14-3-3 proteins function in the initiation and elongation steps of DNA replication in *Saccharomyces cerevisiae*

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Summary

14-3-3s are highly conserved abundant eukaryotic proteins essential for viability, at least in lower eukaryotes. We previously showed that they associate with mammalian and yeast replication origins in a cell-cycle-dependent manner, and are involved in the initiation of DNA replication. Here, we present evidence that 14-3-3 proteins are novel regulators of the initiation and elongation steps of DNA replication in *Saccharomyces cerevisiae*. The results show that the Bmh2 protein, one of the two 14-3-3 homologues in *S. cerevisiae*, interacts with Mcm2 and Orc2 proteins, binds to ARS1 maximally at the G1 phase, is essential for plasmid stability, and is required for normal S-phase entry and progression. Furthermore, during G1 phase, the Bmh2 protein is required for the association of MCM proteins with chromatin and their maintenance at replication origins. The results reveal that 14-3-3 proteins function as essential factors for the assembly and maintenance of the pre-replication complex during G1 phase.

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Key words: 14-3-3, DNA replication, Initiation, Elongation, Origin

Introduction

The control of DNA replication is tightly regulated by the stepwise assembly of the prereplication complex (pre-RC) during the G1 phase of the cell cycle (Diffley, 2004). Pre-RC formation requires the presence of the origin recognition complex (ORC), Cdc6 and Cdt1 proteins, which act as loading factors for the MCM complex (Bell and Dutta, 2002; Mendez and Stillman, 2003; Speck and Stillman, 2007; Tanaka and Diffley, 2002). Origin firing requires additional proteins including Cdc45, Dpb11, Sld2, and the cyclin-dependent (Clb-Cdc28) and the Dbf4-dependent (Cdc7-Dbf4) kinases (Dalton and Hopwood, 1997; Kamimura et al., 1998; Lei et al., 1997; Stillman, 1996; Wohlschlegel et al., 2002; Zegerman and Diffley, 2007; Zou and Stillman, 1998). In addition, other components are required for origin activation, such as the GINS complex, and the Mcm10 and Noc3 proteins (Gambus et al., 2006; Ricke and Bielinsky, 2004; Zhang et al., 2002). Altogether, these proteins activate DNA helicase, which unwinds DNA and allows the recruitment of replication protein A (RPA) and DNA polymerases (Walter and Newport, 2000).

Additional proteins are also involved in the regulation of DNA replication, including members of the 14-3-3 protein family, which bind specifically to DNA cruciforms that form transiently at or near replication origins at the onset of S phase (Alvarez et al., 2002; Novac et al., 2002; Zannis-Hadjopoulos et al., 2002). 14-3-3 proteins, including *Saccharomyces cerevisiae* homologues, are involved in many cellular processes, such as signal transduction pathways, apoptosis, transcription, cytoskeletal organization, response to DNA damage and cell-cycle regulation (Bruckmann et al., 2004; Jin et al., 2004; Lottersberger et al., 2006; Lottersberger et al., 2003; Mhawech, 2005). They are also DNA-binding proteins and associate with origins of DNA replication in a cell-cycle-dependent manner (Callejo et al., 2002; Novac et al., 2002).

Using chromatin immunoprecipitation (ChIP) assays, we previously showed that 14-3-3 proteins associate with origins of DNA replication in *S. cerevisiae* and are involved in the initiation of DNA replication (Callejo et al., 2002; Yahyaoui et al., 2007). In this study, we examined their role in this process, using *S. cerevisiae* as a model. We found that the Bmh2 protein interacts with members of the pre-RC, is required for the initiation and elongation steps of DNA replication, and, together with ORC, is essential for the binding of MCM proteins to replication origins at G1 phase, suggesting an integral role of 14-3-3 in the formation and maintenance of the pre-RC.

Results

Bmh2 protein interacts with Mcm2 and Orc2 and binds to chromatin throughout the cell cycle

To test whether 14-3-3 proteins are components of the initiation machinery, we analyzed the interaction of Bmh2 protein with known initiator proteins as well as the cell-cycle profile of its association to chromatin. Although both Bmh1 and Bmh2 proteins associate with origins of replication (Callejo et al., 2002; Yahyaoui et al., 2007), we concentrated on the Bmh2 protein in order to take advantage of the existing bmh2 temperature-sensitive (ts) mutant, which allows a comparison with the wild type. Whole-cell extracts (WCEs) from asynchronous wild-type cells, containing 1 g of total proteins, were subjected to reciprocal co-immunoprecipitations (co-IPs), using anti-Bmh2, anti-Mcm2 and anti-Orc2 antibodies (Fig. 1A). Mcm2 and Orc2 proteins were immunoprecipitated with anti-Bmh2 antibody (Fig. 1A, left panel), although its interaction with Orc2 was much weaker than with Mcm2, possibly indicating a stable complex of Bmh2 with MCM proteins. In the reciprocal IPs, the Bmh2 and Orc2 proteins were immunoprecipitated by anti-Mcm2 antibody (Fig. 1A, right panel).

