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学 位 論 文 題 目	The Sld5 and Psf1 proteins required for chromosomal DNA replication in *Saccharomyces cerevisiae*
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論文内容の要旨

In *Saccharomyces cerevisiae*, chromosomal DNA replication initiates at a restricted region known as the autonomously replicating sequence (ARS). The origin recognition complex (Orc) binds ARS throughout the cell cycle and the minichromosome maintenance (Mcm) proteins are recruited by Cdc6 from late M phase to G_1 phase to form the pre-replicative complex (pre-RC). Then, the Sld3-Cdc45 complex joins the pre-RC and ARS region is unwound in cooperation with Cdk and Cdc7/Dbf4 protein kinases. Finally, DNA polymerases are recruited to ARS and this step requires Dpb11, which forms a complex with DNA polymerase ε (Pol ε). However, how the Dpb11-Pol ε complex is recruited to ARS has not been known. In this article, we describe novel replication proteins that seem to mediate between the Sld3-Cdc45 and the Dpb11-Pol ε complexes at the initiation step of DNA replication.

To gain an insight into the function of Dpb11, we have isolated *sld* (synthetic lethality with *dpb11-1*) mutations. One of the *SLD* genes, *SLD5* encodes a 34-kDa protein, which amino acid sequence is well conserved among eukaryotic organisms and is essential for cell growth. Since original *sld5-1* mutation itself did not show any defect in cell growth, we isolated four thermosensitive *sld5* mutations. At the restrictive temperature, all the thermosensitive mutant cells arrested with a dumbbell-shape with a single nucleus with a DNA content between 1C and 2C, suggesting that Sld5 is required for DNA replication. Since all of them divided more than once after temperature shift up, we had not characterized them in detail.

To identify factors interacting with Sld5, we isolated *PSF1* (Partner of *SLD* five) as a multicopy suppressor of the *sld5-12* thermosensitive mutation. The *PSF1* gene encodes a

24-kDa protein, which amino acid sequence is well conserved among eukaryotic organisms and is essential for cell growth. To understand the function of *PSF1*, a thermosensitive mutation, *psf1-1*, was isolated and characterized. At the restrictive temperature, *psf1-1* cells arrested with a dumbbell shape with a single nucleus as observed in *sld5* thermosensitive mutants. To investigate whether DNA is synthesized at the restrictive temperature, *psf1-1* cells were arrested in G_1 phase with α-factor and released at the restrictive temperature. DNA content of *psf1-1* cells had not increased for 120 min, and then gradually reached 2C while wild-type cells reached 2C DNA content by 80 min, suggesting that Psf1 is required for an early step of DNA replication. Since cells defective in initiation of DNA replication begin to lose viability immediately after cells start budding at the restrictive temperature, we determined the point at which the cells start losing viability. At the restrictive temperature, *psf1-1* cells as well as *sld5-12* cells started losing viability when cells started budding. It suggests that Psf1 participates in the initiation step of DNA replication. We also arrested *psf1-1* cells in S phase with HU or in M phase with nocodazole and then released them at the restrictive temperature. In both cases, cells entered subsequent G_1 phase and arrested with 1C DNA content while *psf1-1* cells after release from HU-arrest reached 2C DNA content 30 min later than wild type cells. Since HU blocks late-origin firing, it is likely that Psf1 is essential for all origin-firing during S phase progression and chromosome DNA replicates only from early-firing origins in *psf1-1* cells released at the restrictive temperature from HU.

The transcript-level of *PSF1* is reported to fluctuate during the cell cycle and to peak at G_1/S phase boundary. We thus examined the protein-level of Psf1 by western blotting using tagged Psf1 and found that the Psf1 protein level is roughly constant during the cell

cycle. Then, we determined cellular localization of the Psf1 protein during the cell cycle by indirect immuno-fluorescent microscopy and revealed that the Psf1 protein is localized in nucleus throughout the cell cycle. As Psf1 is a nuclear protein required for DNA replication, we examined whether the Psf1 protein associates with ARS region *in vivo* using chromatin immunoprecipitation (CHIP) assay. The CHIP assay revealed that Psf1 associates with early ARSs, ARS1 and ARS305 from 60 min after release from α-factor and 75 min with late ARS, ARS501. Psf1 also reassociated with non-ARS fragments in later period. We further showed that Sld5 associates with ARS1 at the same timing as Psf1, suggesting that Sld5 and Psf1 associate with ARS together. During the CHIP assay, we also found that Psf1 coimmunoprecipitates with Rfa when Psf1 associates with chromosome DNA. Rfa binds single-stranded DNA, which appears in unwound origins and at replication forks during DNA replication. Therefore, these results suggest that Psf1 associates with replication origins and folks.

