT MUT (S251A,T233A,S236A), a quadruple point mutant (S203A,S251A, T233A,S236A) (4 PT MUT), the 5 PT MUT (S203A,S222A,S251A, T233A,S236A), and a 1–200 truncation mutant of hCDC34 (1–200). Cell lysates were immunoprecipitated with anti-HA antibody (12CA5) or with nonspecific normal mouse ascites (AsCites). The samples were then subjected to a kinase assay. The percent phosphorylation (% REL PHOS) was determined by Phosphorimagery analysis and was normalized relative to the phosphorylation of the WT CDC34 protein (lane 2). Right panel (WESTERN): In parallel, transfected cell lysates were analyzed by Western blotting with 12CA5 ascites.

Fig. 6. CDC34 immunopurified from mammalian cells is associated with a CK2-like kinase that is sensitive to heparin. A, left panel (IP-KINASE): CDC34 was immunoprecipitated with CDC34 antibody (CDC34 Ig) or nonspecific rabbit immunoglobulin (Rig) from cell lysates derived from proliferating (Cyc) or serum starved (Quiesc) NIH3T3 cells, and kinase assays were performed on the immunoprecipitates. Right panel (WESTERN). Cell lysates were analyzed in parallel by Western blotting using CDC34 antibody. B, cell lysates from exponentially growing 10T-1/2 cells were immunoprecipitated with CDC34 antibody (CDC34 Ig) or rabbit immunoglobulin (Rig) and subjected to kinase assay without (lanes 1 and 2) or with 0.2 μM (lane 3) or 2.0 μM heparin (lane 4). The percent phosphorylation (% REL PHOS) was determined by Phosphorimagery analysis and was normalized relative to the phosphorylation of CDC34 in the absence of heparin (lane 2). Molecular mass markers (M) are as indicated in kilodaltons. C, cell lysates from exponentially growing 10T-1/2 cells were immunoprecipitated with CDC34 antibody, and kinase assays were performed on the immunoprecipitates with buffer (None) or kinase inhibitors heparin, staurosporine (StauRo), and wortmannin (WortM) at the indicated micromolar concentrations (CONC). D, left panel (IP-KINASE): HA-tagged CDC34 wild-type (WT) and mutations were transiently expressed in 10T-1/2 cells. The CDC34 mutants analyzed were the 3 PT MUT (S251A,T233A,S236A), a quadruple point mutant (S203A,S251A, T233A,S236A) (4 PT MUT), the 5 PT MUT (S203A,S222A,S251A, T233A,S236A), and a 1–200 truncation mutant of hCDC34 (1–200). Cell lysates were immunoprecipitated with anti-HA antibody (12CA5) or with nonspecific normal mouse ascites (AsCites). The samples were then subjected to a kinase assay. The percent phosphorylation (% REL PHOS) was determined by Phosphorimagery analysis and was normalized relative to the phosphorylation of the WT CDC34 protein (lane 2). Right panel (WESTERN): In parallel, transfected cell lysates were analyzed by Western blotting with 12CA5 ascites.

influenza hemagglutinin (HA) epitope were transiently expressed in 10T-1/2 cells. Cell lysates were immunoprecipitated with 12CA5 antibody, subjected to kinase assays, and re-immunoprecipitated. Western blot analysis of the cell lysates indicated that similar amounts of HA-tagged CDC34 proteins were analyzed (Fig. 6E, right panel). The results show that the CDC34 4 PT MUT exhibits a small decrease in CDC34 phosphorylation, whereas phosphorylation of the CDC34 5 PT MUT and 1–200 is reduced to background levels (Fig. 6E, left panel, lanes 1, 5, and 6). These studies demonstrate that a kinase, which exhibits the characteristics of CK2, associates with CDC34 in proliferating mammalian cells and phosphorylates CDC34 predominantly at residues Ser-203 and Ser-222. Mutation of CDC34 residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236 abolishes the phosphorylation of CDC34 by the associated kinase.

In Vivo Phosphorylation of CDC34 Maps to Five Carboxyl-terminal CK2 Consensus Sites at Residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236—Our studies suggest that re-combiant CK2, HeLa cell-derived kinases, and a CK2-like CDC34-associated kinase all phosphorylate CDC34 in vitro within the acidic carboxyl-terminal tail of CDC34 at five residues (Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236). To identify the in vivo CDC34 phosphorylation sites, 10T-1/2 cells were transiently transfected with HA-tagged versions of CDC34 WT, 5 PT MUT, and 1–200 followed by metabolic labeling with [32P]orthophosphate and immunoprecipitation with 12CA5 antibody. Labeling of untransfected cells indicates that endogenous CDC34 in 10T-1/2 cells is readily phosphorylated (Fig. 7A, lane 2). Immunoprecipitation of equivalent tri-chloroacetic acid-precipitable counts with 12CA5 antibody demonstrates that, although HA-CDC34 WT was readily phosphorylated, the in vivo phosphorylation of HA-5 PT MUT and HA-1–200 was severely reduced to near background levels (Fig. 7A, right panel, lanes 4, 6, and 8). Western analysis of lysates from unlabeled cells transfected in parallel, indicated that HA-tagged WT, 5 PT MUT, and 1–200 CDC34 proteins were expressed at equivalent levels (Fig. 7B). These results demonstrate that mutation of CDC34 residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236 abolishes the phosphorylation of CDC34 in vivo and suggest that phosphorylation of CDC34 in vivo is mediated by CK2.

