Report

Characterization of centrosomal localization and dynamics of Cdc25C phosphatase in mitosis

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Abbreviations: Cdk, cyclin-dependent kinase; FRAP, fluorescence recovery after photobleaching; MPF, mitosis promoting factor; NA, numerical aperture; siRNA, small interfering RNA

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In mammalian cells, three Cdc25 phosphatases A, B, C coordinate cell cycle progression through activating dephosphorylation of Cyclin-dependent kinases. Whereas Cdc25B is believed to trigger entry into mitosis, Cdc25C is thought to act at a later stage of mitosis and in the nucleus. We report that a fraction of Cdc25C localises to centrosomes in a cell cycle-dependent fashion, as of late S phase and throughout G₂ and mitosis. Moreover, Cdc25C colocalises with Cyclin B1 at centrosomes in G₂ and in prophase and Fluorescence Recovery after Photobleaching experiments reveal that they are both in dynamic exchange between the centrosome and the cytoplasm. The centrosomal localisation of Cdc25C is essentially mediated by its catalytic C-terminal domain, but does not require catalytic activity. In fact phosphatase-dead and substrate-binding hotspot mutants of Cdc25C accumulate at centrosomes together with phosphoTyr15-Cdk1 and behave as dominant negative forms that impair entry into mitosis. Taken together, our data suggest an unexpected function for Cdc25C at the G₂/M transition, in dephosphorylation of Cdk1. We propose that Cdc25C may participate in amplification of Cdk1-Cyclin B1 activity following initial activation by Cdc25B, and that this process is initiated at the centrosome, then further propagated throughout the cytoplasm thanks to the dynamic behavior of both Cdc25C and Cyclin B1.

Introduction

Progression through the different phases of the eukaryotic cell division cycle is a tightly regulated process that is coordinated by sequential activation of members of the family of the Cyclin-dependent kinases. Mitosis in particular is orchestrated by Cdk1-Cyclin B1 kinase, also called MPF (for “Mitosis-Promoting Factor”), whose universal and central role ensures timely and coordinated chromosome segregation through activation of a number of substrates and establishment of the appropriate mitotic structures. MPF activation is a complex process, which involves a number of phosphorylation and dephosphorylation events, including phosphorylation of Cyclin B1, phosphorylation of Cdk1 on Thr10, by Cdk-activating kinase CAK, and dephosphorylation of phosphorylated Cdk1 on Thr14 and Tyr15 by dual specificity Cdc25 phosphatases. In human cells three Cdc25 isoforms, A, B and C assume overlapping functions in orchestrating cell cycle progression. Cdc25B and Cdc25C have been more directly implicated in activation of MPF. Active MPF in turn participates in further activation of Cdc25 phosphatases through a positive feedback loop, thereby amplifying MPF activity to a threshold level, which triggers unconditional and irreversible entry into mitosis.

Although it is generally accepted that entry into mitosis requires accumulation of active Cdk1-Cyclin B1 in the nucleus, a number of studies point to initial activation of MPF in the cytoplasm, and more recently to the centrosome, and substrates of MPF are found in both the nucleus and the cytoplasm. The active form of Cdk1-Cyclin B1, in which Cyclin B1 is hyperphosphorylated, first appears at centrosomes in prophase. Moreover, a growing number of reports document the physical association of mitotic regulators with the centrosome, including Plk1 and Cdc25B, thereby pointing to this organelle as a centralizing platform, which coordinates the complex sequence of events leading to MPF activation.

Recent work suggests a dynamic relationship between Cdc25B and Chk1 on the one hand, and Aurora-A and Eg3 on the other, in centrosomal activation of Cdc25B. The state-of-the-art model in mammalian cells implies that Cdk1-Cyclin B1 activation first occurs at centrosomes in prophase, as a consequence of Cdc25B-mediated dephosphorylation of centrosomal Cdk1. Cdc25C is sequestered by 14-3-3 proteins in the cytoplasm throughout interphase, and is then activated and translocated into the nucleus at the G₂/M transition following dissociation from 14-3-3 proteins and hyperphosphorylation by Plk and Cdk-Cyclin kinases. Cdc25C is therefore thought to contribute to amplification of MPF activity well after the G₂/M transition, in the nucleus, where it would counteract the inhibitory function of Wee1.
kinase. However its biological features and functional implication at the G2/M transition are not fully understood.

