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DNA Replication-Initiation Proteins in Eukaryotic Cells

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Abstract

DNA replication is a highly regulated cellular process in proliferating cells, involving cell cycle dependent assembly of DNA replication-initiation proteins (DRIPs) onto origins of replication. The process of pre-replicative complex (pre-RC) formation at the M-to-G_1 transition, also known as replication licensing, requires origin recognition complex (Orc1-6p) that binds and marks replication origins to facilitate the loading of additional DRIPs, such as Noc3p, Ipi3p, Cdc6p, Cdt1p and Mcm2-7p. The subsequent activation of pre-RC at the G_1-to-S transition is dependent upon cyclin-dependent kinases (CDKs) and Dbf4-dependent kinase (DDK). This sequential process ensures that DRIPs are precisely loaded to form pre-ICs and then activated by their regulators so that chromosomal DNA is replicated only once per cell cycle. Despite substantial gains in the study of the mechanisms and regulation of pre-RC, the finite details of the pre-RC assembly and disassembly processes remain unclear and controversial. In this review we describe the present state of understanding on DRIPs and the pre-RC architecture and dynamics.

Abbreviations: CDC6: Cell Division Cycle; CDC14: Cell Division Cycle; CDC45: Cell Division Cycle; CDT1: Cdc10-Dependent Transcript; CMG: Cdc45p-MCM-GINS; GINS: Slf5, Psf1, Psf2, Psf3; IPI3: Involved in processing IST2; MCM: Mini-chromosome maintenance; NOC3: Nucleolar Complex; ORC: Origin Recognition Complex; Pol30/PCNA: Proliferating Cell Nuclear Antigen; pre-IC: Pre-Initiation Complex; pre-RC: Pre-Replicative Complex; DRIP: DNA Replication-Initiation Proteins

Introduction

The DNA double helix structure was first proposed by J.D. Watson & F. Crick [1]. They also presented a semi-conservative hypothesis, which necessitated that two single strands of a DNA double helix separate and each acts as a template for the synthesis of a new strand of DNA [2]. This semi-conser vative model of DNA replication was later confirmed by experiments (Meselson & Stahl 1958). The replicon model for the control of DNA replication first proposed by Jacob, Cuzin and Brenner states that a trans-acting regulatory factor, the initiator protein specifically binds onto the cis-acting sequence, known as the replicator at a specific chromosomal location to sequester other replication factors to initiate replication of the replicon at the origin of replication [3]. The replicon model therefore formed the basis of DNA replication studies that have been carried out in model organisms such as bacteria, budding yeast, fission yeast and metazoans.

Recent advances in molecular and cellular biology, genetics and biochemistry have resulted in the identification of dozens of DNA replication-initiation proteins (DRIPs) and their regulators that control the initiation of DNA replication. However, these discoveries present new challenges and clearly indicate that there are still many aspects of DNA replication initiation that remain elusive. The initiation of eukaryotic DNA replication is a two-step process involving replication licensing and origin activation. Pre-replication complex (pre-RC) assembly at the M-to-G_1 transition (replication/

origin licensing) is stringently controlled, contributing to the once-per-cell cycle control of DNA replication [4-12]. Deregulated replication may lead to aneuploidy, cancer and/or cell death [13-17].

The pre-replication complex is activated by cyclin-dependent kinases (CDKs) and Dbf4p-dependent kinase (DDK) after the cell has passed the restriction point (START in yeast), to form a pre-initiation complex (pre-IC), at an origin of replication [18-20]. Re-formation of the pre-RC within the same cell cycle is prevented by the phosphorylation (inactivation) of certain pre-RC factors [21,22]. Some other components are exported from the nucleus or degraded [23]. Origin activation is therefore irreversible once the cell becomes committed to the cell cycle after passing the restriction point. Activation of the origin leads to the action of helicases unwinding the DNA double helix and forming replication forks. Replication proteins assembled at replication forks incorporate nucleotides into newly synthesized DNA chains. This is done with high fidelity to ensure that replication results in viable genomic duplication.

