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Processing of stalled replication fork under thymine starvation and its relationship with thymineless death in *Escherichia coli*

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In this work we characterized the mechanism by which the stalled replication forks generated under depletion of deoxynucleotides (dNTP) are processed and the relationship of the fork processing with the viability of the cell. RFR occurred at the stalled forks generated by hydroxyurea treatment (chemical inactivation of NDP reductase), was impaired under incubation of *nrdA101* mutant at 42ºC (structural inactivation of NDP reductase), and did not take place under thymine starvation. Viability experiments confirmed RFR model predictions and supported that thymineless death (TLD) is related with the processing of the stalled forks. Furthermore we show that TLD conditions suppressor generated stalled forks processed by RFR process. We suggest is that the connection between DNA replication and TLD is through the fate of the stalled replication forks when thymine is removed.

Keywords replication, TLD, DSB, RFR, NDP reductase

1. Introduction

Impairment of replication fork progression is a serious threat to living organisms and a potential source of genome instability[1, 2]. Several strategies have been proposed for the processing of inactivated replication forks. Most of these require the action of recombination proteins, with different proteins being implicated, depending on the cause of the fork arrest [3]. In several replication mutants, the stalled forks generated upon inactivation of the mutant enzyme are reversed and result in the formation of a Holliday junction (HJ) adjacent to a DNA double strand end, a reaction called 'replication fork reversal' (RFR) (Fig.1A) [4]. In a *rec* proficient background this intermediary could be processed without generating DNA double-strand breaks (DSBs) by using the recombination proteins RecBCD, RecA, and by the HJ-specific resolvase RuvABC (Fig.1 B) [5]. In contrast, in the absence of RecBCD activity (Fig. 1C), resolution of the RFR-produced HJ is done by RuvABC resolvase and leads to fork breakage [6]. These particular DSBs are dependent on RuvABC activity in a *recB* deficient background. In this work we did a comparative study of the ability of the replication forks to be reversed after they have been stalled by depletion of DNA precursosors, deoxyribonucleotides (dNTP). The depletion of dNTP was achieved by inactivation of NDP reductase and by thymine starvation. Inactivation of NDP reductase was attained in two ways: by addition of hydroxyurea (Hu) which is a specific inhibitor of NDP reductase activity [7], or by the incubation of an *nrdA101* thermosensitive mutant strain at 42ºC.

NDP reductase is the only specific enzyme required for the enzymatic formation of dNTP, and has been proposed to be a structural component of the replication hyperstructure in *E. coli* [8,9]. It is a 1:1 complex of two subunits called proteins R1 and R2, coded by genes *nrdA* and *nrdB*, respectively [10]. For many years it has been known that thymine auxotrophic microorganisms undergo cell death in response to thymine starvation, the so-called thymineless death (TLD) [11]. This phenomenon has been researched for over five decades, but the molecular mechanism remains an enigma. Our proposal is that TLD is related with the fate of the stalled replication forks generated when thymine is removed.

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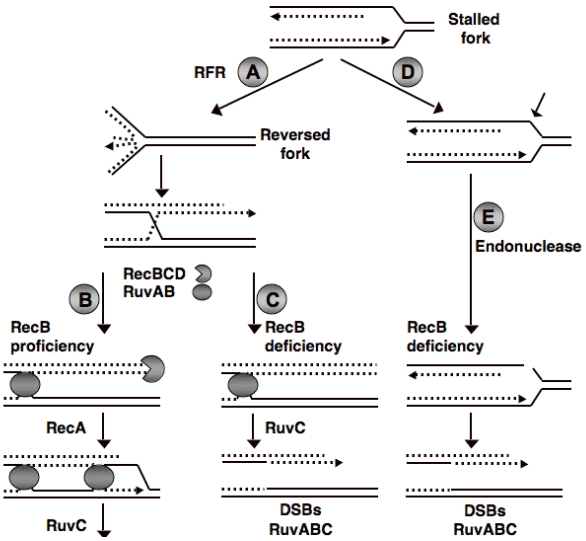

Figure 1. The fate of the stalled forks. In the first step (A), the replication fork is arrested, causing fork reversal. The reversed fork forms a HJ (two alternative representations of this structure are shown – open X and parallel stacked X). In Rec^+ cells (B), RecBCD initiates RecA-dependent homologous recombination, and the resulting double HJ is resolved by RuvABC. In the absence of RecBCD (C), resolution of the HJ by RuvABC leads to DSBs at the stalled replication fork. Alternatively, the replication fork is arrested without being regressed (D) and it is susceptible to be cut by an endonuclease, generating DSBs at the stalled replication fork (E). Continuous line (parental chromosome); dashed lines (newly synthesized strands); disk (RuvAB); incised disk (RecBCD).