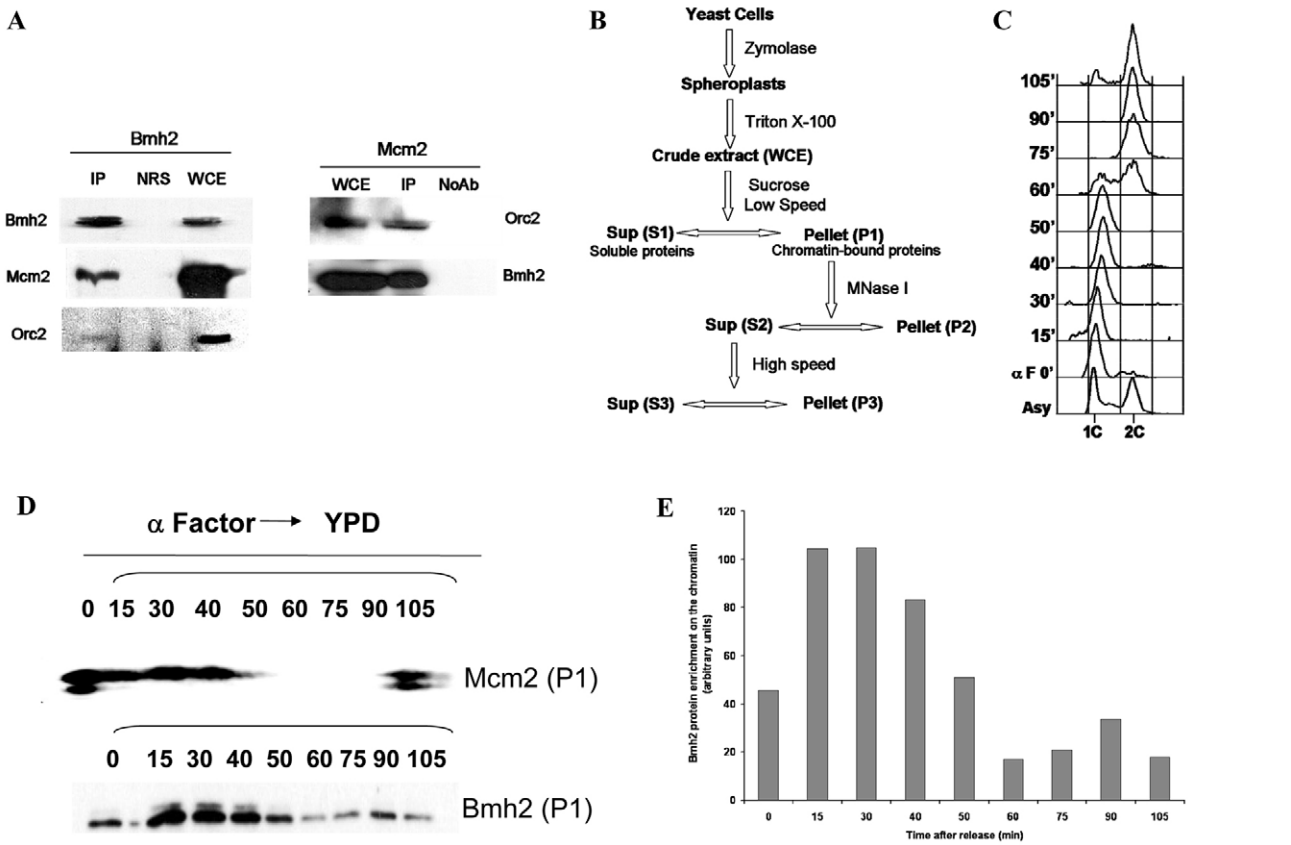

Fig. 1. Interaction of Bmh2 with Mcm2 and Orc2 proteins, and its chromatin-binding profile. (A) Extracts from wild-type cells were immunoprecipitated with anti-Bmh2 antibody (left panel) and immunoblotted with anti-Bmh2, anti-Mcm2, and anti-Orc2 antibodies as indicated. Normal rabbit serum (NRS) was used as a negative control for the IP. Co-IPs were performed with anti-Mcm2 antibody (right panel), and blotted with anti-Bmh2 and anti-Orc2 antibodies. IP without antibody (NoAb) was used as a negative control. (B) Chromatin fractionation protocol. (C) Wild-type cells were synchronized at G1 phase and released in YPD medium. The cell-cycle progression of the cells after release from G1 was monitored by FACS analysis. Asy, asynchronous cells. (D) Aliquots of cells, as described in C, were harvested at the various time points after release and subjected to chromatin-binding assays. The chromatin fractions (P1) were immunoblotted for Mcm2 and Bmh2, as indicated. (E) Quantification of the Bmh2 enrichment on the chromatin as presented in D. The values are presented as arbitrary units.

The specificities of the interactions of Bmh2 with Mcm2 and Orc2 were confirmed by immunoprecipitation with normal rabbit serum (NRS) and no antibody (NoAb), as negative controls (Fig. 1A). Moreover, considering that the interaction of Orc2 with Bmh2 is very weak, we also performed the reciprocal IP of Bmh2 protein by anti-Orc2 antibody in these cells. The data show that a significant amount of Bmh2 protein was immunoprecipitated by anti-Orc2 antibody, although the interaction is still weak (Fig. S1 in supplementary material). These results suggest that Bmh2 is in a complex with other initiator proteins and interacts with essential components of the pre-RC that are assembled at replication origins.

We examined next the chromatin-binding profile of the Bmh2 protein, using the chromatin-binding assay, as described previously (Donovan et al., 1997; Liang and Stillman, 1997). *S. cerevisiae* wild-type cells were subjected to the chromatin isolation protocol shown in Fig. 1B, and the different fractions (S3 and P3) obtained after high-speed centrifugation were analyzed by western blotting to assess whether nuclear Bmh2 binds to chromatin, as Bmh2 is considered to be mainly cytoplasmic (data not shown). For the cell-cycle profile of Bmh2 association with chromatin, cells were synchronized to G1 with α factor and a timecourse experiment was performed after releasing them into S phase. The DNA content at each time point after release was monitored by flow cytometry (Fig. 1C). Chromatin fractions (P1) at different times were subjected to western blot analysis with anti-Mcm2 and anti-Bmh2 antibodies (Fig. 1D). As also previously shown (Liang and Stillman, 1997), Mcm2 protein was present in the pellet fraction during G1 and early S phase (from 0 to 40 minutes after release), dissociated from the chromatin during S phase (from 50 to 90 minutes) and reassociated with it at the end of mitosis (105 minutes) (Fig. 1D, upper panel). By contrast, Bmh2 protein was bound to chromatin throughout the cell cycle (Fig. 1D, lower panel), but its amount in the pellet fractions was not constant, showing an increase at G1 and the beginning of S phase (15 to 40 minutes). The amounts of Bmh2-protein enrichment on the chromatin were quantified and presented as arbitrary units (Fig. 1E). These results show that Bmh2 protein bound to chromatin throughout the cell cycle with an increase in the beginning of S phase, in agreement with our previous findings, whereby 14-3-3 proteins were found to associate with origins maximally at the G1-S boundary (Callejo et al., 2002; Novac et al., 2002).