One trivial explanation for the lack of phosphorylation observed for CDC34 mutants 1–200 and 5 PT MUT is that these mutants no longer efficiently bind to CK2β. To determine whether CK2β can bind effectively to CDC34-(1–200) and 5 PT MUT in vivo, 293 cells were transiently transfected with FLAG-tagged human CK2β and CDC34 WT, 1–200, or 5 PT MUT. An immunoprecipitation with anti-FLAG antibody was performed on the cell lysates followed by Western blot analysis using anti-CDC34 antibody. The results show that CDC34-(1–200) and CDC34 5 PT MUT are efficiently co-precipitated by CK2β in mammalian cells, indicating that the lack of observed CDC34-(1–200) or 5 PT MUT phosphorylation is due to removal or mutation of the target phosphorylation sites (Fig. 7C). Direct Western analyses of the transfected cell lysates indicated that the expression of CDC34-(1–200) was ~2-fold lower than the WT CDC34, whereas the expression of WT and 5 PT MUT proteins was similar (data not shown).

Mutation of CDC34 Residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236 Results in an Altered Localization of Nuclear CDC34 to the Cytoplasm in Transiently Transfected Osteosarcoma Cells—Reports have demonstrated that CK2-mediated phosphorylation plays a role in regulating the subcellular localization of substrate proteins (30, 52). The phosphorylation of large T antigen of SV-40 at serine residues 111 and 112 by CK2 results in a significantly faster rate of large T antigen nuclear uptake (52). Interestingly, Reymond et al. (53) have reported that a truncation mutant of human CDC34-(1–200) results in a strikingly altered subcellular localization in U2OS human osteosarcoma cells from one that is predominantly nuclear to one that is predominantly cytoplasmic. These authors conclude that the carboxyl-terminal tail of CDC34 mediates its nuclear localization (53). In an effort to understand the biological significance of CDC34 phosphorylation by CK2, we transiently
transfected HA-tagged CDC34 mutants into U2OS cells and studied the subcellular localization of the CDC34 mutants. The endogenous CDC34 in U2OS cells localized to the nucleus (Fig. 8A) as has been previously reported (53, 54). Transiently transfected U2OS cells were fixed, permeabilized, and stained with 12CA5 antibody and a Cyanin-3-coupled secondary antibody for immunofluorescence. HA-tagged WT CDC34 localized essentially as endogenous CDC34 to the nucleus (Fig. 8B), whereas the HA-(1–200) mutant localized significantly more cytoplasmically (Fig. 8C). The HA-5 PT MUT exhibited a localization that was noticeably more cytoplasmic, similar to that of the CDC34-(1–200) mutant (Fig. 8D). In five independent experiments, we observed the localization of HA-tagged CDC34 WT to be nuclear with only −0.2% of the transfected cells exhibiting a more cytoplasmic CDC34 localization. By contrast, 40–80% of the cells transfected with CDC34-(1–200) and 5 PT MUT exhibited a more cytoplasmic CDC34 subcellular localization. These studies suggest that the subcellular localization of CDC34 may be influenced by specific CK2 phosphorylation of CDC34 within its tail domain.

