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The Replicon Initiation Burst Released by Reoxygenation of Hypoxic T24 Cells is Accompanied by Changes of MCM2 and Cdc7

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Although MCM2 is obviously important for the initiation of eukaryotic DNA replication, its role in O_2 dependent regulation of replicon initiation is poorly understood. In this report, I analysed the changes of MCM2 during the transition from hypoxically suppressed replicon initiation to the burst of initiation triggered by reoxygenation in T24 cells. A high level of chromatin bound and nucleosolic MCM2 was found under the hypoxic replicon arrest. In contrast low cytosolic MCM2 was noticed. Recovery of O_2 induced phosphorylation and diminution of chromatin bound MCM2, whereas cytosolic MCM2 increased. The level of chromatin bound Cdc7 did not change significantly upon reoxygenation. However, after reoxygenation, significant phosphorylation of Cdc7 and an increase of coimmunoprecipitation with its substrate (MCM2) were observed. This provides a hint that reoxygenation may promote the kinase activity of Cdc7. These changes might be the critical factors in O_2 dependent regulation of replicon initiation. Moreover, phosphorylation of Cdc7 by Cdk2 can be observed *in vitro*, but seems to fail to regulate the level of chromatin bound Cdc7 as well as the changes of MCM2 in response to reoxygenation of hypoxically suppressed cells.

Keywords: Cdc7, Chromatin, Hypoxia, MCM2, Reoxygenation, Replication

Introduction

In eukaryotes, the initiation of DNA replication is a cell cycle regulated process, requiring the formation of a pre-replicative complex (pre-RC) in G1 phase. Pre-RC is assembled through sequential binding of ORC, Cdc6, Cdt1 and MCM proteins. Pre-RC is activated for active replication by two essential kinases Cdk2 and Cdc7 (Dutta and Bell, 1997; Kelly and Brown, 2000; Bell and Dutta, 2002; Wang *et al.*, 2006). Cdk2 is thought to phosphorylate diverse factors including pRB, transcription factors and components of pre-RC such as MCM-proteins and Cdc6 (Ohtani *et al.*, 1998; Angus *et al.*, 2002; Choi *et al.*, 2003b). Cdc7 is a serine/threonine kinase and the activation of Cdc7 requires association with its regulatory protein Dbf4 in S. cerevisiae, Dfp1/Him1 in S.pombe, and ASK in mammals (Kitada *et al.*, 1992; Kumagai *et al.*, 1999; Takeda *et al.*, 1999; Masai and Arai, 2000; Hisao Masai, 2002). In eukaryotes, one of the conserved substrates of Cdc7 kinase is MCM2, an essential component of MCM2-7 complex (Tye, 1999; Kelly and Brown, 2000; Lei and Tye, 2001; Bell and Dutta, 2002; Shechter and Gautier, 2004). Biochemical studies suggest that the presence of MCM2 inhibits the helicase activity of MCM4/6/7 complex (Lee and Hurwitz, 2000; Ishimi *et al.*, 2001). The phosphorylation of MCM2 by Cdc7 is reported to be a critical step in the initiation of DNA replication, eventually leading to release of MCM2 and resulting in activation of the MCM4/6/7 helicase (Ishimi, 1997; Lei *et al.*, 1997; Ishimi *et al.*, 1998; Lei and Tye, 2001). However, these results have been discussed again by the finding that concerted action of Cdk2 and Cdc7 is probably required for the phosphorylation of MCM2 and for the initiation of DNA replication as well (Masai *et al.*, 2000).