Association of Bmh2 protein with replication origins is cell-cycle regulated

In view of the association of Bmh2 with the *S. cerevisiae* chromatin, we analyzed whether it associated specifically with yeast replication origins. ChIP assays were performed with two origins of DNA replication, ARS1 and ARS307, in comparison to their respective non-ARS negative regions, R2.5 and Neg307 (negative controls)

(Zhang et al., 2002; Callejo et al., 2002; Yahyaoui et al., 2007). As previously done in similar studies, we used only one negative region as the negative control for each origin (Homesley et al., 2000; Zhang et al., 2002; Callejo et al., 2002; Yahyaoui et al., 2007). Immunoprecipitated chromatin with anti-Bmh2 antibody, anti-Orc3 antibody, NRS or NoAb were subjected to conventional PCR, using specific primers for the ARS1, ARS307, R2.5 and Neg307 regions. PCR results of Bmh2 and Orc2 IPs showed amplification of specific bands for ARS1, whereas no PCR product was found in the NoAb IP control, or when R2.5 was used as a template, except for the genomic DNA, as expected (Fig. 2A, upper panel). Similar results were obtained for ARS307, using only Bmh2 ChIP this time (Fig. 2A, lower panel).

To further characterize the interaction of Bmh2 with replication origins, we analyzed its binding to the well-known ARS1 replicator by real-time PCR. The association of Bmh2 with ARS307 was previously shown (Callejo et al., 2002). ChIP with each antibody (anti-Bmh2 and anti-Orc3) or without antibody (NoAb control) were quantified by real-time PCR to determine the amount of DNA molecules immunoprecipitated. The specificity of the real-time PCR products for ARS1 was first analyzed by the melting curve of *S. cerevisiae* genomic DNA (Fig. 2B). The same genomic DNA was also used to build the standard curves for ARS1 (Fig. 2C) and R2.5 (not shown) primer sets that are required for the quantification of the PCR products. All ChIP amounts are proportional to the input DNA for each antibody and are determined according to the same standard genomic DNA curve. As shown in Fig. 2D, anti-Bmh2 and anti-Orc3 antibodies immunoprecipitated ARS1 sequence to the same extent, with 5.3×10^{11} and 5.0×10^{11} molecules, respectively. By contrast, the amounts of DNA immunoprecipitated by both these antibodies from the non-ARS sequence R2.5 were at background level, as they also were with the no antibody (NoAb) control. These results show that the Bmh2 protein binds specifically to ARS1, associating with it in a similar manner as the initiator protein Orc3.

To further analyze the involvement of Bmh2 in the initiation of DNA replication, we quantified its association with ARS1 and R2.5 sequences in different phases of the cell cycle. Wild-type cells were synchronized to G1 with α factor, to early S phase with hydroxyurea (HU), or to G2-M with nocodazole. Extracts were subjected to ChIP assays with anti-Bmh2 antibody and quantified by real-time PCR, using ARS1 or R2.5 as templates. The Bmh2 protein was found to associate with the ARS1 in a cell-cycle-dependent manner, whereas it associated with R2.5 at a background level throughout the cell cycle (Fig. 2E). The amount of Bmh2 associated with ARS1 was maximal at G1 phase, with approximately 15.5×10^{11} molecules; the S and G2-M phases had 4.5×10^{11} and 4.3×10^{11} molecules, respectively (Fig. 2E). These results show that the 14-3-3 homologue Bmh2 protein associates specifically with the *S. cerevisiae* ARS1 and its association is cell-cycle regulated, being maximal at G1 phase.

