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Study of the docking-dependent PLK1 phosphorylation of the CDC25B phosphatase

Valérie Lobjois [a,b,1], Carine Froment [d,e,1], Emmanuelle Braud [f,g], Fanny Grimal [h], Odile Burlet-Schiltz [d,e], Bernard Ducommun [a,b,c,*], Jean-Pierre Bouche [a]

[a] Université de Toulouse, LBCMCP, F-31062 Toulouse, France
[b] CNRS, ITAV-UMS3039, F-31106 Toulouse, France
[c] CHU de Toulouse, F-31059 Toulouse, France
[d] CNRS, IPBS-UMR5089, F-31077 Toulouse, France
[e] Université de Toulouse, UPS, IPBS, F-31077 Toulouse, France
[f] INSERM U648, F-75270 Paris Cedex 06, France
[g] Université Paris Descartes, F-75270 Paris Cedex 06, France
[h] INSERM, CPTP-U563, F-31024 Toulouse, France

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ABSTRACT

CDC25 (A, B and C) phosphatases control cell cycle progression through the timely dephosphorylation and activation of cyclin-dependent kinases (CDK). At mitosis the CDC25B phosphatase activity is dependent on its phosphorylation by multiple kinases impinging on its localisation, stability and catalytic activity. Here we report that prior phosphorylation of CDC25B by CDK1 enhances its substrate properties for PLK1 in vitro, and we also show that phosphorylated S50 serves as a docking site for PLK1. Using a sophisticated strategy based on the sequential phosphorylation of CDC25B with ^{16}O and ^{18}O ATP prior to nanoLC–MS/MS analysis we identified 13 sites phosphorylated by PLK1. This study illustrates the complexity of the phosphorylation pattern and of the subsequent regulation of CDC25B activity.

1. Introduction

CDC25 phosphatases are key actors of cell proliferation as they control the activity of cyclin-dependent kinases at key cell cycle transition. Human CDC25 A, B and C are implicated at G1/S, during S-phase, at G2/M and during mitosis [1]. Although they probably all share some degree of redundancy, CDC25B has been recognized a unique role in G2/M checkpoint control which is probably associated with existence of a family of splice variants with specific features controlling their localisation and activity [2].

CDC25B activity is regulated through its phosphorylation by a large number of protein kinases that have been shown to be involved in controlling entry into mitosis in an unperturbed cell cycle as well as in response to checkpoint activation [3]. Inhibiting (CHK1, MK2, p38, pEG3, PKB/AKT) and activating kinases (Aurora-A, CK2, CDKs) have been identified and their phosphorylation sites have been extensively mapped by nanoLC–MS/MS analysis [4–14]. For instance, we have shown that CDC25B is phosphorylated by CDK1/CyclinB on at least 11 SP or TP sites, and 10 of these sites are also phosphorylated in vivo by CDK/Cyclins [14].

Among the putative regulators of CDC25B is the polo-like kinase 1 (PLK1) which has been shown previously to phosphorylate CDC25C within its NES, thus leading to its nuclear translocation [15]. Elia and collaborators have shown that the CDC2–CyclinB activity provides priming phosphorylation of CDC25C for interaction with the polo-box domain (PBD) of PLK1 [16]. Recent studies showed that priming phosphorylation-dependent targeting of PLK1 to specific substrates is a commonly employed mechanism [17,18].

PLK1 is known to play a critical role in the control of entry into mitosis [19] and we recently reported that it also regulates CDC25B-dependent mitosis entry. We demonstrated that PLK1 activity is required for the nuclear translocation of CDC25B at the G2-M transition and for its subsequent activation. However, a detailed description of PLK1 phosphorylation sites on CDC25B and its relation with CDK phosphorylation is still missing. In this report we addressed this issue through a comprehensive proteomic study of the docking-dependent phosphorylation of CDC25B by PLK1 using an elegant combination of two distinct phosphorylation sites isotopic labelling and nanoLC–MS/MS analysis.

