Regulation of Cdc45 in the cell cycle and after DNA damage

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Abstract
The Cdc (cell division cycle) 45 protein has a central role in the regulation of the initiation and elongation stages of eukaryotic chromosomal DNA replication. In addition, it is the main target for a Chk1 (checkpoint kinase 1)-dependent Cdc25/CDK2 (cyclin-dependent kinase 2)-independent DNA damage checkpoint signal transduction pathway following low doses of BPDE (benzo[a]pyrene dihydrodiol epoxide) treatment, which causes DNA damage similar to UV-induced adducts. Cdc45 interacts physically and functionally with the putative eukaryotic replicative DNA helicase, the MCM (mini-chromosome maintenance) complex, and forms a helicase active ‘supercomplex’, the CMG (Cdc45–MCM2–7–GINS [go-ichi-ni-san]) complex. These known protein–protein interactions, as well as unknown interactions and post-translational modifications, may be important for the regulation of Cdc45 and the initiation of DNA replication following DNA damage. Future studies will help to elucidate the molecular basis of this newly identified S-phase checkpoint pathway which has Cdc45 as a target.

Eukaryotic DNA replication
In all organisms, including humans, accurate chromosomal DNA replication is essential to maintain stability of the genome and for normal cell division, with replication of damaged DNA leading to mutations in the genome and to the development of diseases such as cancer [1]. Therefore understanding the molecular mechanisms regulating DNA replication after DNA damage is of great importance to biomedical researchers, and detailed knowledge of these mechanisms may lead to the identification of novel molecular targets for drug therapies in cancer and other diseases.

Eukaryotic DNA replication occurs during S-phase of the cell cycle and is a highly regulated process involving various replication proteins [2–9]. DNA replication is initiated at distinct origins of replication on chromosomal DNA. To activate an origin, the multisubunit ORC (origin recognition complex) must bind to chromatin at origins of DNA replication [2,6,8]. This ORC–DNA complex serves as a landing platform for the binding of Cdc (cell division cycle) 6 and Cdt1 (Cdc10-dependent target 1) [2,6,8]. This multimeric Cdc6–Cdt1–ORC–DNA complex facilitates the loading of the MCM (mini-chromosome maintenance) 2–7 complex to chromatin, forming the preRC (pre-replicative complex) [2,6,8]. Activation of the preRC by CDks (cyclin-dependent kinases) and DDK (Dbf4-dependent kinase) allows the formation of an initiation complex consisting of RPA (replication protein A), Cdc45 and the GINS [go-ichi-ni-san (five-one-two-three)] complex, activating the replicative helicase complex and causing the unwinding of the DNA double helix. MCM10 protein facilitates the chromatin-association of Pol-prim (DNA polymerase α-primase), which coincides with the initiation of DNA replication [3,4]. In addition, topoisomerase 1 is recruited to sites ahead of the progressing replication fork, where it nicks the DNA backbone in order to relieve torsional stress on the DNA caused by supercoiling during replication [2].

Subsequently, the first RNA primer is synthesized by the primase activity of Pol-prim and elongated by its DNA polymerase activity [2,8,9]. This RNA–DNA is recognized by RFC (replication factor C) which loads the PCNA (proliferating-cell nuclear antigen), the replicative sliding clamp, on to DNA [2,8,9]. RFC and PCNA, together with RPA, mediate a polymerase switch from Pol-prim to Pol (DNA polymerase) δ or ε, allowing continuous DNA synthesis on the leading strand and discontinuous DNA synthesis on the lagging strand [2] (Figure 1). On the lagging strand, the repetitive priming of Pol-prim followed by RFC- and PCNA-mediated primerase switch causes the formation of Okazaki fragments, which are matured into a continuous new strand by the actions of RNAses H, Fen1 (flap endonuclease 1), Dna1, Pol δ and DNA ligase 1 [2,8,9].