Our genetic analysis strongly suggests that Psf1 interacts with Sld5 because thermosensitive growth of *psf1- 1* and *sld5-12* was suppressed by high-copy *SLD5* and *PSF1,* and the *psf1-1* mutation was synthetically lethal with *sld5-12.* Two-hybrid analysis also showed an interaction between Psf1 and Sld5. We therefore performed coimmunoprecipitation assay and demonstrated that Psf1 co-immunoprecipitates with Sld5 in G_1, S and M phases. We further showed that the Sld5-Psf1 complex is hardly detected in *psf1-1* cells. Since *psf1-1* cells are defective in DNA replication, it seems likely that complex formation between Psf1 and Sld5 is required for chromosomal DNA replication.

Our two-hybrid assay further showed that Psf1 interacts with Dpb11, Dpb2 and Sld3. The Sld3-Cdc45 complex associates with ARS and this association is required for

unwinding of ARS region. Dpb2 is a second largest subunit of Pol ε that forms a complex with Dpb11 and associates with ARS after unwinding of ARS. Thus, Psf1 interacts with the proteins that associate with ARS before and after its unwinding. We therefore propose that the Sld5-Psf1 complex mediates between Cdc45-Sld3 and Dpb11-Pol ε complexes for recruitment of DNA polymerases to ARS.

論文の審査結果の要旨

真核生物の染色体 DNA 複製開始領域には、M期後期から G1 期にかけて複数の開始タンパク質が集合した後、DNA ポリメラーゼがロードされ、DNA 合成が開始すると考えられている。出芽酵母では、DNA ポリメラーゼの複製開始領域へのローディングには Dpb11 タンパク質が必要である。また、*DPB11* 遺伝子と遺伝学的に相互作用する因子として、複数の *SLD*(Synthetic lethality with *dpb11-1*)遺伝子が分離されているが、それらの機能については明らかではない。本論分では、出芽酵母をモデル系として、真核生物染色体 DNA の複製機構を明らかにするため、*SLD* 遺伝子の１つである *SLD5* 遺伝子及び *SLD5* と相互作用する *PSF1* 遺伝子の解析を行っている。

まず、*SLD5* 及び *PSF1* 遺伝子は増殖に必須であったため、それら遺伝子の温度感受性変異を分離してその性質を調べた。その結果、両遺伝子の変異株とも、非許容温度で DNA 複製に欠損を示したため、両遺伝子は複製に関与していると結論した。さらに、G1 期に同調した *psf1-1* 変異株を非許容温度で細胞周期を開始させると、野生株が DNA 合成を終了する時間でも DNA 合成はほとんど起らず、Psf1 が染色体の複製開始に関与していることが示唆された。また、CHIP(Chromatin Immunoprecipitation)法を用いて、Psf1 と複製開始領域の結合を調べると、Psf1 はＳ期に複製開始領域に結合していることが分かった。このことは、Psf1 が直接、DNA 複製の開始に関与していることを示している。

次に、Psf1 と Sld5 の抗体を作成し、免疫沈降を行ったところ、それぞれの抗体で Psf1 と Sld5 が共沈殿することがわかり、両者が同一の複合体中に存在していることが明らかになった。また、G1, S, G2/ M 期において、この複合体は存在するが、*psf1-1* 変異では、この複合体は検知できなかった。*psf1-1* は複製に欠損を示すため、Psf1 と Sld5 を含んだ複合体形成が複製に必要であることが示唆される。

最後に、Psf1, Sld5 と他の複製タンパク質の間の２ハイブリッド法による結果から、Psf1, Sld5 も DNA ポリメラーゼの複製開始領域へのローディングに関与するというモデルを提案している。

以上ののように高山さんは多数の実験を注意深く行い、複製タンパク質、Sld5, Psf1 の機能について新しい知見を与えたことは、この分野への寄与も大きく、学位授与の要件を満たすものと審査員一同判断した。