2. Materials and methods

2.1. Synthesis of [γ-$^{18}O_4$]ATP

$H_2{}^{18}O$ (97 atom% ^{18}O) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The labeled [γ-$^{18}O_4$]ATP

* Corresponding author at: Centre Pierre Potier, ITAV-UMS3039 CRT-RIV, 141 Route d'Espagne, BP 50624, 31106 Toulouse Cedex 1, France. Fax: +33 5 82 99 10 01.
E-mail address: bernard.ducommun@itav-recherche.fr (B. Ducommun).
[1] These authors equally contributed to this work.

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was synthesized using the procedure reported by Wehrli et al. [20]. The method required the preparation of ADP-morpholidate which was obtained by condensing morpholine with ADP in the presence of dicyclohexylcarbodiimide [21]. $H_3P^{18}O_4$ was synthesized as a tri-*n*-butylammonium salt by slightly modifying as described [22]. The 4-morpholine *N,N′*-dicyclohexylcarboxamidine salt of ADP-morpholidate (0.1 mmol) was condensed under argon with 3 equivalents of tri-butylammonium phosphate in anhydrous dimethylsulfoxide (5 mL) at 35 °C for 38 h. The reaction mixture was diluted with water (40 mL) and purified by ion exchange chromatography on a column of DEAE-cellulose (HCO_3^-) using a gradient of triethylammonium bicarbonate (0.01–0.35 M). The fractions containing ATP were collected and water removed under *vacuo*. The residue was evaporated with methanol (3 × 20 mL) before being dissolved in 2 mL of methanol. A solution of 1 M NaCl in acetone (0.5 mL) was added to precipitate the sodium salt, which was washed with acetone (2 × 10 mL) to give 0.038 mmol of [γ-$^{18}O_4$]ATP.

2.2. Phosphorylation in vitro

Recombinant HA3-CDC25B3-Hisproteinpurified as previously described [14] was used throughout this study and will be designated CDC25B for simplicity.

Phosphorylation by CDK1/CyclinB: recombinant CDC25B (3 μg) was phosphorylated for 2 h at 30 °C with (or without) 500 ng CDK1/CyclinB (Upstate, 800 units/mg) in 25 μl in buffer containing 20 mM MOPS–NaOH (pH 7.5), 25 mM 2-glycerophosphate, 0.5 mM EGTA, 1 mM EDTA, 15 mM MgCl2, 5 mM DTT, 120 μg/ml ovalbumin and 160 μM $^{16}O\gamma$ATP. To inactivate CDK1, incubation was continued for 1 h in the presence of 5′-fluorosulfonylbenzoyladenosine (3 mM). Three hundred microliter of 800 mM KCl/800 mM arginine was added and CDC25B3 was bound to 10 μl of anti-HA beads (Roche). Beads were washed once with the KCl-arginine solution, then four times with PLK1 buffer (20 mM Hepes-NaOH, pH 7.4; 125 mM NaCl; 10 mM $MgCl_2$; 0.02% Triton X-100; 1 mM EGTA; 5 mM DTT, 200 μg/ml ovalbumin).

Phosphorylation of CDC25B3 by PLK1 was performed in 25 μl for 2 h at 30 °C with 20 μM [γ-^{32}P]ATP or 100 μM [γ-$^{18}O_3$]ATP with 200 ng PLK1 (Invitrogen).

Phosphorylated samples were reduced and alkylated by successive incubation in 30 mM dithiothreitol for 30 min at 55 °C and 100 mM iodoacetamide for 30 min at room temperature in the dark. Proteins were separated by SDS–PAGE using a 4–12% gel and coomassie-blue stained gels were analyzed using a Fuji FLA-3000 phosphorofluoromager using fluorescence at 633 nm for normalization of IP (immunoprecipitation) signals. Then, the CDC25B3 corresponding bands were excised and subjected to in-gel tryptic digestion using modified porcine trypsin (Promega, France) at 20 ng/μl as previously described [23] and analyzed by nanoLC–MS/MS.