I carried out an especially elaborated starvation protocol which accumulates T24 cells in a G1 arrest, from which they can be released by medium renewal (van Betteraey-Nikoleit *et al.*, 2003). Normally, the cells then proceed to the S-phase within approximately 5 h, passing through a number of the above mentioned regulatory steps until the start of orderly DNA synthesis at the origins of replicon (scheduled to be activated as the very first of the S-phase). When the cells are subjected to hypoxia directly, after restimulation with fresh medium, the replicon initiation is reversibly suppressed which leads to a "hypoxic pre-initiation state", similar to the "pre-replicative complex" described by Bell and Dutta (Bell and

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Dutta, 2002; van Betteraey-Nikoleit *et al.*, 2003). Reoxygenation after several hours hypoxia causes a synchronous wave of initiation followed normal daughter strand synthesis by elongation and termination (Probst *et al.*, 1984; Probst *et al.*, 1988; Riedinger *et al.*, 1992; Gekeler *et al.*, 1993; Riedinger *et al.*, 2002). Hypoxia interferes with this switching process, from hypoxic pre-initiation state to effective and synchronous initiation of DNA replication (van Betteraey-Nikoleit *et al.*, 2003). The molecular mechanisms involved are still largely unknown.

In this report, I examined the MCM2 and Cdc7 in the course of starvation-refeeding/hypoxia-reoxygenation experiment. I first demonstrated that reoxygenation after several hours hypoxia induces phosphorylation of chromatin bound MCM2 which then dissociates from chromatin. In addition, an increase of phosphorylation of Cdc7 as well as an increase of coimmunoprecipitation of Cdc7 and MCM2 after reoxygenation is reported. On the other hand, it has been shown that the concerted action of Cdk2 and Cdc7 is reported to be important for phosphorylation of MCM proteins (Masai *et al.*, 2000). This report provides evidence that Cdc7 can be phosphorylated by Cdk2 in T24 cells. However, inhibiting the phosphorylation of Cdc7 by Cdk2 seems not to affect the changes of chromatin associated Cdc7 and MCM2 in the course of the replicon initiation burst after O_2 recovery.

Material and Methods

Cell culture, transient hypoxia, reoxygenation and radioactive labeling. T24 cells (ATCC No. HBT-4) were grown in plastic flasks in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (100 U/100 μg · mL^{-1}). The cells were subcultured when they reached confluence. Under these conditions, the cells exhibited a partially tetraploid caryotype. For synchronization, the desired number of glass petri dishes was seeded, 44 h before the start of an experiment, from an almost confluent large culture with 150,000 cells · mL^{-1} (35 mm, 1.5 mL; 145 mm, 25 mL). After 44 h, most of the cells became arrested in G1-phase due to starvation (van Betteraey-Nikoleit *et al.*, 2003). For prelabeling the DNA, the seeding medium was supplemented with 5 nCi · mL^{-1} ^{14}C-thymidine. Experiments started by stimulation of the cells by a complete exchange of the culture medium with prewarmed fresh medium supplemented with 10% (v/v) fetal calf serum. Subsequent gassing of the cell cultures was performed with a continuous flow of humidified artificial air containing 5% (v/v) CO_2 for normoxic incubations, and with 0.0075% O_2, 5% CO_2, and Ar ad 100% for hypoxia. This hypoxic gassing protocol diminished the pO_2 in the cultures within 1.5-2 h to about 0.1% and within 6-7 h to about 0.02%. The equipment and the procedures used have been described in Dreier *et al.*, 1993. For reoxygenation, 0.25 volumes of medium equilibrated with 95% O_2/5% CO_2 (v/v) were added to the hypoxic cultures, and gassing was continued with artificial air. [Methyl-^{3}H]deoxythymidine and inhibitor were added either directly to the cells, or under hypoxic culture conditions by plunging a spatula carrying appropriate quantity in dried form into the culture medium without opening the gassing vessel. To stop the incubations, medium was removed by aspiration and the cells were washed once with ice-cold phosphate buffered saline (PBS: 150 mM NaCl/P_i; 10 mM NaHPO4, pH 7) and either processed for determination of acid insoluble radioactivity as described in Probst *et al.*, 1983 or, otherwise, for analyses described below.

Alkaline sedimentation analyses of growing daughter strand DNA. Cultures on 35 mm glass petri dishes were pulse-labeled for 8 min with 7 μCi [methyl-^{3}H]deoxy-thymidine · mL^{-1}. The cells were trypsinized for 5 min at room temperature and layered onto the top of 10-30% alkaline sucrose gradients (Probst and Gekeler, 1980). After denaturation of the DNA for 6 h, centrifugation was performed at 20 000 r.p.m., 23°C for 10 h in a Beckman SW28 rotor. Fractions of 1.2 mL were collected from the top of the gradients and processed to analyze acid insoluble radioactivity.