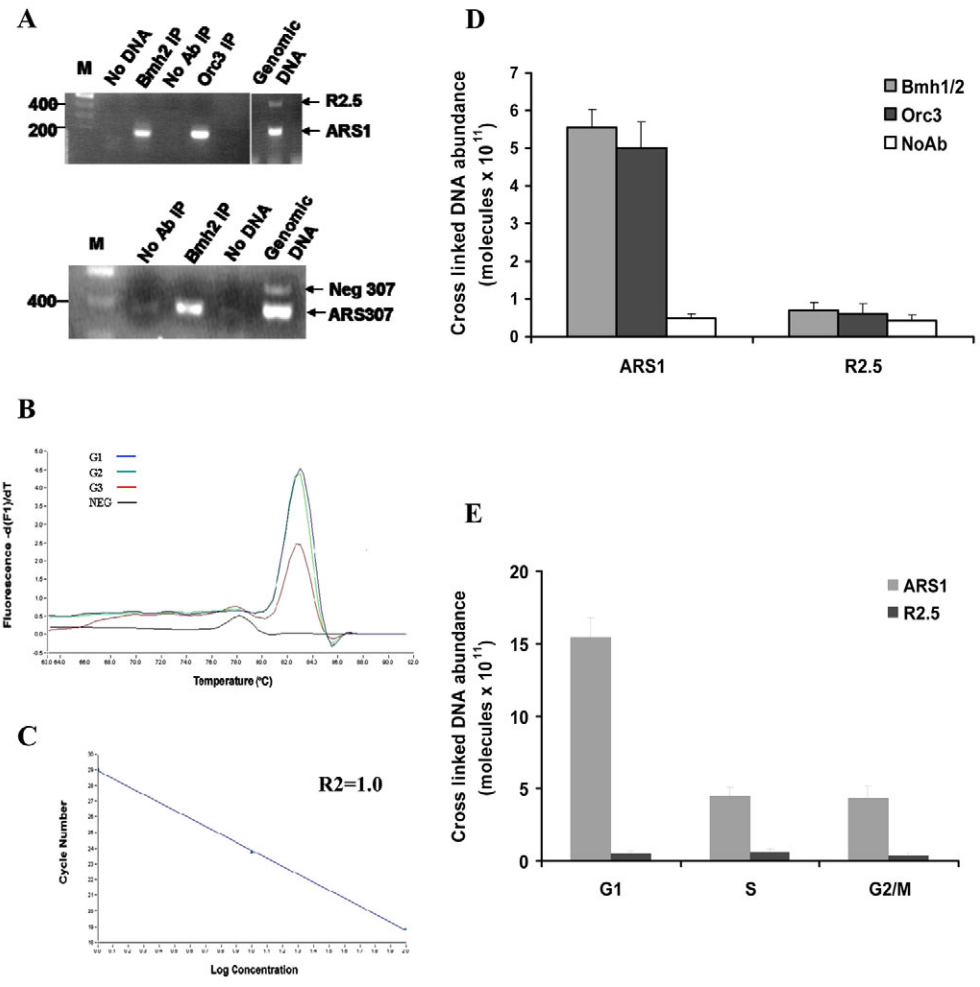

Fig. 2. Association of Bmh1 and Bmh2 proteins with the budding yeast origin of replication ARS1. (A) ChIP assays were performed with asynchronous wild-type cells, using anti-Orc3 and anti-Bmh2 antibodies, or with no antibody and NRS as controls. ChIP materials were subjected to conventional PCR, using the ARS1, ARS307 and their respective non-ARS sequences, R2.5 and Neg307, as templates. (B) A representative melting curve for *S. cerevisiae* genomic DNA, used with the ARS1 primer set. NEG, non-ARS sequence for ARS307. (C) The standard curve of *S. cerevisiae* genomic DNA used for ARS1 real-time PCR quantifications. (D) Real-time PCR measurements of ChIP assays performed with asynchronous wild-type cells, using anti-Orc3 and anti-Bmh2 antibodies or with no antibody as a control. ChIP materials were subjected to real-time PCR using the ARS1 sequence, and the non-ARS sequence, R2.5, as templates. Amounts of DNA molecules immunoprecipitated were quantified according to genomic standard curves and the specificity of products was tested by respective melting curves. (E) Wild-type cells were synchronized at G1, S and G2-M phases and aliquots of each were subjected to ChIP assays with the anti-Bmh1 antibody, and quantified by real-time PCR using ARS1 and R2.5 as templates, with the same conditions as in D.

Bmh2 protein is required for initiation of DNA replication

In view of all the results shown above, we tested next whether the yeast 14-3-3 protein homologue Bmh2 is required for the initiation of DNA replication. For this, we used a Bmh2 temperature-sensitive (*bmh2-ts*) mutant strain and the plasmid stability assay. This assay is largely employed to characterize mutant cells defective in initiation of DNA replication (Hogan and Koshland, 1992; Loo et al., 1995; Zoo et al., 1997; Zhang et al., 2002). It consists of measuring the loss rates of a pair of plasmids in wild-type and mutant strains. The pair of plasmids bears either one or more replication origins. It has been shown that, whereas wild-type cells lose both plasmids at low rates, all previously characterized mutants in initiator proteins, including ORC, Cdc6, MCM, Cdc45 and Noc3, have high plasmid loss rates with plasmids bearing one origin and lower loss rates with plasmids bearing more than one origin (Hogan and Koshland, 1992; Loo et al., 1995; Zoo et al., 1997; Zhang et al., 2002). In the case of ts mutant strains, plasmid loss rates are usually measured at the permissive and semipermissive temperatures of the mutant. This assay requires colony formation and thus cannot be performed at the restrictive temperature.

The *bmh2-ts* strain used in this study was described by Bruckmann et al. (Bruckmann et al., 2004). This mutant was constructed by replacing the *BMH2* gene by *URA3* in the Matα strain CEN-PK113-13D. Subsequently, the *bmh2*::*URA3* allele was replaced by the *bmh2(ts)* allele. *BMH1* was deleted in Matα strain CEN-PK113-7D, the two strains were crossed and, after dissection, the Matα haploid strain, having the *bmh1::kanMX*, *bmh2(ts)* and *URA3* alleles, was selected. Thus, this strain, in which *BMH1* has been deleted, has the *bmh2* mutant allele integrated at the *BMH2* allele locus and expresses mutant Bmh2 at endogenous levels. In the *bmh2* mutant allele, a single point mutation (a serine to a proline residue) at position 189 partly complements the lethal *bmh1 bmh2* double disruption, allowing growth at 23°C and 30°C, but not at 37°C. Expression of Bmh1, Bmh2 and bmh2-ts protein, at the semi-restrictive temperature of 30°C, was analyzed by western blot using anti-Bmh2 antibody. As shown in Fig. 3A, the wild-type cells express Bmh1 and Bmh2 at normal levels, whereas in mutant cells, only bmh2-ts is expressed at a very low level.

Plasmid loss rates in the wild-type and *bmh2-ts* strains were measured using the pair of plasmids pARS-1 and pARS-2, containing one (ARSH4) and two ARS (ARSH4 and ARS307), respectively, as previously described (Yahyaoui et al., 2007). As shown in Fig. 3B, *bmh2-ts* mutant cells were severely compromised in their ability to maintain pARS-1 at 30°C, with an average loss rate of 6.4% per generation. At 23°C, the mutant cells lost pARS-1 plasmid to a lesser extent, with an average rate of 1.8%. By contrast, the plasmid loss rates of pARS-2 at both 23°C and 30°C were very low and decreased to approximately 0.22% per generation. For the wild-type cells, the plasmid loss was equally very low for both plasmids (0.3-0.4% per generation). The loss rates obtained for *bmh2-ts* mutant cells are comparable to those obtained for the initiator proteins mentioned above (MCM, ORC, Cdc6 and Noc3) (Hogan and Koshland, 1992; Loo et al., 1995; Zoo et al., 1997; Zhang et al., 2002). Altogether, the results suggest that the Bmh2 protein is essential for the initiation of DNA replication, as is expected for a component of the pre-RC.