Cdc45
The Cdc45 protein is involved in the regulation of eukaryotic chromosomal DNA replication and is required for the initiation and elongation steps as well as the co-ordination of DNA replication [5–9]. Cdc45 was originally identified in yeast screens as an essential gene involved in the regulation of DNA replication [10]. Additionally, the Cdc45 gene was shown to genetically interact with the replication factors MCM5/CDC46, MCM7/CDC47 and ORC [10]. These

Key words: Cdc45, cell cycle, checkpoint, DNA damage, DNA replication, genome stability.

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; BPDE, benzo[a]pyrene dihydrodiol epoxide; Cdc, cell division cycle; CDK, cyclin-dependent kinase; Cdt1, Cdc10-dependent target; Chk, checkpoint kinase; DDK, Dbf4-dependent kinase; GINS, go-ichi-ni-san (five-one-two-three); MCM, mini-chromosome maintenance; CMG, Cdc45–MCM2–7–GINS; ORC, origin recognition complex; PCNA, proliferating-cell nuclear antigen; Pol, DNA polymerase; Pol-prim, DNA polymerase α-primase; preRC, pre-replicative complex; RFC, replication factor C; RPA, replication protein A.

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interactions implied a role for Cdc45 in the initiation and elongation stages of DNA replication [10] (Figure 1).

**Cdc45 and the initiation of eukaryotic DNA replication**

Cdc45 plays a crucial role in the initiation of DNA replication. Chromatin association of Cdc45 corresponds well with origin activation and the progression of DNA replication [11] (Figure 1), and Cdc45 chromatin association is mediated by the converging actions of CDKs and DDK [12], implying that Cdc45 is also crucial in the regulation of initiation of DNA replication. Moreover, it has been demonstrated that Cdc45 chromatin association is mediated in a DDK-dependent manner, with chromatin association of Cdc45 essential for the progression of DNA replication [12]. As this DDK complex only exists in late G₁ and S-phase of the cell cycle and the assembly of Cdc45 into protein complexes containing MCM2–7 proteins is DDK-dependent, this regulation of Cdc45 protein may then finally control the initiation of DNA replication [12]. Currently, it is anticipated that the association of Cdc45 with the MCM and GINS complexes are the final steps to activate the replicative helicase in eukaryotic cells, a prerequisite to initiate DNA replication [6,7,9,13]. These studies imply that Cdc45 may represent a ‘limiting factor’ in the initiation of DNA replication and a crucial target for signalling pathways regulating cell-cycle progression and DNA replication.

**Cdc45 and the elongation stage of eukaryotic DNA replication**

Co-immunoprecipitation studies in yeast have revealed numerous physical interactions between Cdc45 and proteins involved in the elongation stage of DNA replication, including Sld3 protein [14]. In yeast, Cdc45 was also purified as part of a complex containing MCM2 protein [15] and was shown to interact physically with MCM5 [16], MCM7 [17] and MCM10 [18] proteins.

Protein–protein interaction studies involving the gene product of the human homologue of *CDC45* corroborate these data, showing conserved physical interactions with MCM5 and MCM7 [5], as well as interactions with subunits of Pol δ and ε [5] and of the GINS complex [5]. In addition, Cdc45 interacted physically with TopBP1 (topoisomerase IIβ-binding protein 1) [19] and the p70 subunit of Pol α [20]. In humans, it has also been demonstrated that Cdc45 interacts with MCM3, MCM7, GINS subunits, RPA, and Pol δ and ε subunits in a cell-cycle-dependent manner [21].

The MCM2–7 complex is the proposed candidate for the eukaryotic helicase, and, for some time, helicase activity has only been shown to be present in a subcomplex of the MCM2–7 complex, consisting of MCM4–6–7 [13]. Recent results, however, show that the yeast MCM2–7 complex has helicase activity *in vitro* depending on buffer conditions and other factors [22–30]. Interestingly, Cdc45 was purified in *Drosophila* as part of a ‘supercomplex’ with MCM2–7 and GINS termed the CMG complex, which was shown to have helicase activity [13], lending more credence to the idea that Cdc45 is required for the formation and normal function of the replication fork. In summary, these studies imply a crucial role for Cdc45 protein in DNA replication in human cells, where it is proposed to act as a molecular tether.