Cell fractionation. Cells were washed once with phosphate buffered isotonic saline and twice with hypotonic buffer (50 mM Hepes, pH 7.5, 20 mM β-glycerophosphate; 1 mM DTT, containing Aprotinin (1 μM), Leupeptin (50 μM), AEBSF (1 mM), NaF (10 mM) and Na_3Vo_4 (1 mM)) and then suspended in 10 mL of the hypotonic buffer. After 10 min on ice, the cells were disrupted to free nuclei by 20 strokes with a tight fitting pestle of a dounce homogenisator and were then centrifuged for 5 min, at 1500 *g* and at 4°C to separate the cytosolic supernatant from nuclear pellet. Afterwards, the nuclei were resuspended in extraction buffer (hypotonic buffer containing 0.1% NP40) and centrifuged for 3 min, at 600 *g* and at 4°C. Suspension and centrifugation were repeated thrice. The supernatants were combined and yielded the fraction of nucleosolic proteins. The remaining pellet contains 8.2% of total cellular protein and 96.6% of total DNA and is further referred to as chromatin fraction. This chromatin fraction could be solubilized by incubation in IP buffer (extraction buffer containing 450 mM NaCl/P_i) for 1 h on ice and subsequent centrifugation.

Immunoprecipitation, Kinase assay and phosphatase digestion. For immunoprecipitation of Cdk2, Cdc7 and the subsequent kinase assay, solutions of solubilized chromatin bound proteins were adjusted to equal protein content (300 μg) incubated with 2 μg of Cdc7 antibody (Mo Bi Tec) or Cdk2 antibody (Santa Cruz Biotechnologies) for 40 min on ice. Then, 20 mL of slurry of protein G-agarose beads (Santa Cruz Biotechnologies) equilibrated with IP buffer were added, and incubation was continued overnight under continuous rotation. Subsequently, the beads were washed twice with 400 μL IP buffer and twice with 400 μL kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 20 mM β-Glycerophosphate, containing Aprotinin (1 μM), Leupeptin (50 μM), AEBSF (1 mM), NaF (10 mM), and Na_3VO_4 (1 mM)). Excess fluid was removed completely and the beads were incubated for 45 min at 30°C in a mix of 20 μL kinase buffer containing 0.2 mM ATP; and 10 μCi γ^{33}-P-ATP. Incubation was stopped by adding 20 μL 2× protein sample buffer. After denaturation, the reaction products were separated on a 12% SDS- Polyacrylamide gel, blotted on to a Nylon-P membrane (Amersham) and subsequently exposed to Hyperfilm MP (Amersham). For phosphatase digestion of Cdc7, the chromatin bound proteins were precipitated by the Wessel-Flügge precipitation (Wessel and Flugge, 1984). The precipitate was washed twice with phosphatase buffer (New England Biolabs)

supplemented with protease inhibitors. Phosphatase digestion was performed for 60 min at 30°C with 400 U lambda phosphatase (New England Biolabs). The supernatant was removed and after denaturation, the proteins were separated on a 8% (149 : 1, acrylamide:bisacrylamide) SDS-Polyacrylamide gel, blotted on to Hybond-P (Amersham).

For immunoprecipitation and subsequent phosphatase digestion of MCM2, solutions of solubilized chromatin bound proteins, containing 300 μg of protein were incubated with 2 μg of MCM2 antibody (Santa Cruz Biotechnologies) for 40 min on ice. Then, 20 μL of slurry of protein G-agarose beads (Santa Cruz Biotechnologies) equilibrated to with IP buffer were added, and incubation was continued overnight under continuous rotation. Subsequently, the beads were divided into two equal parts and washed three times with IP buffer. 20 μL of protein sample buffer was added to one part afterwards. The other part was washed three times with phosphatase buffer (New England Biolabs) supplemented with protease inhibitors. Phosphatase digestion was performed for 30 min at 30°C with 200 U lambda phosphatase (New England Biolabs). After a final wash with IP buffer, 20 μL protein sample buffer was added. After denaturation, proteins were separated on a 8% (149 : 1, acrylamide:bisacrylamide) SDS-Polyacrylamide gel, and blotted on to a Hybond-P membrane (Amersham). Proteins were visualized as described below.