Bmh2 protein is required for normal entry into S phase

To further investigate the role of 14-3-3 in the initiation of DNA replication, we used FACS analyses to monitor whether *bmh2-ts* mutant cells are defective in cell-cycle progression. Wild-type and

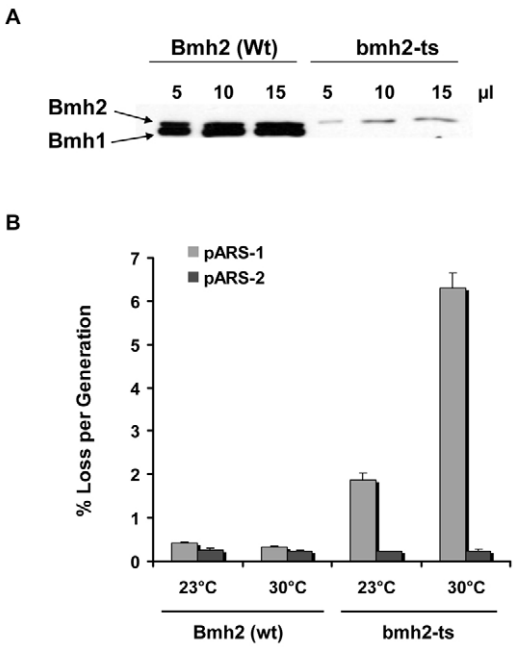

Fig. 3. Bmh2 protein is required for minichromosome maintenance in the budding yeast. (A) The expression of Bmh2 protein was assessed in wild-type and mutant cells at 30°C. (B) The plasmid loss rates were measured for the wild-type (Wt) and the temperature-sensitive mutant (*bmh2-ts*) cells containing either pARS-1 or pARS-2 at 23°C (permissive temperature) or 30°C (semi-permissive temperature). Error bars indicate plasmid loss rates as the average of three separate experiments and one standard deviation.

bmh2-ts cells were synchronized in G1 phase with α factor at 23°C and released in fresh YPD medium at the restrictive temperature of 37°C. The cells were then analyzed for their DNA content at various times after release from G1 block. As shown in Fig. 4 (left panel), the control strain entered S phase at 40 minutes and completed S phase at 80 minutes. By contrast, switching the mutant cells to 37°C resulted in a marked delay in entering S phase, which started only at 100 minutes after release from the block. Although some cells were able to complete S phase and accumulate 2C DNA by 120 minutes at 37°C, enough cells were still in S phase by 160 minutes and the cell population as a whole was unable to enter the next G1 phase even by 160 minutes after release. By contrast, the wild-type cells had completed bulk DNA synthesis by 60 minutes after release, and progressed through the cell cycle to become asynchronous, starting the next G1 phase as early as 60 minutes.

These results indicated that the Bmh2 protein is required for the normal entry into S phase and suggested that 14-3-3 proteins regulate the early steps of DNA replication. However, because enough of the mutant cells completed the S phase, we also analyzed whether the Bmh2 protein is required for S-phase progression and the elongation step of DNA replication. For this, wild-type (control) and *bmh2-ts* cells were pre-synchronized in G1 phase with α factor, subsequently transferred into HU for 2 hours at 23°C and were then released from HU and incubated at 37°C. As shown in Fig. 4 (right panel), the control cells rapidly completed S phase, accumulated 2C DNA by 40 minutes after release and subsequently underwent cell division, entering a new G1 phase by 80 minutes. By comparison, the *bmh2-ts* mutant cells progressed very slowly through S phase and were not able to complete bulk DNA synthesis and enter a new G1 phase even by 160 minutes. The progression

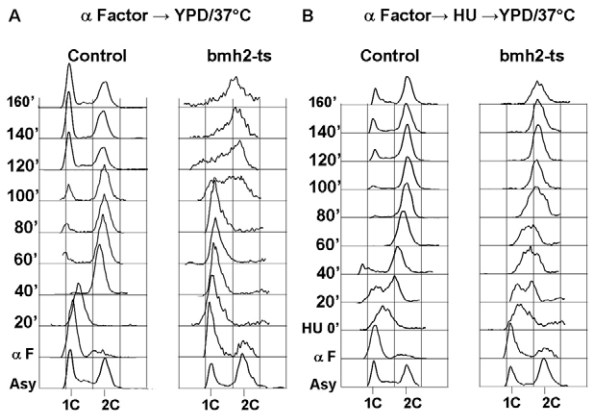

Fig. 4. Bmh2 protein is required for normal cell-cycle progression. (A) Wild-type and *bmh2-ts* cells were synchronized with α factor in G1 phase at 23°C, and released into fresh YPD medium at 37°C. Aliquots were taken from each time point and analyzed by flow cytometry for DNA content. (B) Wild-type and *bmh2-ts* cells were pre-synchronized with α factor at 23°C and transferred into hydroxyurea (HU)-containing medium for 2 hours at 23°C. The cells were then released in fresh medium at 37°C. Cell cycle was monitored by FACS analyses for DNA content.