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**Figure 1** Cdc45 function at the replication fork during the elongation stage of eukaryotic DNA replication

Proposed model for localization and function of human Cdc45 during the elongation stage of DNA replication. Cdc45 associates with replicative DNA polymerases, GINS and with the MCM2–7 complex, probably acting as a molecular tether between the replicative polymerases and the MCM2–7 complex. Adapted from [9], with permission from Blackwell Publishing Ltd.

**Figure 2** Model of the distinct mechanisms of Cdc45 regulation in response to low and high levels of UV damage

Low levels of UV- or BPDE-induced DNA adducts inhibit Cdc45 loading in a Chk1-dependent manner that does not involve changes in Cdc25A and CDK2. In contrast, high levels of DNA adducts that cause global replication blocks activate Chk1/Chk2 and target Cdc25A for degradation. The resulting inhibition of CDK2 activity prevents Cdc45 loading at unfired origins.

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between the MCM2–7 helicase complex and the replicative DNA polymerases facilitating elongation of replication [5].

**Cdc45 in DNA damage-dependent checkpoint response**

Genomic DNA can become damaged via exogenous sources (UV light or ionizing radiation) or endogenous sources (reactive oxygen species and free radicals) [1,8].

In order to maintain genome stability after DNA damage, eukaryotic cells have established various processes to repair DNA lesions and possess mechanisms to initiate DNA damage-dependent signalling pathways and to establish DNA damage-dependent checkpoints throughout the cell cycle, which arrest cells in certain cell cycle phases to facilitate repair of damaged DNA or apoptosis of the cell [1,2,8,31]. These checkpoint pathways are essential to diminish genome instability of eukaryotic cells and tumour development in humans [31–35] (Figure 2).

To this end, cells activate signal transduction pathways mediated by sensor, transducer and effector proteins in response to DNA damage. These pathways control a wide variety of processes including cell-cycle arrest and DNA repair pathways, modulate transcriptional activity and may lead to programmed cell death via apoptosis in some cell types [34]. One class of signal transducers are the PI3K (phosphoinositide 3-kinase)-like kinase family, which include the ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) proteins in mammals and their homologues in yeast [34]. In addition, these transducers act upstream in the regulation of two checkpoint kinases Chk1 and Chk2, which act in a subset of the damage response involved in cell-cycle regulation [34]. These proteins are involved in cell-cycle checkpoints which affect the initiation of replicons via intra-S-phase checkpoint signalling [35] (Figure 2). The ionizing radiation-induced intra-S-phase checkpoint requires signalling from ATM through Chk1 and Chk2 to Cdc25A to inhibit cyclin E/CDK2 following double-strand breaks [35] (Figure 2). Another pathway exists which signals through SMC1 (structural maintenance of chromosomes 1) protein, although this signalling is poorly understood [35]. A different signalling pathway is activated which inhibits replicon initiation after treatment with UV or by the carcinogen BPDE (benzo[a]pyrene dihydrodiol epoxide), with the latter inducing bulky adducts on DNA similar to UV-induced lesions [35,36]. These agents do not directly induce double-strand breaks and do not signal through ATM [35,36]. Instead, ATR signals through Chk1 at low doses of UV and BPDE, which selectively inhibit replicon initiation and have little effect on DNA chain elongation [35]. In addition, under these conditions, no Cdc25A degradation occurs and cyclin E/CDK2 is not inhibited [35].

