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Report

Uncoupling the replication machinery

Replication fork progression in the absence of processive DNA synthesis

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Abbreviations: APH, aphidicolin; Fen1, flap endonuclease 1; Mcm, minichromosome maintenance; PCNA, proliferating cell nuclear antigen; pol, polymerase; RPA34, replication protein A subunit 34; SSB, single-strand binding; ssDNA, single-stranded DNA

Key words: PCNA, DNA polymerase, RPA, DNA ligase I, Fen1, Mcm, replication machinery, aphidicolin

The precise coordination of the different steps of DNA replication is critical for the maintenance of genome stability. We have probed the mechanisms coupling various components of the replication machinery and their response to polymerase stalling by inhibition of the DNA polymerases in living mammalian cells with aphidicolin. We observed little change in the behaviour of proteins involved in the initiation of DNA replication. In contrast, we detected a marked accumulation of the single stranded DNA binding factor RPA34 at sites of DNA replication. Finally, we demonstrate that proteins involved in the elongation step of DNA synthesis dissociate from replication foci in the presence of aphidicolin. Taken together, these data indicate that inhibition of processive DNA polymerases uncouples the initiation of DNA replication from subsequent elongation steps. We, therefore, propose that the replication machinery is made up of distinct functional sub-modules that allow a flexible and dynamic response to challenges during DNA replication.

Introduction

Duplication of the genome is an essential aspect of eukaryotic cell division. This process is mediated during S phase by the replisome, a large protein complex that assembles in a regulated, step-wise fashion at sites throughout the genome known as origins of replication.[1] The assembly and activity of the replisome is conserved throughout evolution and can be divided into three phases: activation, origin initiation and elongation.

Activation of DNA replication requires the specific association of protein complexes with the origins of replication. A number of proteins have been implicated in this process, and more are likely to be identified. Two groups of proteins, the origin recognition complex (ORC) and the minichromosome maintenance proteins (Mcm) are known to play a critical role in mediating origin activation.[2-4] This initial step serves to mark the origins and promotes the recruitment of further replisome components. The next phase, origin initiation, involves the establishment of a replication fork. The Mcm proteins, in conjunction with additional factors, are thought to function as a replicative helicase that opens up the DNA duplex.[5-8] The resulting single-stranded (ss)DNA is coated by the single-strand DNA binding (SSB) replication protein A complex (RPA), which consists of three distinct subunits.[9-12] This complex associates with the primase subunit of the DNA polymerase (pol) α/primase complex, which synthesizes short RNA/DNA primers that are able to initiate DNA replication.[13-17] Upon completion of this step, a functional, replication fork is now present at the origin of replication. The subsequent loading of the polymerase clamp PCNA promotes the final phase in replisome assembly. PCNA functions as a loading platform for the highly processive DNA polymerases (such as DNA pol δ) that are primarily responsible for replicating the genome.[18] In addition, PCNA recruits additional elongation factors such as the Okazaki fragment maturation proteins DNA Ligase I and the Flap endonuclease1 (Fen1) which associate via their PCNA binding motif (PBD).[19-21]

Much of the current knowledge of the eukaryotic DNA replisome has been gained from in vitro studies and in vivo genetic analysis performed in *S. cerevisiae*. However, less is known about the assembly and coordination of the metazoan replication machinery in vivo. One particularly important issue is whether the replisome functions as a single holoenzyme, or if it consists of distinct functional submodules. In this work, we have investigated this question by examining the interactions between components of the mammalian replication machinery following treatment with the DNA polymerase inhibitor aphidicolin.

Aphidicolin has been shown to specifically inhibit DNA pol α[22,23] and DNA pol δ[24,25] by competing with dNTPs for binding to the polymerase, with DNA pol δ being roughly 10 times more sensitive than DNA pol α.[26,27] Addition of aphidicolin to isolated nuclei