**Results**

Cdc25C localises to centrosomes in G2 and during mitosis. We reinvestigated the subcellular localisation of Cdc25C in HeLa cells using an antibody raised against a specific C-terminal sequence of Cdc25C (C-20) and found, in agreement with another recent study, that whilst most Cdc25C localises to the cytoplasm in asynchronous cells, a fraction of endogenous Cdc25C localises to centrosomes, co-localising with structures staining for pericentriolar material and γ-tubulin (Fig. 1A and C). This localisation persists throughout G2 and mitosis, from prophase through telophase and is clearly specific, as centrosomal staining disappears upon treatment with Cdc25C siRNA, whereas neither Cdc25A nor Cdc25B levels are affected (Fig. 1B and C), or upon incubation of the antibody with its corresponding immunization peptide (Suppl. Fig. 1). The centrosomal localisation of Cdc25C was also observed in U2OS osteosarcoma and in non-transformed, non-immortalized NHF and HS68 human diploid fibroblasts, further supporting its biological relevance (data not shown). Moreover, Cdc25C co-fractionates with centrosomal γ-tubulin in centrosomal preparations purified from synchronized cells in G2, implying it is physically associated with centrosomes (Fig. 1D).

To address whether centrosomal localisation of Cdc25C was cell cycle-dependent, we used a HeLa cell line which stably expresses centriolar GFP-centrin, and examined when Cdc25C appeared at centrosomes. We found that Cdc25C first appears at duplicating centrosomes and further accumulates when centrosomes are fully duplicated and begin to separate, in G2 (Fig. 2A and B). To further assess at which stage endogenous Cdc25C localizes to centrosomes, we compared the kinetics of centrosomal Cdc25C accumulation with those of Cyclin A, which revealed a general trend indicative of initial appearance of Cdc25C at centrosomes as of late S phase, then accumulating throughout G2 (Fig. 2C and D).

Cdc25C and Cyclin B1 colocalise at centrosomes in G2 and in prophase and are highly dynamic. In mammalian cells Cyclin B1 accumulates at centrosomes in G2, and is first activated and hyperphosphorylated at this organelle in prophase. Indirect immunofluorescence performed on HeLa cells revealed that the endogenous form of Cdc25C colocalised with Cyclin B1 both at centrosomes and in the cytoplasm in G2, and in prophase (Fig. 3A). Likewise, ectopically expressed mRFP-Cdc25C and EGFP-Cyclin B1 colocalised in both the cytoplasm and at centrosomes (Fig. 3B). EGFP-tagged Cyclin B1 and Cdc25C (localisation of which is identical to that of mRFP-Cdc25C) were microinjected into HeLa cells in G2, and fluorescence recovery after photobleaching (FRAP) experiments were performed by photobleaching the centrosomal fraction of these proteins, to characterise their mobility (Fig. 3C and D). These studies revealed that centrosomal EGFP-Cdc25C was highly dynamic, the

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*Figure 1.* Cdc25C localises to centrosomes in G2 and mitosis. (A) Indirect immunofluorescence of endogenous Cdc25C with the C-20 antibody. Cells in prophase (A), prometaphase (B), metaphase (C), anaphase (D), telophase (E) and cytokinesis (F). Centrosomes were stained with the anti-CTR453 antibody, DNA with Hoechst 33342. Bar: 10 μm. Images were acquired with a 63X objective. (B) Western blot with anti-Cdc25A, B or C, of cell extracts prepared from untreated cells (NT) or cells treated with control siRNA (Neg) or siRNA targeting Cdc25C (25C). (C) Indirect immunofluorescence performed with anti-Cdc25C C-20 and anti-gamma-tubulin antibodies on HeLa cells treated with siRNA targeting Cdc25C (lower) or control siRNA (upper). Bar: 20 μm. Images were acquired with a 40X objective (D) Western blot with anti-Cdc25C and anti-gamma-tubulin antibodies of centrosomal fractions purified on a sucrose gradient.
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Molecular basis for centrosomal localisation of Cdc25C. To gain insight into the molecular basis for centrosomal localisation of Cdc25C, we asked whether it might be mediated through specific sequence determinants, functional domains or protein partners. The N-terminal, regulatory domain of Cdc25 phosphatases harbours residues involved in substrate and partner recognition, including motifs involved in Cyclin B1 and 14-3-3 binding. Moreover, the catalytic domain of Cdc25B bears several “hot spot” residues, which are conserved in all three Cdc25 isoforms, as well as a C-terminal stretch of amino acids involved in recruitment of its Cdk substrate. We therefore generated a series of constructs expressing mRFP-tagged Cdc25C variants, deleted for its N-terminal domain (construct 286–473), or for its C-terminal domain with or without the consensus 14-3-3 binding site and adjacent NLS (constructs 1–285 and 1–180, respectively). A schematic representation of the different deletion variants and mutants of mRFP-Cdc25C generated for the purpose of this study is shown in Figure 4A. These constructs were microinjected into synchronized HeLa cells in G2 and microinjected cells in which the different mRFP-Cdc25C variants localised to centrosomes were scored (Fig. 4B–D). We found that only the C-terminal domain localised to centrosomes with similar efficiency to full-length mRFP-Cdc25C, and preferentially in cells in G2. However, the longer N-terminal construct (1–285), but not the shorter one, partially relocalised to centrosomes in prophase, indicating that the region between residues 180 and 285 contributes to centrosomal localisation of Cdc25C in prophase. To further investigate whether catalytic activity, Cdk or 14-3-3 binding had any influence on centrosomal accumulation, we generated a catalytically inactive form of mRFP-Cdc25C (C377S), two substrate-binding “hotspot” mutants R392L and Y401A (corresponding to R488 and Y497 in Cdc25B), and a form which no longer binds 14-3-3 (S216A). As shown in Figure 4E, none of these single amino acid substitutions impeded centrosomal localisation of Cdc25C.