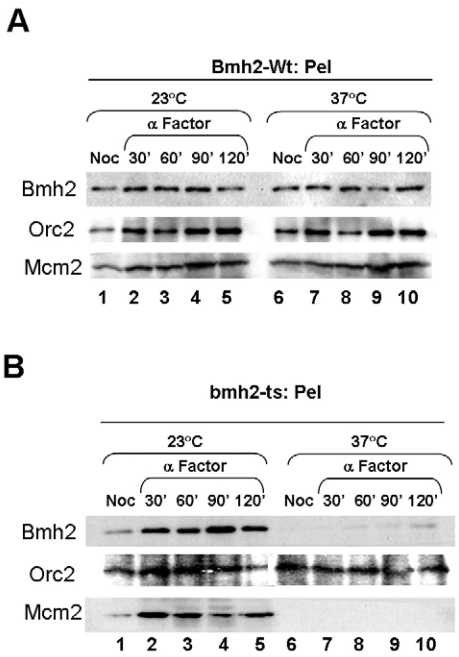

Fig. 5. Bmh2 protein is essential for the loading of the pre-RC onto chromatin at G1 phase. Wild-type (A) and *bmh2-ts* (B) cells were pre-synchronized in G1 phase at 23°C for 3 hours and released in YPD medium for 75 minutes at 23°C. Nocodazole was then added to block cells in G2-M phase, and the cells were kept at 23°C (lane 1) or shifted to 37°C (lane 6) for 100 minutes. The cells were then released from nocodazole arrest into α-factor-containing medium at 23°C (lanes 2-5) or at 37°C (lanes 7-10). The cells were harvested at various time points after release and subjected to chromatin-binding assays. The chromatin fractions were immunoblotted for Bmh2, Orc2 and Mcm2 as indicated. Pel, pellet chromatin fractions.

profile of *bmh2-ts* mutant cells through S phase is typical of mutants defective in the early steps of DNA replication (Hogan and Koshland, 1992; Liang and Stillman, 1997; Zhang et al., 2002).

Altogether, the FACS analysis results show that Bmh2 protein is required for normal entry into S phase, and because it considerably slows down S-phase progression, it might also play a role in fork progression and the elongation step of DNA replication. Bmh2 protein might play such a role by either directly affecting the replisome components, or indirectly by activating an S-phase checkpoint control. Our results of the Bmh2-chromatin binding and chromatin immunoprecipitation in synchronized cells are in agreement with and support this hypothesis. Further studies are needed, however, to test whether Bmh2 is required only for origin firing and replication initiation and is then displaced from the origin, or whether it travels with the fork and regulates the elongation process.

Bmh2 protein is essential for pre-RC formation

In view of the above results, indicating that 14-3-3 proteins are components of the pre-RC and are required for the initiation of DNA replication, we next examined whether they play a role in the formation or maintenance of the pre-RC. During G1 phase, the pre-RC assembles and is maintained until G1-S transition, when it disassembles (Bell and Dutta, 2002; Stillman, 1996). We used the bmh2-ts mutant to examine whether 14-3-3 is required for the association of ORC and/or MCM, two essential components of the pre-RC (Labib et al., 2001). Although MCM proteins require ORC and Cdc6 for their association with the origins at G1 phase (Perkins and Diffley, 1998), the exact mechanism is still poorly understood.

To investigate the relationship between Bmh2, ORC and MCM, we first examined whether Mcm2 or Orc2 would fail to load onto chromatin during the pre-RC assembly at the end of mitosis, when Bmh2 is suboptimal in *bmh2-ts* cells. Wild-type and *bmh2-ts* cells were pre-synchronized to G1 with α factor and released in the cell cycle for 75 minutes at 23°C (permissive temperature). The cells were then arrested at G2-M with nocodazole at 37°C (non-permissive temperature) for 100 minutes, released into the subsequent G1 phase in the presence of α factor at 37°C for 2 hours and samples were harvested every 30 minutes for chromatin-binding assays (Fig. 5). The same procedure was carried out for both strains at 23°C, as controls. In wild-type cells, the levels of chromatin-bound Bmh2 and Orc2 proteins were approximately constant at both 23°C and 37°C (Fig. 5A). The level of Mcm2 protein was low for the nocodazole-arrested cells (Fig. 5A, lanes 1 and 6), and increased when Mcm2 started to load onto chromatin as the pre-RC was formed (lanes 2-5 and 7-10). By contrast, when the level of Bmh2 protein was suboptimal in *bmh2-ts* mutant cells at 37°C (Fig. 5B, lanes 6-10), Bmh2 did not bind to chromatin, whereas the binding of Orc2 was not affected. The Mcm2 protein was also absent from the chromatin fractions at 37°C (Fig. 5B, lanes 6-10), indicating that Mcm2 failed to load onto chromatin under conditions of suboptimal function of the Bmh2 protein. These results suggest that Bmh2 is required for the loading of the MCM proteins onto chromatin at G1 phase, independently of ORC.

Bmh2 is essential for maintenance of the pre-RC

The second level of regulation of the initiation of DNA replication is the maintenance of the pre-RC onto chromatin until the end of G1 (Bell and Dutta, 2002; Donovan et al., 1997). To investigate the role of Bmh2 in this process, we examined whether initiation proteins would be affected by Bmh2 protein depletion in G1-arrested cells. Wild-type and *bmh2-ts* cells were synchronized in G1 with α factor at 23°C, and then maintained at 23°C (permissive) or transferred to 37°C (non-permissive) for 6 hours, in the presence