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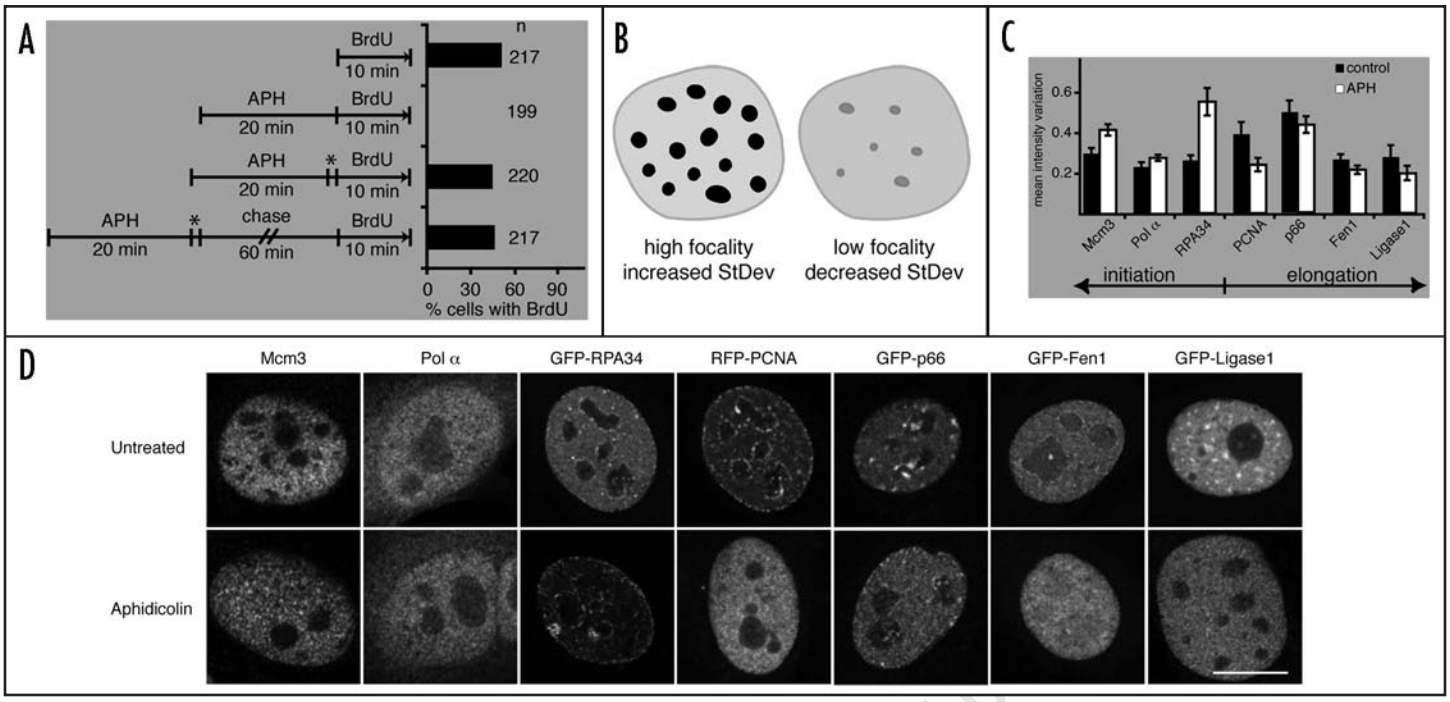

Figure 1. Aphidicolin causes a reversible inhibition of DNA synthesis and leads to an altered localization of replication factors. (A) Cells were incubated with 50 μg/ml aphidicolin (APH) for 20 minutes. Inhibition and resumption of DNA synthesis were assayed by BrdU pulse labelling. BrdU was added during the aphidicolin incubation, immediately following an aphidicolin wash out (asterisk) or 60 min after removal of aphidicolin. BrdU positive cells were counted in untreated and treated populations with the total number (n) of evaluated cells stated next to the respective bars. (B) Schematic illustrating the basis for our measurement of focality in control versus aphidicolin treated cells. (C) To quantify the changes in focal accumulation following treatment with aphidicolin, we calculated the mean intensity variation of fluorescence intensities in control (black bars) and aphidicolin treated cells (white bars) (see Methods). (D) Following a 20 minute treatment with either DMSO (untreated; top row) or 50 μg/ml aphidicolin (bottom row), we examined the localization pattern of replication proteins in S-phase cells. To ensure analysis of S-phase cells, endogenous DNA polymerase α and Mcm3 were visualized by immunofluorescence microscopy in cells stably expressing GFP-PCNA. Similarly, GFP-RPA34, GFP-p66 (66 kDa subunit of DNA polymerase δ), GFP-Fen1 and GFP-Ligase I were detected in cells co-expressing RFP-PCNA. Scale bar, 10 μm.

did not inhibit the initiation of replication or the formation of short primers below 40 nucleotides in length. However, these primers were not elongated into Okazaki fragments.[28,29] These results indicate that while aphidicolin is a potent inhibitor of DNA replication, it has little effect on the activation or initiation of replication origins.

Our results demonstrate that replication fork progression is not stalled in the presence of aphidicolin; rather the initiation and the elongation machineries become uncoupled. We observed no significant changes in the distribution of initiation factors such as Mcm3, while the accumulation of RPA34 increased at replication foci. This suggests that replication fork progression continued in the absence of processive DNA synthesis, creating long patches of ssDNA covered with RPA34. In contrast, the polymerase clamp PCNA, the p66 subunit of DNA polymerase δ and the Okazaki fragment maturation factors (DNA Ligase I and Fen1) all dissociated from RF following aphidicolin treatment. The differential behaviour of these factors following cessation of DNA polymerization highlights the dynamic and modular composition of the replication machinery.