2.3. NanoLC–MS/MS analysis and database searches

The dried peptide extracts were dissolved in 15 μl of 0.05% trifluoroacetic acid in 2% acetonitrile and analysed by online nanoLC using an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) coupled to a nanospray LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Five microliter of each peptide extract were loaded on a 300 μm ID × 5 mm PepMap C18 precolumn (LC Packings, Dionex, USA) at 20 μl/min in 2% acetonitrile, 0.05% trifluoroacetic acid. After 5 min desalting, peptides were online separated on a 75 μm ID × 15 cm C18 PepMap™ column (LC Packings, Dionex, USA). The flow rate was set at 300 nl/min. Peptides were eluted using a 0–50% linear gradient of solvent B in 60 min (solvent A was 0.2% formic acid in 5% acetonitrile and solvent B was 0.2% formic acid in 80% acetonitrile). Data were acquiredin data-dependent scan with the XCalibur software (version 2.0 SR2, Thermo Fisher Scientific), using a 60 s dynamic exclusion window. The survey scan MS was performed in the Orbitrap on the 300–2000 *m*/*z* mass range with the resolution set to a value of 60,000 at *m*/*z* 400. The three most intense ions per survey scan were selected for MS/MS fragmentation and the resulting fragments were analysed in the linear trap (parallel mode).

The Mascot Daemon software (version 2.2.0, Matrix Science, London, UK) was used to perform database searches in batch mode against SwissProt and Trembl Human databases implemented with the HA3-CDC25B3-His6 recombinant protein sequence. Peak lists extraction from XCalibur raw files were automatically performed through the Mascot Daemon interface using ExtractMSN macro (Thermo Fischer Scientific). Cysteine carbamidomethylation was set as a fixed modification, methionine oxidation and [γ-^{16}O]ATP (mass increment of 79.9663) and [γ-^{18}O]ATP (mass increment of 85.9790) serine/threonine phosphorylation as variable modifications. Up to two missed trypsin cleavages were allowed. Mass tolerances in MS and MS/MS were set to 10 ppm and 0.8 Da for LTQ Orbitrap analyses. Phosphorylated site identification was confirmed by manual interpretation of corresponding MS/MS data.

Unphosphorylated calibration peptides were used as internal standards to calculate normalized ratios of phosphorylated peptide peak intensities between the two PLK1 phosphorylation experiments performed without and with prior CDK1/CyclinB phosphorylation.

3. Results and discussion

3.1. CDC25B phosphorylation by PLK1 is dependent on phosphorylation by CDK1 on serine 50

To examine whether phosphorylation by CDK/Cyclins complexes affects the substrate properties of CDC25B for PLK1, recombinant CDC25B [14] was phosphorylated in vitro by CDK1/CyclinB and compared to mock-phosphorylated protein as a substrate for PLK1. Prior phosphorylation by CDK1/CyclinB led to about threefold increase in PLK1 phosphorylation. In addition, PLK1-phosphorylated CDC25B migrated substantially more slowly on SDS–PAGE gel, suggesting that either CDC25B was phosphorylated on different sites, or that CDC25B molecules carrying a shifting phosphate were selected as substrates (Fig. 1A). These data indicated that

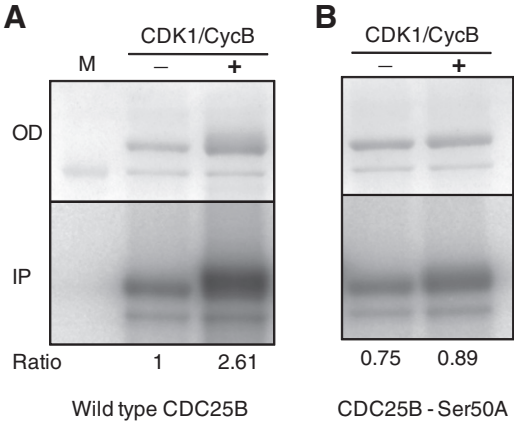


Fig. 1. CDC25B phosphorylation by PLK1 is dependent on the pre-phosphorylation of serine 50 by CDK1/CyclinB wild type (A) or S50A mutant (B) CDC25B was incubated with (+) or without (–) CDK1/CyclinB complex prior to phosphorylation by PLK1. Quantification of protein level and phosphorylation was achieved as indicated in the material and methods section. OD: fluorescence at 633 nm. IP are the immunoprecipitates and M is the molecular weight markers lane. Ratio is the IP–OD ratio normalized to 1 for the wild-type control phosphorylated by PLK1 only.