2. Results and Discussion

2.1. Experimental approach

In this work we have studied the fate of the replication forks stalled using different ways of depleting DNA precursors. To verify whether the RFR process is occuring after a treatment, a *recB* deficient background should be used. According to the RFR model, the occurrence of this process at the stalled forks can be verified by testing whether there is an increase of DSBs in a *recB* deficient background, and determining whether these DSBs are dependent on RuvABC resolvase activity by measuring the amount of DSBs in a *recB* and *recB ruvABC* deficient background (Fig. 1 C) [6]. The occurrence of RFR at the stalled forks has been verified by this system in several replication mutants [4]. If RFR does not take place at the stalled fork, at least two situations may arise. In one hand, there would be an increase of DSBs independent of RuvABC activity and generated by another unknown endonuclease (Fig.1E) [1]. On the other hand, there would be no increase in the amount of DSBs probably because the stalled forks are not susceptible to the endonuclease activity, and the restarting of the forks would take place without the generation of fork breakage [4].

2.2. Starvation of DNA precursors induces DSBs

Cultures of strains JK626 (*thyA arg his recB258::Tn10*), JK707 (*thyA arg his recB258::Tn10 ΔruvABC::Cm*) and their isogenic *nrdA101* counterparts JS628 and JS705 respectively, were grown at 30ºC in M9 minimal medium containing 5 µg/ml thymidine, 5 µCi/ml [*methyl*-^{3}H] thymidine (100 Ci/mmol), 20 µg/ml of required aminoacids and 0.2% of casaminoacids. By the time the cultures reached 0.2 OD_{450nm} one portion of each of the JK626 *(recB)* and JK707 *(recB ruvABC)* cultures was treated with Hu 50 mM (freshly prepared at 1M in MM9), and a second portion was thymidine starved at 30ºC by collecting the cells on a Millipore filter, and washing and resuspending them in M9 medium free of thymidine. To achieve the thermal inactivation of NDP reductase, cultures of JS628 and JS705 *nrdA101* strains growing at 30ºC were transferred to 42ºC at 0.2 OD_{450nm}. To determine the extent of DSBs, the amount of linear DNA was quantified before beginning the treatment, and after two hours of treatment for Hu addition and for 42ºC incubation, and after 30 min for the thymidine starvation treatment.

Cells labeled with [*methyl* ^{3}H]-thymidine were gently lysed in agarose plugs and their DNA was analyzed by pulse field gel electrophoresis (PFGE), in which only linear chromosomes enter the gels while circular molecules remain in the wells [2, 6].

The results show that inactivation of NDP reductase by Hu addition increases by up to twofold the amount of linear DNA in the strain JK626 (*recB)* and JS628 (*nrdA101 recB*), indicating the increase of DSBs (Table 1). However, when we tested whether incubation of the strain JS628 (*nrdA101 recB*) at 42ºC led to DSBs formation, we only found a slight increase of DSBs relative to 30ºC (Table 1, compare JS628 at 30ºC and at 42ºC). To investigate the occurrence of RFR at stalled forks generated by TTP depletion without altering the NDP reductase, we measured DSBs in JK626 (*recB)* under thymine starvation using a similar approach to that performed with the other conditions. We found induction of DSBs in the strain JK626 (*recB*) after 30 min of thymidine starvation (Table 1).