Results

Fast and reversible effect of aphidicolin on DNA synthesis. In order to probe the coordination of different components of the replication machinery in vivo, we treated mammalian cells with aphidicolin, an inhibitor of DNA polymerases δ and α. As an initial characterization of this treatment, we incubated unsynchronised mammalian cells with 50 μg/ml aphidicolin and assayed for ongoing DNA replication with a 10 min pulse of BrdU, a halogenated nucleotide analog. DNA synthesis was completely inhibited after 20 minutes of aphidicolin treatment and resumed within 10 minutes removal of the aphidicolin (Fig. 1A). These observations indicate that treatment with aphidicolin at these concentrations interferes with the replication process in a fast, efficient but also reversible manner.

Differential effect of aphidicolin on localization and binding of proteins involved in the various steps of DNA replication. To study the behavior of replisome components following aphidicolin treatment, we first analysed their localization in fixed cells. The factors that we focused on are displayed according to their time of action during DNA replication, from the DNA duplex unwinding to the ligation of Okazaki fragments. We visualized the various factors via either immunostaining detection of endogenous proteins (DNA pol α and Mcm3) or through ectopic expression of fluorescently tagged proteins (GFP-RPA34, RFP-PCNA, GFP-p66, GFP-Fen1 and GFP-Ligase I). We took two steps to ensure that we limited our analysis to S-phase cells. First, all proteins were detected in cells stably expressing RFP/GFP-PCNA or GFP-RPA34 as a marker for DNA replication. Second, to facilitate comparison, we chose representative cells in mid and late S-phase, but early S-phase cells showed similar results.

In untreated cells we identified two distinct localization patterns (Fig. 1D). Mcm3 and pol α were both detectable in a highly

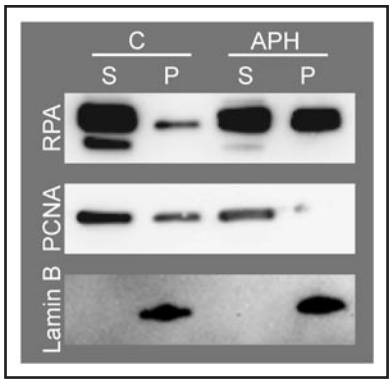

Figure 2. Inhibition of DNA replication with aphidicolin differentially affects chromatin binding of PCNA and RPA34. Western blot analysis of cell fractions obtained from control and aphidicolin treated cells. Following extraction with Triton X-100 and centrifugation, equivalent amounts of the supernatant and pellet fractions from each population of cells were run on SDS-PAGE gels. While RPA accumulated at high levels on the DNA, PCNA dissociated upon aphidicolin treatment.

punctuate pattern evenly distributed throughout the nucleus. While these proteins play an integral role in initiating DNA replication, they do not exhibit co-localization with sites of ongoing processive DNA synthesis (data not shown and ref. 30). In contrast, the other replication factors (RPA34, p66, PCNA, Fen1 and DNA Ligase I), in addition to diffuse nuclear staining, were present in bright nuclear foci that correlate with sites of active DNA replication as visualized by pulse labelling with nucleotides.[19,31-34] Following a 20-minute treatment of cells with aphidicolin, we observed an altered localization pattern in a subset of these proteins (Fig. 1D). While early-acting replication factors such as Mcm3 and DNA pol α exhibited very little change, the single stranded DNA binding protein RPA34 showed an increased focal accumulation. In contrast, the focality of the proteins involved in the elongation phase of DNA replication (PCNA, p66, Fen1 and DNA Ligase I) decreased in the presence of aphidicolin. Several factors (PCNA, Fen1 and DNA Ligase I) were no longer present in discrete foci, but instead diffusely distributed throughout the nucleus, while p66 appeared in smaller, less intense foci. This observation suggests that treatment with aphidicolin disrupts the replication machinery by specifically promoting the dissociation of factors involved in the elongation step of DNA synthesis.

We quantified the changes in focal accumulation following aphidicolin treatment to more accurately describe this effect. Nuclei with very bright foci will exhibit a broad range of pixel intensities and hence calculating the standard deviation across the nucleus reflects this distribution. In contrast, nuclei with a less pronounced focal pattern will have a more uniform and narrow distribution of pixel intensities and exhibit a smaller standard deviation (illustrated in Fig. 1B). Therefore, we measured the mean intensity variation of the fluorescence intensities in control versus treated cells (Fig. 1C and Materials and Methods). We could find minor changes in the focal localization of Mcm3, RPA34 (both focal accumulation) and PCNA (delocalization). The differences for Fen1, DNA Ligase I and p66 were relatively small, which can be explained by an overall higher nucleoplasmic fraction of these proteins compared to factors such as PCNA.[19]

It has previously been observed that PCNA loaded at DNA replication sites remain bound to chromatin following extraction with detergent.[35] If aphidicolin were able to disrupt the association of PCNA with sites of DNA replication, we predict that this factor would then become susceptible to detergent treatment. To test this hypothesis, we extracted cells with Triton X-100 in the presence or absence of aphidicolin, and assayed for the ability of both PCNA and RPA34 to remain bound to chromatin. Following centrifugation to separate soluble proteins (cyto- and nucleoplasm) from insoluble chromatin-bound proteins, these fractions were examined by Western blot analysis (Fig. 2). Our results show that the fraction of unextractable, and thus chromatin-bound, RPA34 increased in aphidicolin treated cells whereas PCNA become extractable upon aphidicolin treatment.