Phosphatase-dead and substrate-trapping mutants of Cdc25C accumulate at centrosomes with phospho-Tyr15 Cdk1. Previous studies report that Cdc25C does not play any role in mitotic entry, in contrast to the Cdc25B isoform, which is responsible for triggering activation of MPF at the G2/M transition. However, we
found that microinjection of a construct expressing mRFP-Cdc25C into synchronized HeLa cells in G2 (and only in G2), lead to a net induction of mitosis compared to mRFP (Fig. 5A and B), although much less significant than Cdc25B at this stage (data not shown). In contrast, the C377S, R392L and Y401A mutants were defective in their ability to promote entry into mitosis, whether or not coinjected with Cyclin B1, therefore behaving as dominant negative mutants. Moreover, we found that these mutants accumulated at centrosomes and promoted accumulation of phospho-Tyr15 Cdk1 at centrosomes, before cells die (Fig. 5C), inferring that the defect in mitotic induction was related to a defect in Cdk1 activation, which is initiated at the centrosome. Closer examination of these cells revealed that several were still capable of separating their centrosomes before dying (Suppl. Fig. 2), indicating that initial Cdc25B-dependent activation of Cdk1-Cyclin B1 required for centrosomal separation \(^ {51} \) was probably not affected.

**Discussion**

In mammalian cells, Cdc25C is sequestered in the cytoplasm during interphase by 14-3-3, \(^ {32-35} \) and then translocates into the nucleus at the G2/M transition, following a series of phosphorylation and dephosphorylation events. \(^ {37-42} \) In this study we report that a fraction of Cdc25C localises to centrosomes in a cell cycle-dependent fashion, as of late S phase and throughout G2 and mitosis, inferring a biological function for Cdc25C at this organelle during these stages. Moreover, we find that Cdc25C and Cyclin B1 colocalise at centrosomes in G2 and in prophase, and that they both undergo a dynamic exchange between the centrosome and the cytoplasm, suggesting a functional interaction between these two partners. It is widely accepted that Cdc25B triggers initial activation of Cdk1-Cyclin B1 at the centrosome, \(^ {18} \) whereas Cdc25C has been thought to act subsequently to amplify MPF activity during mitosis in the nucleus, \(^ {12,13,16,20} \) However, we find that catalytically inactive or substrate-binding “hotspot” mutants of Cdc25C promote centrosomal accumulation of phospho-Tyr15-Cdk1 and impair entry into mitosis. Taken together these findings suggest that centrosomal localisation of Cdc25C is associated with substrate recognition and dephosphorylation, and infer that Cdc25C contributes to activation of Cdk1-Cyclin B1 at the G2/M transition. We propose that Cdc25C may participate in amplification of Cdk1-Cyclin B1 activity following initial activation by Cdc25B, and that this process is initiated at the centrosome, but then further propagated throughout the cytoplasm thanks to the dynamic behavior of both Cdc25C and Cyclin B1.

In addition to being the major microtubule-organizing center of mammalian cells, the centrosome acts as an integrative scaffold which facilitates interactions between mitotic players by increasing their local concentration. In fact, a growing number of reports document the recruitment and functional activation of several mitotic...
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regulators at the centrosome, including Cdk1-Cyclin B1, Cdc25B, Polo-like kinases, Aurora kinases and Pin1. Initial appearance of active Cdk1-Cyclin B1 at centrosomes in prophase is thought to involve Plk1-mediated Cyclin B1 phosphorylation, and a recent model for the interaction between Plk1 and Cdc25C suggests that their interaction is likely to occur at the centrosome. This report is perfectly in line with our findings and provides a functional rationale for the centrosomal localisation of Cdc25C.