Table 1. *Amount of linear DNA in nrdA+ and nrdA101 strains under dNTP depletion conditions. Data are expresed in % linear DNA (mean±SD)*

Strain	*Relevant genotype*	*30ºC*	*42ºC*	*+Hu*	*-TdR*
JK626	*nrdA+recB*	15.18±2.83	13.97±7.10	30.33±3.20	24.14±7.12
JK707	*nrdA+ ruv recB*	6.74±2.60	2.63±0.70	3.68±2.10	26.26±9.83
JS628	*nrdA101 recB*	24.79±7.05	32.09±6.02	40.88±2.81	-
JS705	*nrdA101 ruv recB*	5.94±2.19	3.34±2.28	5.55±0.93	-

2.3. Stalled forks created by thymidine starvation are not reversed

In order to test whether these DSBs resulted from the action of RuvABC, PFGE was performed with JK707 (*recB ruvABC*) in the presence of Hu or thymidine starvation, and with JS705 (*recB ruvABC*) in the presence of Hu or after incubation at 42ºC. The level of linear DNA dramatically decreased in the presence of Hu or thymidine starvation (Table 1), indicating that the DSBs induced by dNTP depletion resulted from the RuvABC resolvase activity in a *recB* deficient background. Therefore we conclude that RFR is induced at the stalled forks generated by depletion of dNTP. Incubation of *nrdA101* strain at the restrictive temperature induced only a small increase of RuvABC-dependent DSBs. This would indicate that stalled forks generated under thermal inactivation of the NDP reductase have much lower propensity to be regressed and cut by RuvABC resolvase than the stalled forks generated under chemical inactivation of the enzyme. Surprisingly, we found that the DSBs level was not reduced under thymidine starvation in *recB ruvABC* strains (Table 1). Consequently these DSBs were not dependent on RuvABC resolvase activity as they occurred under RuvABC inactivation, indicating that replication forks stalled by thymine starvation do not undergo RFR process. The DSBs found under this treatment could have been generated either (i) by direct endonucleolytic cleavage of single strand breaks at or near stalled forks (Fig. 1D), or (ii) by cleavage of single strand breaks at places not related with replication fork, or (iii) by both.

2.3. Reversed replication forks prevent lethality

In addition to the formation of RuvABC dependent DSBs in a *recB* deficient context, RFR model would imply differences on the viability of *rec*-deficient strains under Hu treatment and thymidine starvation. In one hand, if RFR occurred after Hu addition it would be expected: 1) a lethality in the *recB* mutant strain due to the unrepaired DSBs, and 2) a suppression of this lethality by the inactivation of RuvABC resolvase in a *recB ruvABC* deficient strain. On the other hand, if RFR was not taking place under thymidine starvation, TLD should not be alleviated in the *recB* mutant strain by the deficiency of the RuvABC resolvase; as DSBs induced by thymidine starvation are not avoided in a *recB ruvABC* deficient strain.

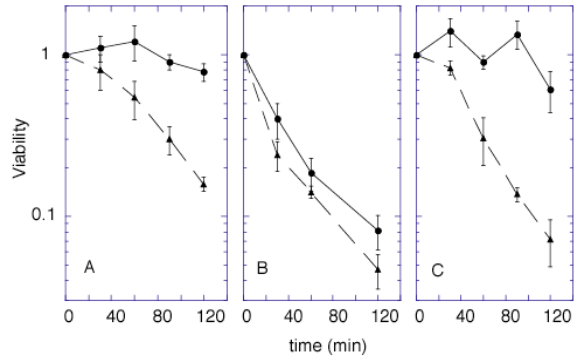

Figure 2. Effect of Hu addition (●) and thymidine starvation (▲) on the viability of (A) the *rec*+ strain, JK607; (B) the *recB* deficient strain, JK626, and (C) *recB ruvABC* deficient strain, JK707. Bold lines indicate the standard deviation from at least four independent experiments.

Viability experiments were performed by growing the strains JK607 (*rec*+), JK626 (*recB*) and JK707 (*recB ruvABC*), up to 0.2 OD and treated by Hu addition or by thymidine starvation in the same experimental conditions as PFGE experiments. At indicated time intervals 0.05-ml aliquots were removed, appropriately diluted, and plated on rich medium plates for determination of viability. We found the viability of the *recB* deficient strain to be highly sensitive to the addition of Hu and thymidine starvation compared with wild type (Fig. 2A, B), as expected by the increase of un-repaired DSBs in the absence of RecBC activity. This detrimental effect was recovered by the inactivation of RuvABC resolvase only in the case of Hu addition but not under thymidine starvation (Fig. 2C). These results support that RFR occurs when DNA replication was inhibited by Hu treatment, but not when it was performed by thymidine starvation.