Eukaryotic Replication-Initiation Proteins (DRIPs)

Eukaryotic DNA replication has been most extensively studied in budding yeast Saccharomyces cerevisiae [4,5,9,24]. Most of the eukaryotic DRIPs were first discovered in budding yeast, and their homologs in metazoans were then identified by sequence homology. Budding yeast cells replicate their DNA from defined origins of replication that were first defined as autonomously replicating sequences (ARSs). The hetero-hexameric origin recognition complex (ORC) and Noc3p (nucleolar complex-associated protein) are bound to chromatin throughout the cell cycle, forming the post-replicative complex (post-RC) during S, G_2 and most of M phases of the cell cycle [9,12,22,24-27].

After dephosphorylation of DRIPs such as ORC, Cdc6p and Mcm3p by Cdc14p during the M-to-G_1 transition, the sequential loading of Ipi1-3p, Cdc6p, Cdt1p, and MCM proteins at replication origins establishes the pre-replication complex (pre-RC) [9,22,25,27,28]. This process of origin licensing is followed by origin activation [18-20].

Origin Recognition Complex (ORC)

Yeast proteins were fractionated to identify DRIPs, and this led to the discovery of Orc1-6p as a hetero-hexameric protein complex, which was characterized and identified as an ATP-dependent ARS binding protein complex [29,30]. Components of ORC are conserved in eukaryotes, and this facilitated the identification of ORC in other eukaryotic organisms. It is evident now that ORC binds and protects replication origins throughout the cell cycle and is essential for loading other DRIPs onto replication origins [6,10,13,23,26,28-30]. However, the dynamics of ORC at replication origins during DNA replication still remains to be elucidated. All ORC subunits barring Orc6p belong to the AAA+ ATPase super family of ring shaped P-loop NTPases. These proteins bind and hydrolyze ATP to facilitate energy-dependent processes such as remodeling of macromolecules or translocation. Orc1-5p form the critical sub complex that possess DNA binding affinity, but Orc1-6p together, are essential for the assembly and maintenance of pre-RCs.

Orc6p is directly involved in the origin association of Cdt1p-Mcm2-7p [23,32-35]. Some reports have suggested that multiple MCM subunits have direct interactions with ORC during the loading of Cdt1p-Mcm2-7p [9,24,36]. Recent cryo-electron microscopy (cryo-EM) and single molecule studies using purified proteins show that the architecture of the Orc1-6p complex is asymmetric, while the Mcm2-7p complex forms a symmetric double-hexamer during pre-RC formation [36-43]. These interesting structural characteristics have spurred debates in the DNA replication field with several different models being proposed [8,38,40-44].

The "One-ORC" model proposes that a single ORC hexamer recruits one Mcm2-7p single hexamer which in turn recruits the second Mcm2-7p complex [10,41,44]. The "two-ORC" model suggests that two Mcm2-7p complexes are loaded onto origins in the same manner by two ORC single-hexamers, each bound at a single origin [43]. This is based on the finding that increasing ORC concentrations lead to a sigmoidal, rather than linear, increase in MCM loading and that the presence of two ORC binding sites supports MCM loading 10-fold more efficiently than a single ORC binding site [43]. It is interesting to note that most cryo-EM studies support the one ORC model, while there is evidence for the existence of ORC dimers, stated as <10% of the purified ORC [37]. Our recent protein interaction study of budding yeast and human DRIPs shows that several yeast and human ORC subunits self-interact [24].

Our study also suggests that ORC single-hexamers dimerize to form double-hexamers at replication origins before MCM loading at the M-to-G1 transition *in vivo* (unpublished). Importantly, our study suggests that the ORC dimer separates into two single-hexamers, each binding and protecting one of the two newly replicated origins from histone invasion. These findings uncovered an essential, cell cycle-dependent, and likely semi-conservative 'dimerization cycle' of ORC that regulates DNA replication.