Electrophoresis of proteins and western blotting. Cytosolic, nucleosolic and chromatin bound proteins were precipitated by the Wessel-Flügge method (Wessel and Flugge, 1984). Chromatin bound proteins for western blot analyses were either separated from the DNA by treatment with extraction buffer containing 450 mM NaCl/P_i. The chromatin fraction was either directly or after Wessel-Flügge precipitation denatured and solubilized with SDS electrophoresis sample buffer. After determination of the protein concentration (Biorad DC protein assay), equal amounts were separated on an appropriate SDS-Polyacrylamide gel, blotted on to Nylon-P membrane (Amersham) and subsequently immunodetected using the ECL western blotting procedure (Amersham) according to the manufacturer's instructions. Dilution of antibodies used was as follows: PCNA (Santa Cruz, Clone PC10) 1 : 10000, Cdc7 (Mo Bi Tec, Clone DCS-342) 1 : 2500, Cdk2 (Pharmingen, Clone 55) 1 : 2500, MCM2 (Santa Cruz, Clone N-19) 1 : 500. All the experiments were performed at least three times with similar results.

Results

Changes of MCM2 and Cdc7 after reoxygenation of hypoxic T24 cells. The experiment was started through medium renewal of starved T24 cells under normoxic and hypoxic conditions and 5 min, 30 min, and 1 h after reoxygenation incubation conditions. The cell samples were fractionated as described in to cytosolic, nucleosolic, and chromatin bound proteins. Equal amounts of above indicated fractions were separated on a 8% SDS-Polyacrylamide gel and probed with anti-MCM2 antibody. The MCM2 migrated to a gel position of approximately 116 kDa. In Fig. 1A, a significant amount of cytosolic, nucleosolic, and chromatin bound MCM2 was detectable under normoxic conditions without medium renewal (N-). This result indicates that MCM2 existed in all 3 fractions at the end of the G1-phase arrest caused by starvation. An elevated signal of chromatin bound and nucleosolic MCM2 was found under hypoxia. A gradual decrease of chromatin bound as well as of nucleosolic MCM2 occurred after 5 min and 30 min of reoxygenation. MCM2 was barely detected after 1 h following reoxygenation and under normoxic incubation condition after medium renewal (N+). In contrast, an increase in the cytosolic MCM2 was seen after reoxygenation and under normoxia after medium renewal. Only few cytosolic MCM2 was detectable under hypoxic incubation condition.

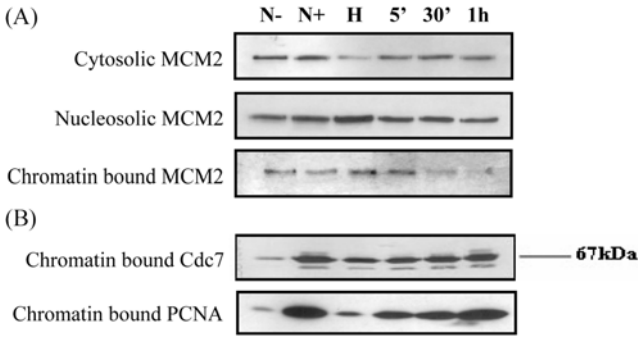

Fig. 1. Changes of MCM2 and Cdc7 after reoxygenation of hypoxic T24 cells. (A) Western blot analysis of cytosolic, nucleosolic, and chromatin bound MCM2 from normoxic, hypoxic, 5 min, 30 min, and 1 h reoxygenated cells. (B) Western blot analysis of chromatin bound Cdc7 and PCNA from normoxic, hypoxic, 5 min, 30 min, and 1 h reoxygenated cells. N-, 7 h normoxic incubation without medium renewal (i.e. beginning of the experiments); N+, 7 h normoxic incubation after medium renewal; H, 7 h hypoxic incubation after medium renewal; 5', 5 min reoxygenated after 7 h hypoxic incubation; 30', 30 min reoxygenated after 7 h hypoxic incubation; 1h, 1 h reoxygenated after 7 h hypoxic incubation.