Figure 4. Molecular basis for centrosomal localisation of Cdc25C. (A) Schematic representation of the mRFP-Cdc25C mutants and truncation constructs generated: full-length wildtype (FL); S216A, C377S, R392L, Y401A, N-terminal domains 1–180 and 1–285 ; C-terminal domain 286-end. (B) Statistical analysis of the centrosomal localisation of the different mRFP-Cdc25C variants described in (A) following microinjection into HeLa cells in G2. For each construct, approximately 120 cells were scored. Results are shown +/- SE bars (n = 3). (C) Typical micrographs of HeLa cells in G2 and prophase illustrating the subcellular localisation of mRFP and wild-type Cdc25C, (D) of different mRFP-Cdc25C truncation constructs (E) and of single-site mutants: S216A, R392L, Y401A and C377S. Images were acquired with a 63X objective. Bar 10 μm.
Our attempts to characterise the molecular basis for centrosomal localisation of Cdc25C reveal that it is primarily dependent on its C-terminal domain, in line with its role in substrate recognition, and altogether independent of the first 180 N-terminal residues. This region is involved in interactions with numerous partners, including cyclins\(^{45,46}\) and Plk1.\(^{52}\) Therefore our findings infer that centrosomal targeting of Cdc25C is not mediated through Plk1 or cyclin binding, and that interactions between these partners at the centrosome itself occur following their recruitment to this organelle. In contrast, the region between residues 180 and 285 clearly participates in recruitment of Cdc25C to centrosomes in prophase. Interestingly this region harbours the NES and the NLS, a Cyclin B1 binding site, the 14-3-3 binding site, which bears the S216 phosphorylation site, and the adjacent S214 mitotic phosphorylation site. In particular, phospho-S216-Cdc25C is dephosphorylated upon 14-3-3 dissociation at the G2/M transition, and this is followed by activating phosphorylation of Cdc25C on several residues, including S214, which prevents rebinding of 14-3-3.\(^{39,40,53}\) In this study, we have shown that centrosomal localisation of Cdc25C is independent of 14-3-3 binding. As such, it seems reasonable to surmise that phosphorylation of S214 may further contribute to localization of Cdc25C at the centrosome in prophase, although further investigations will be required to address this issue.

In conclusion, our study provides insight into the molecular features and dynamics of the centrosomal localisation of Cdc25C, thereby suggesting an unexpected function for this phosphatase at the G2/M transition. In an integrated model, we propose that complete activation of Cdk1-Cyclin B1 required for robust and timely entry into mitosis is the result of the cooperation between Cdc25B and Cdc25C, the former triggering initial activation of centrosomal Cdk1-Cyclin B1, the latter ensuring amplification and propagation of kinase activity. Issues that remain to be addressed include understanding why a fraction of Cdc25C remains localised to centrosomes once entry into mitosis is triggered, nuclear envelope breakdown, and spindle assembly have proceeded, and identifying what triggers initial association of Cdc25C to the centrosome, and what primes its dissociation at the end of mitosis.

**Materials and Methods**

**Cell culture, synchronisation, transfection and microinjection.** HeLa cells were cultured in DMEM supplemented with 10% FCS, 1 mM penicillin and 1 mM streptomycin at 37°C in an atmosphere containing 5% CO\(_2\). NHF cells were graciously provided by J. Piette (CNRS, Montpellier, France). HeLa cells stably expressing GFP-Centrin were a kind gift from M. Bornens (Institut Curie, Paris, France) and were cultured with 0.5 mg/ml G418 (Calbiochem). siRNA transfections were performed twice at 24 h interval at a 50 nM concentration with Oligofectamine\(^\text{TM}\) (Invitrogen) and cells collected after 72 h. HeLa cells were synchronized with 1 mM hydroxyurea (Sigma) and released for 6 h to reach G2. Microinjections were performed with a semi-automatic Eppendorf microinjection apparatus.