**DISCUSSION**

We have shown that in proliferating mammalian cells, CDC34 is a phosphoprotein that is phosphorylated in vivo within its carboxy-terminal tail domain. We also identify the regulatory subunit of human CK2 as a novel CDC34-interacting protein and show that CDC34 and CK2β interact in both in vitro and in vivo. Our studies indicate that CDC34 is a specific substrate of recombinant CK2, a CK2-like kinase present in HeLa cell nuclear extract, and a CK2-like kinase associated with CDC34 in proliferating mammalian cells. Significantly, the phosphorylation of CDC34 by each of the aforementioned kinases is inhibited by the CK2-specific inhibitor, heparin. The phosphorylation of CDC34 by recombinant CK2, the CK2-like kinase present in HeLa cell nuclear extract, and the CDC34-associated kinase can be mapped to five sites (Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236) resulting in an altered localization of nuclear CDC34 to the cytoplasm. Immunofluorescence analysis of human U2OS osteosarcoma cells expressing transiently transfected HA-tagged wild-type and mutant human CDC34 proteins. U2OS cells were either not transfected (A: ENDO hCDC34) or transfected with HA-tagged WT CDC34 (B: HA-CDC34 WT), HA-tagged CDC34-(1–200) truncation mutant (C: HA-CDC34-(1–200)), and HA-tagged quintuple CDC34 point mutant (S203A,S222A,S231A,T233A,S236A) (D: HA-CDC34 5 PT MUT). Cells were stained with CDC34 antibody to stain endogenous CDC34 protein (ENDO hCDC34) or with 12CA5 antibody. Cells were then stained with a donkey anti-rabbit or anti-mouse Cyanin-3 secondary antibody (DAPI/M-CY3, left panels) and Hoechst 33342 (HOECHST, right panels) followed by fluorescence microscopy.

**FIG. 7.** Orthophosphate labeling indicates all in vivo CDC34 phosphorylation sites map to the carboxy-terminal 36 amino acids of CDC34. A, left panel. 32P labeling of endogenous CDC34 (ENDO). Exponentially growing 10T-1/2 cells were metabolically labeled with [32P]orthophosphate, and equivalent trichloroacetic acid-precipitable counts were immunoprecipitated with normal rabbit serum (NRS) or CDC34 antiserum (α-CDC34). The asterisk demarcates the endogenous CDC34 protein band. Right panel: 10T-1/2 cells were transiently transfected with HA-tagged WT CDC34 (HA-WT), HA-tagged CDC34 5 PT MUT (HA-5 PT MUT), or HA-tagged CDC34-(1–200) truncation mutant (HA-1–200). Cell lysates at equivalent trichloroacetic acid-precipitable counts were immunoprecipitated with anti-HA ascites (12CA5) or nonspecific mouse ascites (MA). The percent phosphorylation (% REL PHOS) was determined by PhosphorImager analysis and was normalized relative to the phosphorylation of the WT CDC34 protein (lane 4). The HA-WT and 5 PT MUT protein bands are indicated by the solid arrow, and the HA-1–200 protein band is indicated by the dashed arrow. Molecular mass markers (M) are as indicated in kilodaltons. B, Western analysis was performed in parallel on cells transfected in A using 12CA5 ascites. C, CDC34 wild-type and mutants interact in vivo with casein kinase 2β. 293 cells were transiently transfected with FLAG-tagged human CK2β, WT human CDC34 (WT), and either a 1–200 truncation mutant of human CDC34-(1–200) (left panel, lane 3) or the CDC34 5 PT MUT (S203A,S222A,S231A,T233A,S236A) (right panel, lane 5). Cell lysates were immunoprecipitated (IP: FLAG-CK2β) with anti-FLAG (α-FLAG) or nonspecific mouse (MIg) immunoglobulin and analyzed by Western blotting with CDC34 antibody (WESTERN: α-CDC34). The solid arrow indicates the WT and 5 PT MUT CDC4 protein bands, the dashed arrow indicates the human CDC34-(1–200) truncation mutant protein band, and the asterisks indicate the immunoglobulin light-chain protein bands. Molecular mass markers (M) are as shown in kilodaltons.

**FIG. 8.** Mutation of CDC34 residues Ser-203, Ser-222, Ser-231, Thr-233, and Ser-236 result in an altered localization of nuclear CDC34 to the cytoplasm. Immunofluorescence analysis of human U2OS osteosarcoma cells expressing transiently transfected HA-tagged wild-type and mutant human CDC34 proteins. U2OS cells were either not transfected (A: ENDO hCDC34) or transfected with HA-tagged WT CDC34 (B: HA-CDC34 WT), HA-tagged CDC34-(1–200) truncation mutant (C: HA-CDC34-(1–200)), and HA-tagged quintuple CDC34 point mutant (S203A,S222A,S231A,T233A,S236A) (D: HA-CDC34 5 PT MUT). Cells were stained with CDC34 antibody to stain endogenous CDC34 protein (ENDO hCDC34) or with 12CA5 antibody. Cells were then stained with a donkey anti-rabbit or anti-mouse Cyanin-3 secondary antibody (DAPI/M-CY3, left panels) and Hoechst 33342 (HOECHST, right panels) followed by fluorescence microscopy.
An important specificity determinant for CK2 phosphorylation is that physiologically relevant CK2 sites are commonly found embedded within acidic residues. The phosphorylation sites of CDC34 are embedded within highly acidic residues and do not resemble the consensus sites for any other kinases with the exception of casein kinase 1 (CK1) (47). Although all five phosphorylated residues within the tail domain of CDC34 do match consensus sites designated for CK1, we believe it unlikely that these residues are targeted by CK1 for the following reasons. First, the kinase that is associated with and phosphorylates CDC34 from mammalian cells is sensitive to heparin and can utilize GTP as a phosphate donor, both firmly established characteristics unique to CK2, but not CK1. Second, the CK2 kinase present in vertebrate cell extracts is sensitive to heparin, again implying the phosphorylation of CDC34 observed in cells is mediated by CK2, rather than CK1.