In T24 cells, chromatin bound Cdc7 exists as three bands, one middle major band at 67 kDa, a lower minor band, and an upper minor band (Fig. 1B). Cdc7 was barely detectable under the starved condition which suggests that Cdc7 becomes bound to chromatin after restimulation with fresh medium. The relatively high level of Cdc7 is reported under hypoxia, only slight increase was detectable after the reoxygenation. Chromatin bound PCNA was analysed to confirm that the experiment was correct. PCNA is the processivity factor of DNA-Polymerase δ and its chromatin associated fraction can serve as a direct measure for the intensity of DNA synthesis. A high level of chromatin bound PCNA under hypoxia would be indicative for an incorrect experiment; for example, undesirable entry of oxygen to cells during hypoxic incubation (van Betteraey-Nikoleit *et al.*, 2003). The very low signal intensity of chromatin bound PCNA under normoxic condition without medium renewal (Fig. 1B) indicates that the cells are not in a replicative state and that they proceed after medium

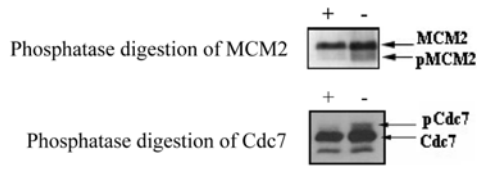

Fig. 2. Phosphorylation of MCM2 and Cdc7 after reoxygenation. Lambda protein phosphatase digestion of chromatin bound MCM2 and Cdc7 from 30 min reoxygenated T24 cells. (+), after phosphatase digestion according to the manufacturer's instructions (New England Biolabs: Lambda protein phosphatase); (–), prior phosphatase digestion.

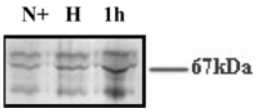

Fig. 4. *In vitro* phosphorylation of immunoprecipitated Cdc7. Chromatin bound Cdc7 was immunoprecipitated with anti-Cdc7 antibody from salt eluted chromatin bound proteins, normoxia, hypoxia and reoxygenation. Immunoprecipitation and subsequent kinase assay were performed as described. The kinase reaction was stopped by boiling in protein sample buffer and the proteins were separated by SDS/PAGE (12% polyacrylamide). After blotting, the membrane was autoradiographed. N+, 7 h normoxic incubation after medium renewal; H, 7 h hypoxic incubation after medium renewal; 1 h, 1 h reoxygenated after 7 h hypoxic incubation.

renewal to S-Phase. An essentially low signal of PCNA indicates that under hypoxia no replication was initiated. However, chromatin bound PCNA increased strongly as soon as 5 min after reoxygenation and continued to increase after 30 min and until 1 h. This expression pattern of PCNA confirms the hypoxic reversible replication arrest and synchronous initiation after few minutes reoxygenation.

***In vivo* phosphorylation of MCM2 and Cdc7 after reoxygenation.** To test whether MCM2 undergoes phosphorylation after reoxygenation, I conducted a λ protein phosphatase digestion of chromatin bound MCM2 from 30 min reoxygenated T24 cells. MCM2 was separated by 8% (149 : 1, acrylamide:bisacrylamide) SDS-Polyacrylamide gel. Fig. 2 shows that the lower band of MCM2 disappeared in the δ protein phosphatase treated sample. This result suggests that the lower band of chromatin bound MCM2 represents its phosphorylation band. In contrast to MCM2, the upper minor band represents phosphorylation band of chromatin bound Cdc7 in T24 cells. The upper minor band disappeared after phosphatase treatment of Cdc7 from 30 min reoxygenated cells (Fig. 2). However, In Fig. 1B, the phosphorylation band of Cdc7 was barely detected under hypoxia and increased significantly after reoxygenation.