These results indicate that components of the replisome exhibit an array of responses following aphidicolin treatment. Some early replication factors, such as Mcm3 and pol α, remain relatively unaffected. RPA34 accumulates to a greater extent at sites of DNA replication, while PCNA, p66, and the lagging strand maturation factors DNA Ligase I and Fen1 dissociate from replication foci. Taken together, these data suggest that inhibition of DNA polymerase by aphidicolin promotes an uncoupling of initiation and elongation factors of the replication machinery. Previous in vitro studies have suggested this idea,[36] but up to this point it had not been observed in cells.

Temporal order of the processive elongation factor disassembly. An advantage of examining DNA replication in situ is that it allows for time-lapse analysis of protein dynamics in living cells. Neither PCNA, Fen1 nor DNA Ligase I remain bound to replication foci following aphidicolin treatment. However, it was unclear whether the dynamics of this dissociation was the same for all factors. To investigate this question we compared the behaviour of fluorescently tagged PCNA, DNA Ligase I (Fig. 3A), and Fen1 (data not shown) in living S-phase cells following aphidicolin treatment. One minute after addition of aphidicolin, DNA Ligase I and Fen1 exhibited significant disassembly from the majority of replication sites. Furthermore, this disassembly lead to an increased nucleoplasmic concentration of these factors as described above. Both DNA Ligase I and Fen1 dissociated completely from replication foci within 5 minutes, which is in agreement with previous experiments demonstrating that these factors have more transient interactions with the replisome.[19] In contrast, PCNA persisted at replication foci much longer, with some signal still present following 15 minutes of drug treatment (Fig. 3A). To confirm that the PCNA signal represented previously loaded PCNA that persisted throughout exposure to aphidicolin, as opposed to PCNA that was loaded de novo onto newly formed adjacent replication sites, we performed time overlay analysis (Fig. 3B). The complete co-localization of the final PCNA signal (green) with the initial PCNA population (red) demonstrates that no assembly of new replication machinery took place.

The disassociation of PCNA, DNA Ligase I and Fen1 from replication foci upon cessation of DNA synthesis demonstrates that these fusion proteins are recruited to the replisome in a DNA elongation-dependent manner and only bind to sites of ongoing processive DNA synthesis. Furthermore, it indicates that the presence of PCNA is not sufficient to recruit Okazaki fragment maturation factors to replication foci and some other factor or condition must be involved. This data, along with the fact that these proteins exhibit such different