PLK1 phosphorylates CDC25B and that pre-phosphorylation of CDC25B by CDK1/CyclinB enhances its substrate properties for PLK1 in vitro, suggesting that CDK1/CyclinB could provide priming phosphorylation for PLK1.

Most substrates of Polo Kinase 1 consist of proteins containing a phosphorylated SpSP site which serves as a docking site for PLK1 polo domains [16], allowing the shielding of the catalytic domain by the polo domains to be lifted [18]. In CDC25B, serine 50 belongs to an SSP motif and was found to be phosphorylated by CDK1 in vivo and in vitro [14]. When recombinant CDC25B Ser50A mutant protein was phosphorylated by CDK1/CyclinB, no stimulation of the subsequent phosphorylation by PLK1 was observed. In addition, the shift in gel migration previously seen for wild-type CDC25B was essentially abolished (Fig. 1B).

Theses results indicate that the SpSP site containing serine 50 serves as a docking site for PLK1 and stimulates phosphorylation of CDC25B by PLK1 on other sites of the protein.

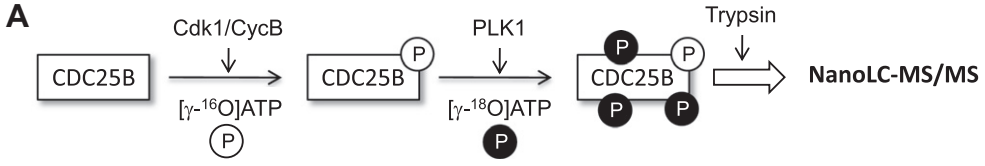
3.2. Identification of docking-dependent sites phosphorylated by PLK1 on CDC25B

In order to get a deeper insight in the regulation of CDC25B by PLK1, nanoLC-MS/MS was used to identify sites phosphorylated by PLK1. In order to discriminate the sites that are specifically phosphorylated as a result of PLK1 docking on SpS50P, a two-step phosphorylation was carried out as above. In addition, to distinguish between the sites phosphorylated by CDK1/CyclinB and the ones phosphorylated by PLK1, a labelling strategy was developed based on the use of [γ-^{16}O]ATP and [γ-^{18}O]ATP in the phosphorylation reaction performed with these two kinases, respectively. The rational of this approach was that when analysed by mass spectrometry after trypsin digestion,^{18}O- and ^{16}O-phosphorylated peptides will have a mass increment of 85.9790 Da and 79.9663 Da compared to the corresponding non-phosphorylated peptide, respectively (Fig. 2A).

Thirteen PLK1 sites were identified in this study: S50, T58, T127, T167, S209, T265, S291, S353, S375, S397, T404, S465 and S513 (Fig. 2B). Representative MS/MS spectra of ^{18}O-phosphorylated peptides for these sites are shown in Supplementary Figs. S1 (S50), S2 and S3 (T58), and S4–S14 (T127–S513). In addition, a minor species was phosphorylated on either T53, T54 or T56 (not shown).

Preliminary experiments using [γ-^{32}P]ATP indicated a 3.5-fold increase in phosphorylation by PLK1 with CDK1/CyclinB-prephosphorylated CDC25B compared to the mock-phosphorylated protein. To identify CDK1-priming dependent PLK1 sites, peak intensities from extracted ion chromatograms (XIC) for short, highly detected, unequivocally cleaved peptides were used to calibrate the amounts of CDC25B subjected to nanoLC–MS/MS analysis as described in the Materials and Methods section. Then peak intensities were measured for all ^{18}O-containing peptides and a normalized ratio indicating the effect of CDK1/CyclinB prephosphorylation was determined.