Immunoprecipitation and coimmunoprecipitation of MCM2 and Cdc7. Chromatin fraction was subjected to immunoprecipitation (IP) using anti-MCM2 antibody and fractionated by 8% SDS/PAGE. Proteins were transferred to a Nylon-P membrane and immunoblotted with anti-MCM2 and anti-Cdc7 antibodies. Similar results were obtained using anti-Cdc7 immunoprecipitation and following western blot analysis with anti-Cdc7 and anti-MCM2 antibodies. In Fig. 3, a high amount of immunoprecipitated MCM2 was detected under hypoxia and which decreased gradually after reoxygenation up to 1 h. In normoxia and after reoxygenation, Cdc7 was strongly coimmunoprecipitated with MCM2 and vice versa. The phosphorylation band (upper minor band) of Cdc7 was weak in immunoprecipitated as well as in coimmunoprecipitated Cdc7. In contrast to MCM2, the immunoprecipitated Cdc7 was almost constant, irrespective of the different incubation conditions. Reoxygenation and thereby phosphorylation of Cdc7 caused no dissociation of Cdc7 from chromatin. Although MCM2 decreases gradually after reoxygenation, the amount of coimmunoprecipitation of MCM2 and Cdc7 increased after reoxygenation as well as under normoxia after restimulation with fresh medium (Fig. 3). This capability of coimmunoprecipitation of MCM2 and Cdc7 was barely detected under hypoxia.

***In vitro* phosphorylation of immunoprecipitated Cdc7.** It has been shown that MCM2 possesses phosphorylation sites for Cdk2 kinase and the concerted action of Cdk2 and Cdc7 may be essential for the phosphorylation of MCM2 (Masai *et al.*, 2000). I examined, whether a relationship between these

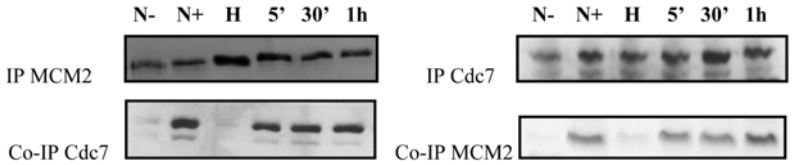

Fig. 3. Coimmunoprecipitation of MCM2 and Cdc7. Chromatin fraction was subjected to immunoprecipitation (IP) using anti-MCM2 antibody, as indicated, and fractionated by 8% SDS/PAGE. Proteins were transferred to a Nylon-P membrane and immunoblotted with anti-MCM2 and anti-Cdc7 antibodies. Similar results were obtained using anti-Cdc7 immunoprecipitation and following western blot analysis with anti-Cdc7 and anti-MCM2 antibodies. N-, 7 h normoxic incubation without medium renewal; N+, 7 h normoxic incubation after medium renewal; H, 7 h hypoxic incubation after medium renewal; 5', 5 min reoxygenated after 7 h hypoxic incubation; 30', 30 min reoxygenated after 7 h hypoxic incubation; 1 h, 1 h reoxygenated after 7 h hypoxic incubation.

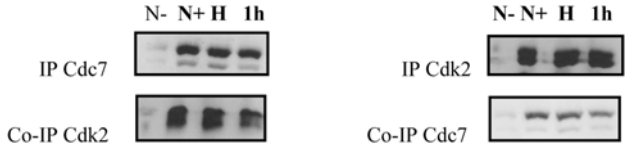

Fig. 5. Coimmunoprecipitation of Cdc7 and Cdk2. Chromatin fraction was subjected to immunoprecipitation (IP) using anti-Cdc7 antibody, as indicated, and fractionated by 8% SDS/PAGE. Proteins were transferred to a Nylon-P membrane and immunoblotted with anti-Cdc7 and anti-Cdk2 antibodies. Similar results were obtained using anti-Cdk2 immunoprecipitation and following western blot analysis with anti-Cdk2 and anti-Cdc7 antibodies. N+, 7 h normoxic incubation after medium renewal; H, 7 h hypoxic incubation after medium renewal; 1 h, 1 h reoxygenated after 7 h hypoxic incubation.