Noc3p

Noc3p is a basic helix-loop-helix (bHLH) protein that was initially identified as a component of the heterodimeric Noc2p-Noc3p complex involved in ribosome biogenesis in budding yeast [45-47]. This complex is involved in the formation of the 40S ribosomal subunits and its nuclear export [45]. Studies have indicated that the Noc complex mediates the maturation of 60S ribosomal precursors and the intra-nuclear transport of the 60S ribosome subunit [45]. Noc3p's essential role in DNA replication has been established in budding yeast, where it is required for the chromatin association of Cdc6p and MCM proteins during pre-RC formation [25]. Noc3p is conserved in eukaryotic organisms, and the human homolog FAD24/hNOC3 is involved in DNA replication, differentiation and adipogenesis [48-52,12].

Noc3p in both budding yeast and human cells interacts with ORC, Cdt1p, MCM and other DRIPs, and is required for the recruitment of Ipi3p (involved in processing IST2), Cdc6p and MCM proteins to replication origins for pre-RC formation during the M-to-G_1 transition [9,12,24,25]. Both human and budding yeast Noc3p binds chromatin at replication origins throughout the cell cycle [12,25]. Silencing or depletion of human or budding yeast Noc3p resulted in pre-RC formation failure, S-phase entry defects and eventual cell death, without affecting the cellular levels of other known DRIPs within the experimental timeframe [12,25]. Over-expression of Noc3p results in dosage lethality in Cdc6p mutant yeast cells, just as overexpression of other DRIPs do, which further elaborates Noc3p's role as a DRIP [54]. The specific inhibition of RPA194 (a ribosome biogenesis pathway protein) did not result in pre-RC assembly defects, indicating a separation of function similar to that reported in the budding yeast [12,25].

Results from fission yeast experiments demonstrating significantly impaired S-phase entry and cell cycle progression with eventual G_2/M phase arrest (likely due to incompletion DNA replication) following Noc3p depletion (inevitably incompletion depletion), similar to the phenotypes observed following ORC or MCM depletion, are consistent with current and previous findings regarding Noc3p's role in DNA replication [12,25,54]. Noc3p is therefore a conserved, multifunctional protein. In addition to Noc3p, ORC, Ipi3p and Cdc6p have also been implicated in ribosome biogenesis, as well as DNA replication, suggesting that the processes of DNA replication and ribosome biogenesis are intricately linked and coordinated [10,13,26,28,46,56].

Ipi3p

Ipi3p is a component of the Rix1 complex (Ipi1p-Ipi2p/Rix1p-Ipi3p), which is required for processing of ITS2 sequences from 35S pre-rRNA and ribosome biogenesis [56-58]. A yeast functional proteomic screen identified Ipi1p, -2p and -3p as novel DRIPs [9]. Ipi3p binds chromatin at ARS sequences in an ORC-, Noc3p- and cell cycle-dependent manner [9]. Ipi3p is required for loading Cdc6p, Cdt1p and MCM2-7p onto chromatin during the M-to-G_1 transition and also for the maintenance of the pre-RC in G_1 phase [9]. Ipi3p is therefore required for pre-RC assembly and maintenance, and this is independent of its role in ribosome biogenesis. Interestingly, as Ipi3 also interacts with Noc3p, it serves to connect ORC and Noc3p to the other pre-RC components [9,24]. Like Noc3p and other DRIPs, Ipi3p is also conserved in eukaryotes, including humans [24,27]. The Ipi3p homolog in fission yeast crb3 was reported to interact with cut5 (a protein involved in DNA replication and check point control) [59].

Interestingly the cut5 homolog in budding yeast (Dpb11p) is required for replication initiation after pre-RC assembly [60,61]. Our study has established human hIPI3 as being required for human DNA replication licensing, cell cycle progression and cell proliferation [27]. Like the budding yeast Ipi3p, hIPI3 interacts with several human ORC and MCM subunits [27]. In particular, the interactions of hIPI3 with hORC2, hNOC3 and hMCM2 are consistent with those previously observed in budding yeast [9,27]. Furthermore, hIPI3, like hNOC3p, also preferentially associates with known human replication origins [27]. hIPI3 silencing experiments also suggest that hIPI3 role in DNA replication is independent of its role in ribosome biogenesis.