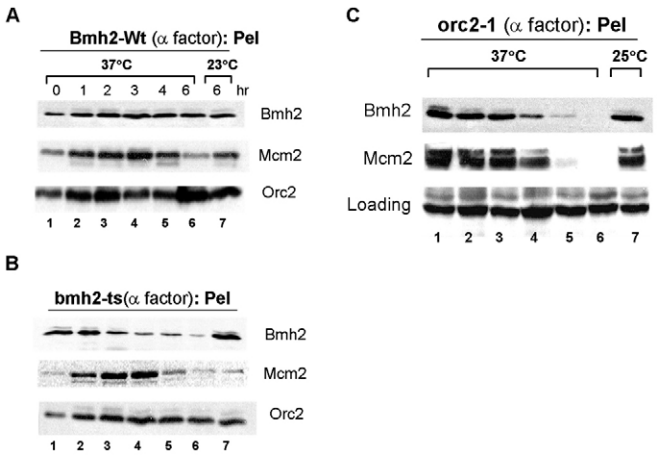

Fig. 6. Bmh2 protein is required for the maintenance of MCM proteins on chromatin in G1, and requires ORC for its maintenance. Wild-type (A) and *bmh2-ts* (B) cells were synchronized in G1 at 23°C for 3 hours (lane 1), and then shifted to 37°C (lanes 2-6) or maintained at 23°C (lane 7) for 6 hours in the continuous presence of α factor. The cells were harvested every hour and subjected to chromatin-binding assays. The pellet chromatin fractions were immunoblotted for Bmh2, Orc2 and Mcm2, as indicated. (C) *Orc2-1* mutant cells were treated as above except for the control temperature which was at 25°C. The pellet chromatin fractions were immunoblotted for Bmh2 and Mcm2, as indicated. The supernatant fractions (Loading) were immunoblotted with anti-Bmh2 antibody as a control of Bmh2 protein expression. Pel, pellet chromatin fractions.

of α factor. Cells were harvested every hour and subjected to chromatin-binding assays. As expected, at both 23°C and 37°C the levels of Bmh2, Orc2 and Mcm2 proteins in wild-type cells were approximately constant (Fig. 6A). For the mutant cells, the level of Bmh2 protein decreased gradually after shifting to 37°C (Fig. 6B, lanes 1-6), as Bmh2 protein level became suboptimal. Whereas this decrease in Bmh2 protein had no effect on the binding of Orc2 proteins, Mcm2 proteins started to load at 0 hours after α-factor synchronization (Fig. 6B, lane 1), were present on the chromatin for the subsequent 3 hours (lanes 2-4), and started to dissociate gradually from the chromatin after that (lanes 5 and 6). Under permissive growth conditions (23°C), all Bmh2, Orc2 and Mcm2 proteins were present at the chromatin, although the amount of Mcm2 was low (Fig. 6B, lane 7). Overall, the data from the last two sections (Figs 5 and 6) indicate that Bmh2 protein is essential for pre-RC assembly, and is required for MCM loading and maintenance onto chromatin during G1 phase.

Another level of regulation of replication initiation is the requirement of ORC for pre-RC assembly and maintenance on the chromatin during G1 phase. In light of the finding that Bmh2 protein is required for pre-RC assembly, we similarly examined whether ORC is essential for the maintenance of Bmh2 onto chromatin during G1. The same analysis was performed as above, this time using *orc2-1* temperature-sensitive (*orc2-1*) mutant cells, which were synchronized in G1 phase and then shifted to 37°C in the continuous presence of α factor. In agreement to what was previously shown (Homesley et al., 2000), removal of ORC from the chromatin during G1 resulted in the dissociation of MCM from the chromatin (Fig. 6C, lanes 5 and 6). The levels of Bmh2 protein on the chromatin also similarly decreased, following a similar pattern of dissociation as Mcm2 (Fig. 6C, lanes 5 and 6). These data indicate that, similar to MCM, ORC is required for the maintenance of the Bmh2 protein on the chromatin. To confirm that the expression of Bmh2 in *orc2-1* cells was not affected at 37°C, the supernatant fractions of these cells were immunoblotted for Bmh2 proteins and showed that it was present in the *orc2-1* cells at the same level throughout the 6 hours of G1 arrest (loading control) (Fig. 6C, lanes 1-7). This result suggests that Bmh2 protein is only displaced from the chromatin by Orc2.

Discussion

In this study, we present evidence for a novel function of 14-3-3 proteins as regulators of the initiation and elongation steps of DNA replication. We show that one of the *S. cerevisiae* 14-3-3 protein homologues, Bmh2, interacts with essential components of the pre-RC and forms complexes with the ORC and MCM proteins. It associates with the budding yeast replication origin ARS1 in a cell-cycle-dependent manner and requires ORC in order to be maintained on the chromatin during G1 phase. It is also essential for normal cell-cycle progression and required for G1-S transition. Thus, our data show that 14-3-3 proteins play an integral role in the regulation of DNA replication, being essential for MCM loading and maintenance onto chromatin at G1 phase.

14-3-3 proteins were previously linked to DNA replication in several studies. They bind to both cruciform DNA and origins of DNA replication in a cell-cycle-dependent manner, with a maximal association at the G1-S boundary (Novac et al., 2002), suggesting that they are involved in the regulation of the initiation of DNA replication. Furthermore, 14-3-3 proteins were found in several proteomic studies to interact with replication-initiation proteins, including Mcm3, Mcm5, Mcm10 and Ku antigen (Meek et al., 2004; Pozuelo Rubio et al., 2004; Satoh et al., 2006), although no function related to these interactions was shown. 14-3-3s are also required for G1-S transition, as demonstrated for several *bmh1-ts* mutant *S. cerevisiae* strains (Lottersberger et al., 2006). This cell-cycle defect was partially due to a reduction of the levels of the G1 cycling *Cln2* transcripts, suggesting that 14-3-3 proteins might regulate the late G1 transcriptional programme. In addition, two global proteomic studies (Bruckmann et al., 2004; Bruckmann et al., 2007) revealed that 14-3-3 proteins regulate the transcription and the post-transcription processes in the budding yeast, as well as having an effect in the activation, repression and translation of many genes. However, in both studies, no proteins directly related to the initiation of DNA replication were found to be regulated. Taking into consideration all the above findings, our results suggest that 14-3-3 proteins are playing a direct role in the initiation of DNA replication and that they are directly responsible for the initiation defect, as *bmh2-ts* mutants have a defect in maintaining extra-chromosomal plasmids.

