Phosphorylation of Human CDC25B Phosphatase by CDK1-Cyclin A Triggers Its Protease-dependent Degradation*

(Received for publication, October 10, 1997, and in revised form, October 29, 1997)

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In eukaryotes the activity of CDK1 (CDC2), a cyclin-dependent kinase that initiates the structural changes that culminate in the segregation of chromosomes at mitosis, is regulated by the synergistic and opposing activities of a cascade of kinases and phosphatases. Dephosphorylation of threonine 14 and tyrosine 15 of CDK1 by the CDC25 phosphatases is a key step in the activation of the CDK1-cyclin B protein kinase. Little is currently known about the role and the regulation of CDC25B. Here we report in vitro and in vivo data that indicate that CDC25B is degraded by the proteasome. This degradation is dependent upon phosphorylation by the CDK1-cyclin A complex but not by CDK1-cyclin B. These results indicate that CDK1-cyclin A phosphorylation targets CDC25B for degradation and that this might be an important component of cell cycle regulation at the G2/M transition.

The eukaryotic cell cycle is controlled by a family of cyclin-dependent kinases that regulate its key transitions. The precise timing of the activation of these enzymes is a central issue of cell cycle regulation. A major regulatory mechanism is provided by the wee1/mik1- and myt1-dependent phosphorylation on threonine 14 and tyrosine 15 of CDK1 (cdc2). This inhibitory phosphorylation keeps the kinase inactive until it is dephosphorylated by the dual specificity CDC25 phosphatase at the G2/M transition (1). In human cells, three homologues of CDC25C, CDC25B, and CDC25C have been identified (2–4). In HeLa cells, expression of CDC25B is low throughout the cell cycle with an increase in G2, CDC25B is predominantly expressed in G2 (2,3), and CDC25A is abundant both at the mRNA and protein levels in late G1 (5). Phosphorylated CDC25C by CDK1-cyclin B was shown and proposed to be part of the self-amplification mechanism of CDC1-cyclin B at mitosis (6). CDK2-cyclin E-dependent phosphorylation of CDC25A was also demonstrated, indicating that a similar feedback loop might regulate the progression in S phase (7). The mechanisms that regulate CDC25B activity and the precise role of that phosphatase and its splicing variants (8) in the control of entry into mitosis remain unclear.

Here we report in vitro and in vivo evidences indicating that CDC25B is degraded by the proteasome and that this process is dependent on the phosphorylation by the CDK1-cyclin A kinase. We propose that the rapid degradation of CDC25B is an important regulatory mechanism that ensures the timely coordinated activation of CDK-cyclin complexes.

MATERIALS AND METHODS

Production of Recombinant Proteins—The CDC25B coding sequence corresponding to the B1 splicing variant (8) was cloned in the pET14b (Novagen) vector. In vitro transcription and translation were performed using the TNT system (Promega) in the presence or the absence of [35S]methionine. S9 insect cells were co-infected with recombinant baculovirus encoding for human CDK1 (CDC2) or CDK2 and cyclin B or cyclin A. Insect cell extracts were prepared as follows: cells were lysed with lysis buffer (10 mM Tris, pH 7.5, 25 mM NaCl, 50 mM NaF, 0.1 mM sodium orthovanadate) in a Dounce homogenizer, then diluted into 4 volumes of solubilization buffer (25 mM NaF, pH 7.5, 250 mM NaCl, 10% glycerol, 0.02% Tween 20), and centrifuged at 100,000 × g for 1 h at 4 °C. The supernatants were assayed for their specific activity toward histone H1. Recombinant GST-p21cIpl was produced and purified as described previously (22).

Cell Culture—HeLa cells and human lung primary fibroblasts IMR90 were cultured as already described (23) and treated for 18 h with 250 and 50 ng/ml of nocodazole respectively. Cells were harvested and lysed as described (23).