With two exceptions, all sites showed enhanced phosphorylation by PLK1 following prior phosphorylation by CDK1/CyclinB (Fig 2B). This was particularly noticeable for sites S397–S513 within the catalytic moiety of CDC25B (range: 3.3–4.2), for T167 and S209 (6.4 and 12.9-fold stimulation, respectively), and less



B

Peptide sequence and phosphorylation sites	Position	Normalized ratio	Sup Fig
AAAS^{18O}pSPVTTLTQTMHDLAGLGSETPK	S50	0.8	S1
AAASpSPV^{18O}p(TTLT)QTMHDLAGLGSETPK	T53, 54 or 56	2.6	
AAASSPVTTLTQ^{18O}pTMHDLAGLGSETPK	T58	1.7	S2
AAAS^{16O}pSPVTTLTQ^{18O}pTMHDLAGLGSETPK			S3
MAEQ^{18O}pTFEQAIQAASR	T127	1.5	S4
NI^{18O}pTNSQAPDGR	T167	6.4	S5
MPWKPTHP^{18O}pSSTHALAEWASR	S209	12.9	S6
FSLTPAEGD^{18O}pTEEDDGFVDILESDLK	T265	2.0	S7
DDDAVPPGME^{18O}pSLISAPLVK	S291	1.5	S8
^{18O}pSVTPPEEQQEAEEPK	S353	1.6	S9
R^{18O}pSVTPPEEQQEAEEPK			
^{18O}pSLCHDEIENLLDSDHR	S375	0.4	S10
SK^{18O}pSLCHDEIENLLDSDHR			
ELIGDY^{18O}pSK	S397	3.4	S11
AFLLQ^{18O}pTVDGK	T404	4.2	S12
DAE^{18O}pSFLLK	S465	3.4	S13
DAE^{18O}pSFLLK^{16O}pSPIAPCSLDK			
AVNDYP^{18O}pSLYYPEMYILK	S513	3.3	S14

Fig. 2. Identification of docking-dependent PLK1 sites of CDC25B3. (A) Scheme of the experimental procedure to differentially label CDK1 and PLK1 phosphorylation sites based on the use of[γ-$^{16}O_3$]ATP and [γ-$^{18}O_3$]ATP. NanoLC–MS/MS analysis after trypsin digestion can then distinguish ^{16}O-phosphorylated peptides and ^{18}O-phosphorylated peptides having a mass increment of 79.9663 Da and 85.9790 Da compared to the corresponding non-phosphorylated peptides, respectively, and phosphorylation sites can be identified. (B) Summary table of the results of identification of docking-dependent PLK1 sites of CDC25B3. Peptide sequences and phosphorylated positions are indicated. Unphosphorylated peptides were used as internal standards to calculate normalized ratios of phosphorylated peptide peak intensities between the two PLK1 phosphorylation experiments performed without and with prior CDK1/CyclinB phosphorylation. All MS/MS spectra are available as supplementary figures (figure numbers indicated in the last column). (1) Data for reduced and sulfoxidized peptides are summed up in this table.

pronounced for other sites. Although the large number and scattering of docking-stimulated sites likely reflects the flexibility of the regulatory region, these variations may result from a hitherto uncharted subdomain topology of the regulatory region of CDC25B. Another evidence for the docking effect came from the fact that the relative abundance of the peptide doubly phosphorylated by CDK1/CyclinB on S50 and by PLK1 on T58 (Supplementary information Fig. S3) was much higher than the combined abundances of [^{16}O]pS50 and of [^{18}O]pT58 (data not shown). Surprisingly, PLK1 phosphorylated its own docking site in vitro (Supplementary information Fig. S1). Not surprisingly, this phosphorylation was insensitive to prior phosphorylation by CDK1/CyclinB (Fig. 2B).

In contrast to the above sites, phosphorylation by CDK1/CyclinB strongly reduced subsequent phosphorylation of S375 by PLK1, suggesting that one or several unidentified sites phosphorylated by CDK1 exerted a negative interference on S375 phosphorylation.

Altogether, these data illustrates the high level of complexity of the PLK1-dependent phosphorylation pattern on CDC25B. The strategy developed in this study represents an attractive and efficient way of studying sequential phosphorylation events. Deciphering the specific role of these modifications in frame with other modifications and regulations that occur on CDC25B at the onset of mitosis remains a very complex challenge. Point mutations of these phosphorylation sites have been generated but had little if no effects and did not yet provide any clue toward a global understanding of the functional consequences of PLK1-dependent phosphorylation of CDC25B. Further investigations are required to achieve that goal. This is especially important as several PLK1 and CDC25 inhibitors are undergoing preclinical or clinical development for anticancer therapy [1,24,25].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.110.

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