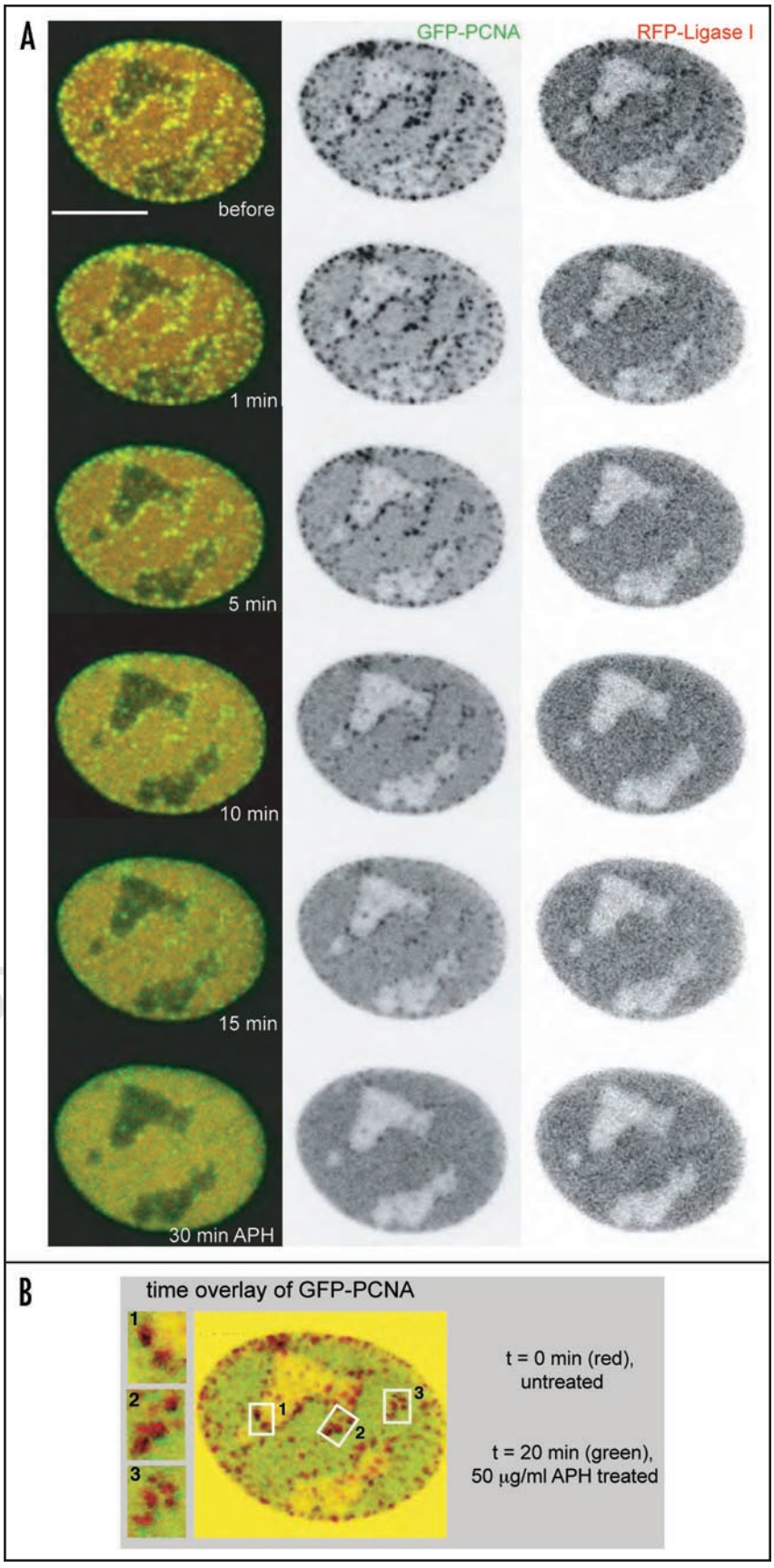

Figure 3. PCNA and DNA Ligase I exhibit different dissociation kinetics following aphidicolin treatment. (A) We undertook time-lapse analysis of cells expressing fluorescently tagged PCNA (green) and Ligase I (red) after addition of 50 μg/ml aphidicolin. We observed a rapid dissociation of DNA Ligase I from sites of replication within 1 min of drug addition. In contrast, PCNA disassembled to a large extent within 15 minutes, but was still present at few remaining replication foci after 30 minutes. (B) A time overlay of the same cell nucleus prior to and 20 min after addition of aphidicolin demonstrates that the PCNA foci remaining after 20 min (green) of aphidicolin incubation co-localize (black) with the initial signal (red). Scale bar, 10 μm.

disassembly kinetics suggests that the replisome is not composed of a single holoenzyme complex, but rather individual submodules that all bind to the replication site with varying affinities.

Accumulation of RPA34 upon aphidicolin inhibition. Time-lapse analysis of cells with fluorescently tagged PCNA and RPA34 further illustrated the diverse reactions of initiation and elongation proteins following inhibition of DNA synthesis (Fig. 4A). In contrast to PCNA, RPA remained bound to chromatin following aphidicolin treatment, and the amount of this protein present at replication sites increased significantly within 5 minutes of drug addition. The enhanced accumulation of RPA at replication foci in aphidicolin could reflect an increase in its recruitment to replication forks, or a decrease in its turnover within the replisome. To distinguish between these possibilities, and to describe how the dynamic behaviour of RPA changes at replication sites following inhibition of DNA polymerases by aphidicolin treatment, we performed dual colour FRAP experiments, including PCNA as a comparison (Fig. 4B–D and Suppl. Movie 1). Prior to aphidicolin treatment, PCNA reassociation was very slow, taking several minutes, while the turnover of RPA was much faster as previously shown in single colour FRAP experiments.[31] Following the addition of aphidicolin, PCNA dissociated from replication foci and was freely mobile in the nucleoplasm as shown by the almost instant recovery. This result is consistent with our results obtained from fixed cells and following detergent extraction (Figs. 1D and 2). Photobleaching of RPA foci showed that this factor was being recruited to replication foci in the presence of aphidicolin, albeit at a slower rate than in untreated cells. Therefore, an increased recruitment of RPA cannot explain its accumulation at replication foci in the presence of aphidicolin, suggesting that there is also a decrease in its dissociation. To directly investigate this question, we fused RPA34 with photoactivatable GFP (paGFP).[37] After photoactivation of paGFP-RPA34 molecules, we monitored their dissociation from replication foci (Fig. 4E). For orientation, replication foci were visualized by RFP-PCNA in control cells and by RFP-RPA34 in aphidicolin treated cells. Due to the high mobility of RPA34 in control cells, even after