Cdc6p and Cdt1p

Cdc6p has been extensively studied in budding yeast. The protein belongs to the AAA+ ATPase superfamily and shares a high degree of homology with Orc1p [62]. Deregulation of this protein in humans has been linked to tumorigenesis [63,64]. Cdc6p functions as a DRIP by facilitating Mcm2-7p chromatin binding [28]. Mutations in the Cdc6p ATP binding domain significantly abrogate chromatin binding [65]. Defective Cdc6p ATP hydrolysis results in Mcm2-7p loading failure [65]. Furthermore, Cdc6p is also involved in the maintenance and activation of the checkpoint mechanisms in the cell cycle by coordinating the S and M phases of the cell cycle [66]. When over-expressed, Cdc6p in S. pombe can induce several rounds of DNA replication without cell division [67]. Furthermore, CDC6 gene expression is cell cycle dependent. Cdc6p accumulates during late M and G_1 phases of the cell cycle, owing to the low Cdc28p-Clb5p-Clb6p CDK activity [68].

Cdc6p is degraded at the onset of S phase mediated by CDK phosphorylation, triggering proteolysis through the SCF/Cdc4 pathway [69,70]. This mechanism contributes to the once-per-cell cycle control of DNA replication [68]. Structure studies have shown that Cdc6p binding to the DNA-bound ORC forms the ORC-Cdc6p-DNA complex [38,71]. EM studies suggest that Cdc6p closes the crescent shaped ORC structure, forming a ring [72]. This process renders a conformational change in ORC which facilitates Mcm2-7p loading [10]. Cdt1p (Cdc10-dependent transcript) was first identified in S. pombe as having Cdc10p dependent, cell cycle regulated expression [73]. It was later characterized as a key licensing factor in pre-RC formation in Xenopus [74].

Cdt1p is required for MCM nuclear localization and subsequent chromatin loading in budding yeast [75,23]. Cdt1p depletion abrogates origin firing, but does not affect S phase progression [76]. CDT1 expression is at its highest in G_1 phase followed by a steady decline upon entry into S phase in human cells during [77]. In budding yeast, Cdt1p accumulates in G_1 phase and is subsequently exported into the cytoplasm before the START checkpoint [76]. A number of cryo-EM studies using purified proteins have revealed the complex structure of Cdt1p together with ORC, Cdc6p and MCM proteins [36,42,54,78,79]. Further *in vitro* and *in vivo* studies are required to fully elucidate the structure and interactions of these complexes.

Minichromosome Maintenance (MCM) Proteins

Originally identified through a genetic screen for factors involved in plasmid maintenance in budding yeast [80], the Mcm2-7p complex comprise of six conserved, structurally related proteins

[80-82]. The MCM complex is a critical component of the pre-RC and is involved in replication initiation and elongation [83]. Mcm2-7p chromatin association occurs during late M to early G1 phase, where it migrates with the replication fork in S phase in accordance with its function as a putative DNA helicase [84]. It has been shown that a complex of Cdc45p-Mcm2-7p-GINS (CMG) is the active helicase [85,86].

Regulation of the MCM complex is controlled by the nuclear export sequence (NES) within Mcm3p and the nuclear localization sequence (NLS) found in Mcm2p and Mcm3p [87]. These sequences are in turn regulated by B-type cyclin-CDK phosphorylation and Cdc14p dephosphorylation [22,87,88]. Mcm2-7p belong to the AAA+ ATPase family of proteins. However, none of the subunits actually exhibit any ATPase activity as determined by in vitro studies [89]. MCM complex subunits interact with one another to form a head-tail hetero-hexamer [9,24,89,82].

Cryo-EM studies have now shed further light onto the structure of the MCM complex. A study identified that interlocked amino-terminal interactions form a central channel within the MCM double-hexamer [40]. This channel, comprising of four concentric rings consisting of interior β-hairpins, creates a passage specifically for duplex DNA [40]. The passage is flanked by two pairs of gate-forming Mcm2p and Mcm5p subunits [40]. Although each MCM subunit has unique features and functions, the DNA channel structure forms the catalytic core of the MCM dimer in the budding yeast replicative helicase [11,42].