Further evidence showing that 14-3-3 proteins function directly as regulators of DNA replication initiation comes from their roles in regulating cell-cycle progression, especially the exit from mitosis. Two studies reported that 14-3-3 proteins are acting as key regulators of factors such as Cdc25 and Cdh1 (Chen et al., 2003; Dial et al., 2007). First, 14-3-3 proteins inhibit Cdc25 protein activity during interphase, and block entry to mitosis (Chen et al., 2003). The suboptimal level of Bmh2 protein in the *bmh2-ts* strain would then promote entry to mitosis, because less Cdc25 would be sequestered in the cytoplasm. Another level of mitosis control is the interaction of 14-3-3 with Cdh1, which is a cofactor of the APC/C ubiquitin-mediated proteolysis system (Dial et al., 2007). Cdh1 interaction with 14-3-3 inhibits APC/C activity and blocks the mitotic exit. APC/C is also required to establish and maintain the G1 state

(Martinez et al., 2006). The low level of 14-3-3 proteins in *bmh2-ts* cells would promote APC/C activation by Cdh1 and cause mitotic exit. Therefore, the replication-initiation defects of the *bmh2-ts* cells observed in this study would not be an indirect result of one of the various regulatory steps discussed above, but rather a direct effect on the regulation of other initiator proteins during late M and G1 phases, namely the MCM proteins, as this study showed.

On the basis of the results of this study and their coordinated activities as cruciform- and origin-binding proteins in vivo, we propose that 14-3-3 proteins participate in the recruitment of MCM proteins onto chromatin and their maintenance at replication origins during the initiation of DNA replication. 14-3-3 proteins also play a role in normal S-phase progression and the elongation step of DNA replication. As shown by FACS analysis, suboptimal amounts of Bmh2 protein considerably slowed down the progression of S phase, suggesting a role of 14-3-3 in the completion of chromosome replication in budding yeast after origin activation. Although the exact mechanism by which 14-3-3 is involved in S-phase regulation cannot be inferred at present, several correlations can support such a role. First, we envisage a direct role by association to MCM proteins. Indeed, we show that Bmh2 protein is bound to chromatin throughout the cell cycle, which can justify its presence at the replication fork during S phase. Moreover, we also show that the interaction of Bmh2 with Mcm2 protein is much stronger compared with that with Orc2, which might allow 14-3-3 to travel with the fork in a stable complex with MCM proteins. As S phase proceeds, 14-3-3 proteins remain bound to ORC, whereas MCM proteins are displaced from the chromatin (Donovan et al., 1997; Liang and Stillman, 1997). As shown in this study, consistent with the fact that, during G1, Bmh2 is maximally bound to the origin and requires Orc2 in order to be maintained on the chromatin, it is likely that most of the 14-3-3 proteins are displaced from the chromatin with MCM during S phase and only a fraction might remain associated with ORC. As the yeast cells enter a new cell cycle, more 14-3-3 proteins are being recruited to the chromatin and participate in the initiation process during G1. Furthermore, the finding that 14-3-3 proteins interact with several MCM proteins (Meek et al., 2004; Pozuelo Rubio et al., 2004) and are required for G1-S transition (Lottersberger et al., 2006) strengthens the hypothesis of their involvement in the elongation step of DNA replication.

Alternatively, 14-3-3 proteins might affect S-phase progression through indirect mechanisms, such as acetyltransferase and deacetylase regulation (Lottersberger et al., 2007), or through other as-yet-unknown factors involved in this process. Further analytical and structural studies on the interaction of 14-3-3 proteins with origins of DNA replication and with other DNA-replication proteins are needed for better understanding of the mechanisms that control the regulation of eukaryotic DNA replication.

Materials and Methods

Strains and plasmids

The *S. cerevisiae* wild-type strain CEN-PK113-7D (*MATa*; Kötter, Göttingen, Germany), the *bmh2-ts* mutant GG3096 [*MATa bmh1::kanMX bmh2* (*ts*)] (Bruckmann et al., 2004), and the *orc2-1* mutant strain in the W303-1A background (Archer et al., 1995) were used in this study. All yeast strains express proteins at endogenous levels. All yeast strains were cultured in glucose complete or minimal media, as indicated. The strains for plasmid stability assays were generated by inserting separately in CEN-PK113-7D and in GG3096 either pARS-1 (pRS313) or pARS-2 (pRS313-ARS307) plasmids (Yahyaoui et al., 2007).

Co-immunoprecipitation and western blotting

Yeast WCEs were prepared as described (Zhang et al., 2002). Lysates were pre-cleared with protein-G agarose beads and immunoprecipitated with anti-Bmh1, anti-Mcm2, anti-Orc3 or anti-Orc2 antibody. Extract with NRS or NoAb added was used in the same immunoprecipitation conditions as controls. Western blotting was carried out as previously described (Yahyaoui et al., 2007).

Cell synchronization, flow cytometry and chromatin-binding assay

Cell-cycle synchronization with α factor, HU or nocodazole, and chromatin-binding assays were performed as described (Liang and Stillman, 1997; Weinreich et al., 1999). FACS analyses were modified from those previously described (Liang and Stillman, 1997) by using Sytox green (Molecular Probes) dye to quantify DNA contents.

ChIP assay and real-time PCR

The ChIP assay was modified from that in Zhang et al. (Zhang et al., 2002). In brief, cells were subjected to chromatin immunoprecipitation with salmon sperm DNA-coated beads (Upstate, Temecula, CA). After the washes, beads were resuspended in 100 µl of 10% Chelex100 resin and the rest of the procedure was carried out as described (Nelson et al., 2006). PCR and real-time PCR reactions were performed as described (Yahyaoui et al., 2007), with 3 µl of each ChIP material as template DNA.

Plasmid loss assay

Plasmid loss assays were carried out as described (Yan et al., 1991). Cells containing either pARS-1 (carrying a single ARSH4) or pARS-2 (carrying ARSH4 and ARS307) were grown to early log phase in SCM-His medium at 23°C, diluted ten times in YPD medium, and grown at 23°C or 30°C for ten generations. Cultures were spread into complete or minimal media plates in triplicate. Plasmid loss rates per generation were determined as described (Yahyaoui et al., 2007).

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