Phosphorylation and Degradation Assay—To assay the phosphorylation of CDC25B, in vitro translated protein was immunoprecipitated with an anti-CDC25B polyclonal antibody directed against recombinant CDC25B protein and incubated either with 15 μg of cellular extract or with recombinant cyclin-dependent kinase extract (3 pmol/min/mg of histone H1/μl lysis) in kinase assay buffer (6) containing 5 μCi of [γ-32P]ATP for 60 min at 30 °C. The precipitates were subjected to SDS-PAGE 8% electrophoresis. When CDC25B phosphorylation and/or level was monitored by the change in electrophoretic mobility, the assay was performed with 1 mM cold ATP, and the samples were analyzed without immunoprecipitation.

Human primary fibroblasts IMR90 were treated for 2 and 4 h with 50 μM LLaL or with the same concentration of Me2SO as described (10). Cell extracts were subjected to Western blot analysis using polyclonal anti-CDC25B antibodies. Extracts from S9 insect cells expressing recombinant CDK1, cyclin A, and CDC25B were prepared as described in the case of IMR90 cells (23) and subjected to Western blot analysis. All Enhanced ChemiFluorescence (ECF) and radioactive isotope detection and quantification were performed using a STORM 840 image (Molecular Dynamics). Digitalized images were used for artwork.

Chemicals—FSBA, LLnL, LLM, MG132, lactacystin, chloroquine, and E64D were solubilized in Me2SO.

RESULTS AND DISCUSSION

Phosphorylation of CDC25B—To analyze the phosphorylation of CDC25B, we produced this protein by in vitro translation in rabbit reticulocyte lysate. As detected both by 32P incorporation (Fig. 1a, upper panel) or by change in electrophoretic mobility of the [35S]methionine-labeled protein (Fig. 1a, lower panel), CDC25B was readily phosphorylated after incubation with cellular extracts from nocodazole-arrested human IMR90 primary fibroblasts and HeLa cells. We then determined the ability of different human CDK-cyclin com-

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* This work was supported by grants from the CNRS, l’Université Paul Sabatier, l’Association pour la Recherche sur le Cancer, and la Ligue contre le Cancer Comité de la Haute-Garonne. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: LLaL, N-acetyl-Leu-Leu-Nleucinal; FSBA, p-fluorosulfonylbenzoyl adenone; LLM, N-acetyl-Leu-Leu-g-thioninone; MG132, Z-Leu-Leu-Leu-CHO; E64D, (2S,3S)-trans-epoxy-succinyl-L-leucylamido-3-methyl butan ethanol ester; PAGE, polyacrylamide gel electrophoresis.
pleses produced in baculovirus co-infected Sf9 cells to phosphorylate CDC25B. Equal specific activities (determined by histone H1 phosphorylation) were used to assay the in vitro phosphorylation of CDC25B. As seen in Fig. 1b (upper panel), CDK1-cyclin B, CDK1-cyclin A, and CDK2-cyclin A complexes efficiently phosphorylated CDC25B, although CDK1-cyclin B led to higher \(^{32}P\) incorporation (1.5-fold greater than CDK1-cyclin A). The number and location of the residues phosphorylated by the three kinase complexes are probably different because distinct changes in electrophoretic mobility of the \(^{35}S\)-labeled CDC25B protein were observed (Fig. 1b, lower panel).

**Degradation of CDC25B**—When the calpain I inhibitor LLnL (9) was omitted from the reaction buffer, a rapid degradation of the CDC25B phosphatase was observed following incubation with CDK1-cyclin A (Fig. 2a). After 30 min of incubation about 50% of CDC25B was degraded (Fig. 2b). Control incubations with the same amount of control Sf9 cell lysate had little effect on CDC25B stability, as did incubation with CDK1-cyclin B. \(p27^{kip1}\), a cell cycle inhibitor that has been shown to be degraded by the proteasome (10–12), was unstable when incubated with all tested Sf9 cell lysates (Fig. 2c). By contrast, an unrelated control protein (i.e., luciferase) was not degraded upon incubation with CDK1-cyclin A (data not shown). When Sf9 cells were simultaneously co-infected with CDC25B, CDK1, and cyclin A encoding baculoviruses as shown in Fig. 3c (upper panel), a major 53-kDa CDC25B degradation product accumulated, similar to that is observed upon incubation of in vitro translated CDC25B with CDK1-cyclin A lysate (Fig. 4a). Together, these results indicate that the instability of CDC25B in both in vitro and in vivo assays reflects a specific degradation that is dependent upon the presence of the CDK1-cyclin A complex.