2.4. Reversed replication forks prevent TLD

Recently, Morganroth and Hanawalt have observed that TLD occurs under Hu addition [12]. According to our proposal it could be expected a supression of TLD under Hu addition, but it has to be pointed out that our prediction would be only in the case the stalled fork underwent RFR under these conditions. In the mentioned work Hu addition was performed at the same time as the culture was thymine starved, and under these conditions the fate of the stalled forks is, at this moment, unknown. In this work we performed the experiments separating in time both treatments. In one hand, Hu was added first, in order to generate reversed stalled replication forks, and five minutes later the culture was starved for thymidine in the presence or absence of Hu (Fig. 3A). These results show that TLD was supressed by the addition of Hu to the culture prior to the starvation of thymidine. In the other hand, the culture was starved for thymidine for 30 minutes first, in order to generate stalled replication forks no-reversed and then the culture was treated with Hu in the presence or absence of thymidine (Fig. 3B). These results show that Hu addition becames lethal if the culture was previously starved for thymidine.

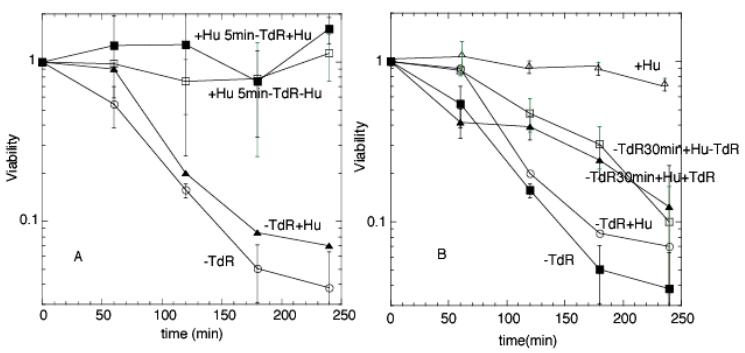

Figure 3. Viavility of rec+ strain JK607 under thymidine starvation and Hu addition. (A) Effect of 5 min of Hu addition prior to thymidine starvation in the presence or absence of Hu. (B) Effect of 30 min of thymidine starvation prior to Hu addition in the presence or absence of Hu.. Bold lines indicate the standard deviation from at least four independent experiments.

Knowing that addition of rifampicin prevents TLD [11], we determined the origin of the DSBs generated under thymidine starvation in the presence of rifampincin. The results (Table 2) showed that the mayority of the DSBs under this condition are originated by RuvABC endonuclease, indicating that the inhibition of transcription permited the reversion of the replication forks stalled by thymidine starvation, and furthermore that there is a relationship between the fate of the stalled replication forks and the effect of the treatment on the cell viability.

Table 2 *. Amount of linear DNA in nrdA strains under thymidine starvation in the presence of rifampicin. Data are expresed in % linear DNA (mean±SD)*

Strain	*Relevant genotype*	*30ºC*	*-TdR*	*-TdR +Rif*	*+Rif*
JK626	*nrdA+recB*	15.18±2.83	24.14±7.12	24.50±2.05	13.97±1.31
JK707	*nrdA+ ruv recB*	6.74±2.60	26.26±9.83	10.46±3.11	3.82±0.98

3. Concluding remarks

The present data show that stalled replication forks display a differential capacity to undergo RFR after depletion of deoxynucleotides brought about in three different ways. Replication fork reversal occurred at the stalled forks generated by Hu addition, was impaired with thermally inactivated NDP reductase, and it did not take place with thymine starvation. Besides the absence of RFR at the replication forks under thymine starvation, the occurrence of DSBs independent of RuvABC endonuclease needs to be explained. We suggest that these DSBs (or at least a number of them) were originated by endonucleolytic cleavage at or near stalled replication forks (Fig.1 D, E), explaining the connection between replication forks stalled by thymine starvation and TLD proposed by Maaløe and Hanawalt in the 1960s. Our proposal is that this connection is not due to the replication process it self, as it has been shown not to be required [12], but through the fate of the stalled replication forks when thymine is removed.

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