**Antibodies, western blotting and indirect immunofluorescence.** Antibodies against Cyclin B1 (GNS1, sc-245) and Cdc25C (C-20, sc-327) were purchased from Tebu-Bio (Santa Cruz, anti-Cdc25A (CS-3656), anti-Cdc25B (CS-9525) and anti-pTyr15-Cdk1 were from Cell Signalling Technology. These primary antibodies were used at 1:50 dilution for indirect immunofluorescence, 1:500 for Western blotting. A 40 fold excess (2 \(\mu\)M) of immunization peptide (Santa Cruz, sc-6392 P) was used to titrate the C-20 antibody. Anti-gamma tubulin (GTU-488, Sigma), was used at a 1:1000 and 1:10000 dilution for Western blotting and for indirect immunofluorescence, respectively. Monoclonal anti-CTR453 antibody raised against
AKAP-450 was kindly provided by M. Bornens (Curie Institute, France) and used at a 1:10 dilution for indirect immunofluorescence. Horseradish peroxidase-coupled secondary antibodies for Western blotting were purchased from GE Healthcare and Alexa488 or Alexa594-coupled secondary antibodies for indirect immunofluorescence were from Molecular Probes.

For Western blotting, cell extracts were prepared in lysis buffer containing 50 mM TrisHCl, pH 7.4, 150 mM NaCl, 0.1% NP40, 0.1% Deoxycholate, 2 mM EDTA, 1 mM PMSF, Complete™ protease inhibitors (Roche), 50 mM NaF, 40 mM β-Glycerophosphate, 1 mM Na2VO4 and dosed in a BCA assay (Pierce).

For indirect immunofluorescence, cells were plated onto glass coverslips coated with fibronectin (Sigma). Cells were fixed with PFA 4% (Electron Microscopy Science) for 10 min and extracted with methanol for 5 minutes at -20°C. All incubations and washes were performed with PBS/BSA 1%, 0.1% Tween 20. Nuclei were stained with Hoechst 33342 (Sigma), and coverslips were mounted in Prolong Antifade Reagent (Invitrogen).

**Microscopy.** Epifluorescence images were acquired on a ZEISS Axiolmager Z1 microscope with a Plan Apo 20x objective/0.8 NA (numerical aperture), an EC Plan Neofluar 40x objective/1.3 NA, a PlanApo 63x objective/1.4 NA or an EC Plan Neofluor 100x objective/1.3 NA, equipped with a Coolsnap HQ camera (Photometrics) and piloted by the Metamorph software (Universal Imaging). Confocal images and FRAP experiments were performed with a ZEISS LSM 510 confocal microscope. EGFP was excited with the 488-nm line of the Argon laser. After 20 prebleach scans (2 scans/s), a region of interest (ROI) was bleached with 25 iterations at 100% laser power, and fluorescence recovery was sampled for 2 min every 500 milliseconds. For time-lapse microscopy, cells were observed with a Zeiss Axiovert microscope with an incubation chamber containing 5% CO2 and thermostated at 37°C, equipped with a Zeiss Plan Neofluor objective 10x/0.3 NA or with a Zeiss LD A-Plan objective 20x/0.8 NA. Fluorescence intensity quantifications were performed essentially as described by Lindqvist et al., (2007) on 10 laser power, and fluorescence recovery was sampled for 2 min every 500 milliseconds. For time-lapse microscopy, cells were observed with a Zeiss Axiovert microscope with an incubation chamber containing 5% CO2 and thermostated at 37°C, equipped with a Zeiss Plan Neofluor objective 10x/0.3 NA or with a Zeiss LD A-Plan objective 20x/0.8 NA. Fluorescence intensity quantifications were performed essentially as described by Lindqvist et al., (2007) on 10 image stacks with a 1 μm spacing, using the Metamorph software (Universal Imaging). Regions of interest (ROI) were selected in the nucleus, in the cytoplasm and outside the cell to obtain background values. For centrosomes, maximal intensity was measured and cytoplasmic intensity subtracted to correct protein level variations.

**Centrosome purification.** Centrosomal fractions were purified on a sucrose gradient from HeLa cells synchronised in G2, according to the original protocol developed by Moudjou and Bornens.54

**Molecular biology.** cDNA encoding human Cdc25C was cloned into pcDNA3.1-MRFP1 (kindly provided by T.Ng, King's College London, UK), and all mutants were generated according to standard procedures. Plasmids expressing EGFP-Cyclin B1 and EGFP-Cdc25C were generously provided by D. Chang (Hong Kong University of Science and Technology, Hong Kong, China) and P.J. Stambrook (University of Cincinnati College of Medicine, Cincinnati, USA), respectively. Smart-Pool™ siRNAs targeting Cdc25C and control siRNA pools were obtained from Dharmacon.

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**Supplementary materials can be found at:**

www.landesbioscience.com/CellCycleSupp/2008/09/1381517_Centrosomal_loc...
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