Our results indicate that the CDC34 5 PT MUT is devoid of phosphorylation in vivo and exhibits an altered subcellular localization from the nucleus to the cytoplasm, suggesting unphosphorylated CDC34 is not as efficiently transported or relocalized from the nucleus to the cytoplasm, suggesting unphosphorylation in vivo. We postulate that the phosphorylation of CDC34 may result in its more efficient localization to the nucleus in a manner similar to the SV-40 large T antigen, CDC34 may result in its more efficient localization to the nucleus. We postulate that the phosphorylation of CDC34 kinase present in vertebrate cell extracts is sensitive to heparin, again implying the phosphorylation of CDC34 observed in cells is mediated by CK2, rather than CK1.

Although CDC34 is localized predominantly to the nucleus in mammalian cells (53, 54), the mechanism by which CDC34 is transported to and retained in the nucleus has not been determined. CDC34 does not appear to contain a canonical basic nuclear localization sequence, although deletion analyses and immunofluorescence in U2OS cells suggest that the localization of CDC34 to the nucleus is somehow mediated by the carboxy-terminal 36 amino acids of CDC34 (53). CDC34 may contain a non-canonical nuclear localization sequence within its carboxy-terminal tail domain through which it binds importin, or it may localize to the nucleus through the binding of other nuclear localized proteins. The subcellular localization of CK2 has been reported to be not only cytosolic and nuclear, but also membrane-bound, mitochondrial, cytoskeletal, nuclear, nucleosomal, and centrosomal (56). We speculate that phosphorylation of CDC34 may alter its conformation, increasing the efficiency of CDC34 nuclear import either through a more stable interaction with importin or with another protein. Alternatively, the phosphorylation of CDC34 may result in its more efficient nuclear retention through the binding of a nuclear protein.

Our preliminary studies indicate that the acidic tail domain of CDC34 and presumably the phosphorylation of residues within the CDC34 tail domain do not appear to be absolutely required for the association of CDC34 with p45Skp2 or Roc1 in transiently transfected cells. Presently, the subcellular localization of characterized SCF components has been reported to be predominantly nuclear like that of CDC34 (21, 54), whereas CDC34/SCF substrates and putative substrates have been localized to both the nucleus and the cytoplasm (5, 57–59). How the subcellular localization of CDC34 and SCF components may modulate specific substrate turnover is presently unclear.

Biochemical fractionation studies of X. laevis interphase egg extracts indicate that the CDC34, which functions in the initiation of DNA replication, is present in a large molecular size complex (7). Whether CDC34 in mammalian cells is present in many multiprotein complexes that are functionally diverse and whether such complexes contain CDC34 and SCF components and/or as yet unidentified components is still undetermined. In vivo studies examining the endogenous associations of CDC34 with CK2 and/or SCF complexes may reveal whether the association of CDC34 with CK2 may itself also regulate CDC34/SCF function.

Because the biological function of CDC34 in cells is diverse, it is not immediately apparent what function of CDC34 might be influenced by putative CK2 phosphorylation. We observed CDC34 to be phosphorylated by CK2 predominantly in proliferating cells. The putative regulation of CDC34 phosphorylation by CK2 in quiescent versus proliferating cells may indicate a role for CK2 phosphorylation in modulating CDC34 activity during cell cycle entry and exit. In mammalian cells, CK2 has also been shown to be required for cell cycle progression (34), suggesting CK2 phosphorylation of CDC34 may modify the required function of CDC34 in the onset of DNA replication. In vivo, this would imply that the phosphorylation state of CDC34 might influence the ubiquitination of G1 or S phase CDC34 substrates. The ubiquitination of mammalian p27Kip1 has been reconstituted using only bacterially expressed recombinant human proteins, including CDC34, indicating that the phosphorylation of CDC34 is not an absolute requirement for p27Kip1 ubiquitination (15). However, whether post-translational modifications of CDC34 may increase the activity of CDC34 in vitro or in vivo is unclear. Alternatively, if post-translational modification of CDC34 plays a role in modulating the cell localization of specific pools of CDC34 in association with other proteins, then an in vitro assay could not recapitulate the regulation that would be imposed in vivo due to compartmentalization. In future studies, it will be important to address the role of CDC34 phosphorylation in the regulation of S phase entry during the mammalian cell cycle.

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