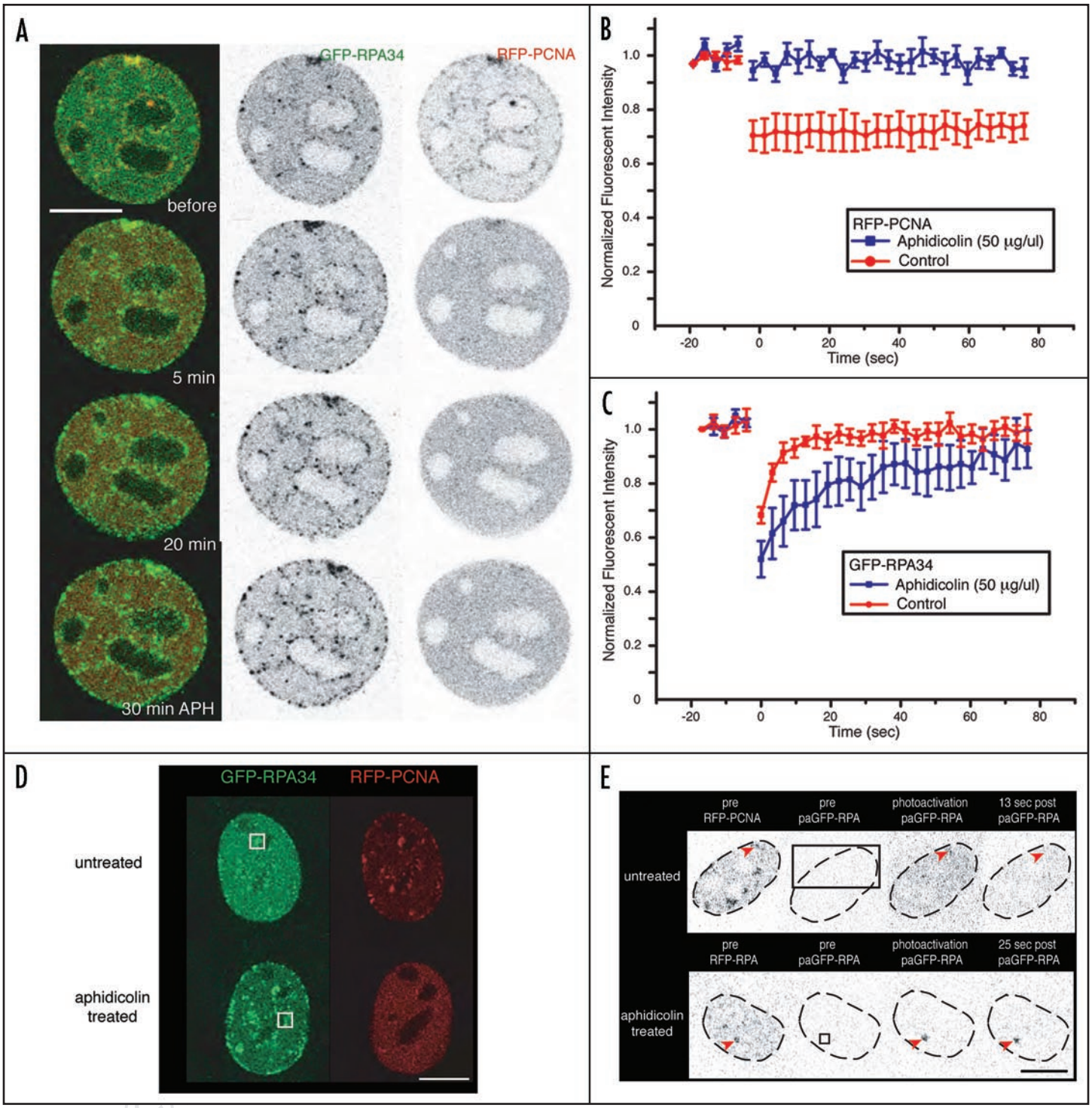

Figure 4. RPA34 accumulates at previous sites of replication in the absence of DNA synthesis. (A) Time-lapse analysis of cells expressing fluorescently tagged RPA34 (green) and PCNA (red) demonstrates that in contrast to PCNA, RPA34 exhibits increased focal accumulation after treatment with 50 μg/ml aphidicolin. (B and C) Mean FRAP curves were obtained from dual colour photobleaching experiments performed in cells expressing GFP-RPA34 and RFP-PCNA. Bleaching was done at time 0 and the data was normalized to the mean initial prebleach value and corrected for total bleaching due to image acquisition. (D) Representative images of a FRAP experiment in control and aphidicolin treated cells (bleached area, white box). RPA34 showed de novo association at replication sites in aphidicolin treated cells albeit at a slower rate, while the disassembled nucleoplasmic PCNA in aphidicolin treated cells showed immediate recovery similar to non S-phase cells. For more information see Movie 1. (E) Photoactivation of paGFP-RPA34 in control cells co-expressing RFP-PCNA and aphidicolin treated cells co-expressing RFP-RPA34 to visualize replication sites (photoactivated area, black box). In control cells RPA34 is rapidly redistributed after photoactivation, while in aphidicolin treated cells RPA did not dissociate from the replication sites (red arrowhead). The photoactivation in control cells was imaged with faster conditions in order to depict the mobility differences. For better visualization of the photoactivation process see Movie 2. Scale bars, 10 μm. Movie 1. RPA34 and PCNA mobility before and during aphidicolin treatment in a single living cell. Movie 2. Photoactivation of paGFP-RPA34 and FRAP of RFP-RPA34 after aphidicolin treatment in a single living cell.