**Cdc25B Is Degraded by the Proteasome in Vitro and in Vivo**—Proteasome inhibitors (13) were used to investigate whether the proteasome pathway was involved in CDC25B degradation. First, as shown above, the presence of LLnL was necessary to avoid CDC25B degradation in vitro (Fig. 2a). Second, when proteasome inhibitors such as LLnL, MG132 (14) (Fig. 3a), or especially lactacystin (15) (Fig. 3b) were added to the phosphorylation assay, the degradation of CDC25B was inhibited but not the phosphorylation, and the protein was stabilized as its lower migrating phosphorylated form (Fig. 4a). Chloroquine, a lysosomal inhibitor, and E64D, a cysteine protease inhibitor, had no effect on CDC25B degradation (Fig. 3a). Similarly, LLM, an inhibitor of calpain II that is a less potent proteasome inhibitor, had a minor protective effect (Fig. 3a). Third, as shown in Fig. 3c (lower panel), in Sf9 insect cells that were simultaneously co-infected with CDC25B, CDK1, and cyclin A encoding baculoviruses, addition of lactacystin led to the inhibition of the degradation and the accumulation of CDC25B. Fourth, human primary fibroblasts IMR90 express only the CDC25B3 variant (8). CDC25B protein accumulated in fibroblasts treated for 2 and 4 h with 50 \(\mu M\) LLnL (Fig. 3d) or 30 \(\mu M\) lactacystin (not shown), although its level remained unchanged in mock cells treated with the same concentration of Me\(_2\)SO. Altogether, this set of in vitro and in vivo observations indicate that degradation of human CDC25B, as also suggested in fission yeast (16), is indeed dependent on the proteasome.
pathway.

**Phosphorylation of CDC25B Is Required for Degradation by the Proteasome**—Finally we assayed whether the degradation of CDC25B was dependent upon phosphorylation by CDK1-cyclin A. Both the ATP analogue FSBA (17) (Fig. 4a) or in the presence of increasing amounts of recombinant GST-p21Cip1 protein (b). The arrowheads indicate the CDC25B translational product, and the bracket indicates the phosphorylated forms. The major degradation product (53 kDa) is indicated with a asterisk. In c, in vitro translated CDC25B was incubated with uninfected SF9 cell lysates (Control) or CDK1-cyclin A cell lysates that were left alone or subjected to immunodepletion with anti-cyclin A polyclonal antibodies (less than 3% of residual kinase activity). Electrophoresis of the sample is shown on the top, and quantification of the experiment on the bottom.

Taken together, our results provide evidence that human CDC25B is degraded by the proteasome in a CDK1-cyclin A phosphorylation-dependent manner. As suggested by the inability of CDK1-cyclin B to trigger CDC25B degradation, a specific and a timely coordinated phosphorylation by the CDK1-cyclin A kinase is probably a key feature of this process. This observation is in agreement with the reported accumulation of CDC25B in late G2 and its rapid disappearance in mitosis (19). CDC25B activity appears therefore to be regulated at multiple levels including a targeted degradation that might take place in the fine tuning of CDK1-cyclin A activity at early stages of the G2/M transition. Together with recent findings, this report indicates that degradation of cyclin-dependent kinase inhibitors (11, 12, 20) and activators (this work) that is triggered by CDK-dependent phosphorylation is a key event in the control of cell proliferation. Abnormal regulation of this mechanism may participate in the oncogenic properties of the CDC25B phosphatase (21).

Acknowledgments—We gratefully acknowledge J. M. Darbon, G. Draetta, I. Jariel-Encontre, J. Hyams, B. B. Rudkin, A. Valette, and G. Villani for advice and critical reading of the manuscript.

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