Cdc45 is involved in one such DNA damage-dependent signal transduction pathway [36]. Recent studies demonstrate that Cdc45 is the main target of a Chk1-mediated DNA S-phase checkpoint, which operates via a Cdc25A/CDK2-independent mechanism [36]. In this case, DNA damage induced by BPDE, there is a demonstrated reduction in chromatin-association of Cdc45, accompanied by Chk1 activation and the inhibition of DNA synthesis [36]. This was accompanied by the inhibition of the association between Cdc45 and MCM7 on the chromatin. Moreover, Cdc45 dissociates from a known origin of replication after BPDE treatment [36]. Chromatin association of Cdc45 was shown to reduce in a dose- and time-dependent manner following BPDE treatment, with low doses inducing Chk1 activation, followed by the recovery of Cdc45 chromatin association and DNA synthesis post-treatment [36]. Conversely, high doses of BPDE induced Chk1 and Chk2 activation, where chromatin association of Cdc45 and hence DNA synthesis were found not to recover post-treatment [36].

These results indicate that Chk1 activation negatively regulates the association of Cdc45 and MCM7 at origins of replication via a pathway which operates independently of Cdc25A/CDK2 following low-dose BPDE-induced DNA damage, and that a separate pathway is activated following high-dose-BPDE treatment [36]. This implies that the Cdc45 protein is an important target of a Chk1-mediated S-phase checkpoint [23]. Thus these findings raise the question of the nature and the molecular basis of this newly identified S-phase checkpoint which has Cdc45 as a target.

**Outlook**

First, the use of a UV-mimetic agent (BPDE) [36] raises the question of whether the same signalling pathway is activated after exposing cells to UV. To address this issue, our group has analysed chromatin association of Cdc45 following low and high dose of UV treatment by Western blotting in HeLa S3 cells, confirming the change in chromatin association of Cdc45 following DNA damage observed previously when using BPDE (R. Broderick and H.-P. Nasheuer, unpublished work).

The Cdc45 protein contains a putative PEST (Pro-Glu/Asp-Ser-Thr) motif, several D boxes and a KEN (Lys-Glu-Asn) box [37], all of which are motifs which can be ubiquitylated. As ubiquitylation may not only regulate protein degradation by the proteasome, but also regulate protein function [38], the question exists whether Cdc45 is regulated by ubiquitylation, what residues are modified, and do any modifications occur in a DNA-damage- or cell cycle-dependent manner. Proteomic analyses of Cdc45 are on the way to address this question and to study Cdc45 modifications in soluble and chromatin-associated fractions in cells synchronized at the various cell cycle stages, and cells treated with low and high doses of UV. These functional studies will then be followed up using directed mutagenesis and ectopic protein expression.

In addition, it is currently unknown whether protein–protein interactions of Cdc45 regulate its function either during the normal cell cycle or after UV damage. Bauerschmidt et al. [5,21] extensively analysed the interactions of Cdc45 with proteins known to be involved in DNA replication throughout the normal cell cycle. However, to date, very little data exist about the nature of protein–protein interactions involving Cdc45 following DNA damage. Also, the use of proteomic analysis to screen...
for novel interaction partners both through the normal cell cycle and post-UV treatment may yield more information about the role of Cdc45 in these processes. In conjunction with this, Bauerschmidt et al. [5] examined the subcellular localization of Cdc45 throughout the cell cycle. They describe a mainly nucleoplasmic signal for Cdc45 with a diffuse staining at G1-phase, punctuate staining at S- and G2-phase and a diffuse signal during mitosis, where the Cdc45 signal appears distinct from DNA as stained by DAPI (‘4′,6-diamidino-2-phenylindole) [5]. However, no analysis of the subcellular localization of Cdc45 following UV treatment has yet been described, which merits further investigation.

Indeed, our group has determined significant rearrangements in the subcellular localization of Cdc45 post-UV damage (R. Broderick and H.-P. Nasheuer, unpublished work).

In summary, the Cdc45 protein plays a crucial role in both the initiation and elongation stages of DNA replication, and hence plays a central role in the maintenance of genome stability. The molecular bases of these processes have yet to be fully elucidated, with the possibilities that post-translational modifications and protein–protein interactions may regulate the function of Cdc45 both during the normal cell cycle and following DNA damage. Further work needs to be carried out to yield answers to these questions. Such studies will shed new light on the processes of eukaryotic DNA replication and the maintenance of genome stability.

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