photoactivation of half of the nucleus, only a very transient accumulation of paGFP-RPA34 was measured at activated replication foci (marked by a red arrowhead). This is consistent with the fast turnover of RPA34 at replication foci revealed by FRAP analysis (ref. 31 and Fig. 4D). In contrast, after aphidicolin treatment paGFP-RPA34 did not dissociate from the replication foci (see also Suppl. Movie 2). This indicates that the accumulation of RPA34 at replication foci in aphidicolin treated cells results from a continued, albeit slower recruitment to the replication fork combined with a decreased dissociation rate.

Discussion

Accurate DNA replication requires the precise coordination of numerous enzymatic activities. As such, it is important to understand how the proteins that mediate these reactions interact with one another. To analyze the interplay and the coordination of replisome components in vivo, we inhibited the elongation phase of DNA replication using aphidicolin and studied the subsequent response of the replication machinery. Our results demonstrate that within 20 minutes of drug treatment, processive DNA synthesis had stopped and changes in the protein composition and assembly kinetics of replication sites could be observed.

We detected little change in the localization or behavior of initiation factors such as Mcm proteins or DNA pol α. Interestingly, we did observe a significant increase in the accumulation of another initiation factor, RPA34, at replication foci in the presence of aphidicolin. Normally, ssDNA are immediately coated and stabilized by RPA, which in turn is displaced by the processive DNA polymerase.[38] This combination of active binding and continuous displacement accounts for the rapid turnover of RPA at replication foci.[31] We propose that aphidicolin treatment causes polymerase stalling, which leads to continued DNA unwinding by the initiation complex in the absence of subsequent DNA synthesis. This generates an excess of single-stranded DNA leading to the increased, stable binding of RPA34 at replication foci.

The homotrimeric PCNA ring forms a stable loading platform for many components of the elongation machinery under normal physiological conditions.[19] However, within minutes of aphidicolin treatment, this factor had mostly dissociated from replication foci. The Okazaki maturation factors Fen1 and DNA Ligase I disappeared from replication foci even more rapidly than PCNA, which is in agreement with previous studies demonstrating that these factors are weakly bound to the replication machinery. This result shows that the presence of the PCNA loading platform is not sufficient to mediate the binding of Fen1 and DNA Ligase I to replication foci. Interestingly, p66, the PCNA binding pol δ subunit[32] remained at a subset of replication foci even after the dissociation of PCNA. This indicates that another interaction must contribute to the retention of p66 at certain replication foci. One possibility is that an alternate polymerase clamp is recruited to aborted replication sites following the dissociation of PCNA. One candidate would be the Rad9-Rad1-Hus1 complex, which plays a major role in mediating the DNA damage response.[39] This idea would not only account for the retention of the p66 subunit of polymerase δ, but could also help explain how cells are able to re-initiate DNA replication so quickly following wash-out of aphidicolin. Alternatively, the p66 subunit could remain bound to the stalled replication fork through an unknown mechanism, and by itself mediate the recruitment of the required replication factors following the removal of aphidicolin.

In conclusion, we suggest that the replication fork is not stalled per se in the absence of processive DNA replication. Rather, inhibition of DNA polymerase activity promotes an uncoupling between the initiation and elongation machineries, causing processive movement of the replication fork unwinding the DNA duplex despite the fact that no DNA synthesis is actually occurring. This idea is in accordance with results from biochemical analysis in cell free systems such as Xenopus egg extracts.[36,40,41] Likewise, electron microscopic and supercoiling results with SV40 DNA indicated that the fork movement may continue after aphidicolin treatment.[42] These results shed new light on the dynamics and coordination of replication factors throughout S-phase in mammalian cells. The different enzymatic activities involved in DNA replication are not statically coupled but surprisingly dynamic as revealed by inhibition of one component of the machinery. This dynamic and modular composition of the replication machinery enables an efficient coordination of its participating enzymatic activities and a flexible response to adverse conditions such as DNA damage.