Pre-Initiation Complex and Proteins at Replication Forks

After pre-RC formation, CDK and DDK promote the loading of Mcm10p, Cdc45p, Sld2p, Sld3p, Dpb11p, GINS and DNA polymerases to the pre-RC, hence forming the pre-initiation complex (pre-IC). These actions are followed by helicase activation and the unwinding of replication origins [84,90-92].

Mcm10p was initially identified through genetic screening [80]. Mcm10p has since been shown to be a requisite for DNA replication initiation and elongation through its interactions with the pre-RC components in budding yeast [93,94]. Mcm10p chromatin association is cell cycle regulated [95]. The Mcm2-7p complex recruits Mcm10p to origins where it coordinates DDK phosphorylation of MCM, resulting in helicase activation [96]. Mcm10p deficiency in budding yeast primarily causes defects in replication fork progression [94]. Studies further implicate that Mcm10p recruits Cdc45p and Pol α/primase to origins [97]. Mcm10p is also a component of the replisome progression complex (RPC) and migrates with the replication fork [98]. Furthermore, Mcm10p recruits Sld3p and Cdc45p to the pre-RC [97]. Sld3p and GINS competitively bind Mcm2-7p [99]. Sld3p dissociates from the MCM complex following DNA unwinding, facilitating GINS-MCM complex interactions for CMG complex formation [99,94].

Subsequently, replication factor A (RFA) binds to protect the single stranded DNA, and new DNA strands are synthesized by DNA polymerases facilitated by other replication proteins. Given the significant advances in our understanding of replication initiation and replication fork progression, future investigations into the mechanisms and regulation of these proteins in normal and cancer cells may lead to discoveries that have significant clinical value.

Concluding Remarks

Eukaryotic genome duplication entails an intricately coordinated process in which the cell enforces a series of steps to ensure the regulated assembly and disassembly of several protein complexes at replication origins. Although there has been significant progress in elucidating the DNA replication initiation control mechanisms, several key questions remain unanswered. At present, several models of DNA replication licensing have been proposed, which although similar in principle, have fundamental differences. For example, the ORC dynamics presents a fundamental dilemma. Is "one-ORC" sufficient to load two MCM single-hexamers in vivo? Or are "two-ORCs" required to allow for the symmetrical and efficient loading of pre-RC proteins? How are new ORC proteins recruited to bind and protect the newly replicated origins? Our recent *in vivo* data suggest that ORC self-interacts and dimerizes to from double-hexamers before MCM proteins are loaded onto replication origins. This process is likely mediated by other DRIP(s) (unpublished). Our model of ORC dimerization presents an essential, cell-cycle dependent and semi-conservative ORC dimerization cycle, in a dynamic cellular system to ensure faithful DNA replication.

Although it has been established that DRIPs are recruited sequentially onto replication origins, their respective biochemical activities have yet to be fully elucidated. For example, most DRIPs are known to bind ATP and have ATP hydrolysis activities. However, how ATP binding and hydrolysis are regulated to facilitate the formation and dissolution of DRIP complexes is not clear.

Another issue concerns the roles of ribosome biogenesis proteins in DNA replication initiation. Ample evidence from studies in yeast and human cells support the dual roles of proteins like Noc3p, Ipi3p, Yph1p and Cdc6p in DNA replication and ribosome biogenesis. Importantly, we have identified separation-of-function Noc3p mutants that are defective in DNA replication without obvious defects in ribosome biogenesis, and vice versa (unpublished). These findings suggest that DNA replication and ribosome biogenesis are coordinated by these multi-functional proteins.

DNA replication is stringently controlled to ensure the integrity of genetic information in cell proliferation. Deregulation of DNA replication may give rise to cancer, for example, the deregulation of Cdt1p and Cdc6p promotes re-firing of the same origin [100]. As a result of active DNA replication and cell division, pre-RC proteins such as Cdc6p and Cdt1p and MCM complex proteins are found to be overexpressed in cancers to promote cancer development,

invasion and metastasis [101,102]. Importantly, it has been shown that silencing of DRIPs results in apoptosis of cancer cells but not normal cells [103-113]. It is therefore important to study DNA replication, particularly DRIPs, to further understand the molecular pathways and mechanisms involved in cancer development, helping to develop novel cancer detection methods and therapeutics.

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