Materials and Methods

Cell culture, expression plasmids and transfection. Mouse C2C12 myoblasts were used for the majority of our experiments. All proteins localized identically in mouse and human cells except for p66, which localized correctly only in human cells. Therefore, for the p66 experiment shown in Figure 1D, HeLa cells were used. Cells were transiently transfected by the calcium phosphate-DNA co-precipitation method as described.[34] C2C12 cells stably expressing GFP-PCNAL2 (human PCNA) have been described before.[33] Cells were incubated with 50 μg/ml (150 μM) aphidicolin (Sigma, Oakville, Ontario) in preconditioned cell culture medium for the indicated times. The aphidicolin stock solution was made in DMSO and hence DMSO alone was used as a control. The fusion constructs GFP-RPA34,[31] GFP-Ligase I,[34] GFP-p66,[32] GFP-Fen1, mRFP-PCNA and mRFP-Ligase I[19] were generated as described. By replacing GFP with the sequence encoding mRFP1[43] we generated RFP-RPA34. With the exception of mouse Fen1, all cDNAs in the fluorescent fusion constructs were human.

Immunofluorescence and in situ DNA replication assay. We performed replication labeling by adding 20 μM BrdU (Sigma, Oakville, Ontario) to the culture medium prior to fixation. For aphidicolin chase experiments, the medium was removed, the cells were rinsed with fresh medium and further incubated in preconditioned medium for the indicated times. These experiments were repeated twice.

The following primary antibodies were used: mouse monoclonal anti-BrdU (clone B44, Becton-Dickinson, San Jose, California), mouse monoclonal anti-DNA pol α (ATCC clone SJK132-20,[44]), and purified rabbit polyclonal TSG23 antibody against Mcm3.[45] Immunostaining was performed as described before.[31]

Cell extracts and western blot analysis. Samples were prepared for Western blot analysis by treating synchronized HeLa cells with either DMSO or 50 μg/ml aphidicolin for 20 minutes. The cells were subsequently trypsinised, counted and pelleted. Equal numbers of cells were extracted with 0.5% Triton X-100 in PBS for 15

min on ice. After centrifugation, the supernatant and pellet fractions were isolated, and equal amounts of the soluble extract and cell pellet were run on 8% or 12% SDS–PAGE gels. Endogenous PCNA and RPA34 were detected using anti-PCNA (clone PC10, Dako, Glostrup, Denmark) and anti-RPA (RPA34-19, Oncogene, Darmstadt, Germany) antibodies. Antibodies against lamin B were used as a loading control.[46]

Confocal microscopy, fluorescence recovery after photobleaching and photoactivation. Confocal images (350 nm optical sections) of fixed cells were acquired using a Leica TCS SP2 AOBS. Live-cell imaging and FRAP analysis was conducted using a Zeiss LSM510 Meta. In both cases a 63x/1.4 oil immersion objective was used. DAPI, GFP, mRFP1, TexasRed and Cy5 were excited sequentially at 405 nm, 488 nm, 543 nm, 594 nm or 633 nm to minimize crosstalk. For live-cell microscopy, cells were cultured in LabTek chambered glass coverslips (Nunc, Wiesbaden, Germany) at 37°C. Care was taken to prevent over- or underexposed image pixels and to use identical microscope parameters. For GFP-RPA34/RFP-PCNA FRAP analysis, the same cells were photobleached before and after aphidicolin treatment. After acquisition of five prebleach images, a region of interest (ROI 30 x 30 pixel; pixel size 37 nm) was photobleached with 100% of the 543 nm and 60% of the 488 nm lasers for 10 iterations (0.5 sec) and 25 postbleach images were acquired without time interval. This procedure was also used for photoactivation and simultaneous photobleaching of paGFP-RPA34/RFP-PCNA in control cells and of paGFP-RPA34/RFP-RPA34 in aphidicolin treated cells. The RFP-PCNA or RFP-RPA34 signals were used as markers of replication foci in S-phase cells. The 488 nm laser was used at low power for imaging to minimize photobleaching or further photoactivation. A 256 x 256 pixel area was imaged in control cells and half the nucleus was photoactivated. In aphidicolin treated cells an area of 512 x 512 pixels was imaged and a ROI of 30 x 30 pixel was photoactivated. These differences in protocol were needed due to the very different mobility of RPA34 before and after aphidicolin treatment.

Image analysis and data processing. Adobe Photoshop CS and Adobe Illustrator CS were used to assemble and annotate all figures. FRAP evaluation, graphs and statistical evaluation were made using Microsoft Excel, Origin and WCIF Image J. Quantification of the focal distribution of replication factors was determined by calculating the mean intensity variation: the standard deviations of fluorescence intensities of 16 ROIs (44 x 44 pixel; pixel size 116 nm) per experiment were normalized to the average intensity of the respective binned image (n = 2) and depicted as a diagram. Our data was confirmed by further analysis using ROIs of various sizes, yielding essentially the same results. The FRAP analysis was conducted as described,[19] and the data normalized to the mean initial prebleach value and corrected for total bleaching due to image acquisition.

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Note

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