two kinases could be involved on the changes of MCM2 triggered by reoxygenation. Anti-Cdc7 Immunoprecipitates of salt eluted chromatin bound proteins from different incubation conditions were incubated in the protein kinase assay mixture described in Material and Methods and separated afterwards on a 12% SDS-Polyacrylamide gel. The autoradiograph of the blot obtained from this gel convincingly shows an *in vitro* phosphorylation of immunoprecipitated Cdc7 (Fig. 4). A slight increase of phosphorylation was found with material prepared 1 h after reoxygenation. The autoradiograph shows three bands of Cdc7. This indicates that Cdc7 possibly has more than one phosphorylation site. However, Fig. 5 shows that Cdc7 is strongly coimmunoprecipitated with Cdk2 and vice versa. Chromatin bound Cdk2 and Cdc7 from normoxic, hypoxic, and reoxygenated cells were immunoprecipitated using anti-Cdc7 and anti-Cdk2 antibodies. Immunoprecipitates were separated on a 8% polyacrylamide gel containing SDS and probed on the blot of the gel with both antibodies. Fig. 5 shows that the different incubation conditions caused no significant differences in coimmunoprecipitation of Cdc7 and Cdk2 in T24 cells. The question arises here, 'Is an action of Cdk2 and Cdc7 essential for the changes of MCM2 after reoxygenation?'

Effect of administration of rottlerin 3 hours before reoxygenation on Cdk2, Cdc7 and MCM2. Hypoxic gassed 20 μM rottlerin was added (without interruption of the hypoxic incubation) 3 h before stop or reoxygenation to hypoxic cells. In controls, inhibitor addition was omitted. The separation of chromatin bound Cdk2, MCM2, and Cdc7 were succeeded by 8% SDS-Polyacrylamide gel. Fig. 6A shows that administration of rottlerin caused inhibition of mobility shift of Cdk2 in hypoxic and reoxygenated cells. This block of mobility shift indicates the Cdk2 inhibition. On the other hand, Fig. 6A reveals that a high amount of chromatin bound MCM2 was detectable in starved cell, decreased significantly after medium stimulation, whereby more MCM2 is accumulated in rottlerin treated and not treated hypoxic cells. After reoxygenation, the amount of chromatin bound MCM2

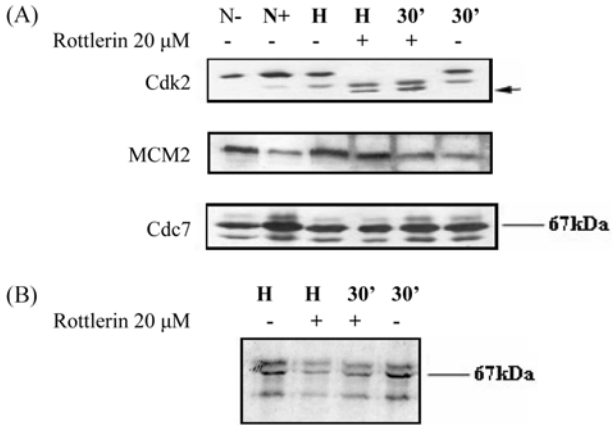

Fig. 6. (A) Inhibition of Cdk2 does not affect the changes of MCM2 and Cdc7 after reoxygenation. Western blot analysis of chromatin bound Cdk2, MCM2 and Cdc7 from 7 h normoxic, hypoxic, and 30 min reoxygenated T24 cells. Rottlerin (20 mM) was added 3 h before reoxygenation where indicated. The black arrow indicates that the mobility shift of Cdk2 is inhibited by rottlerin treatment. N–, 7 h normoxic incubation without medium renewal; N+, 7 h normoxic incubation after medium renewal; H, 7 h hypoxic incubation after medium renewal; 30', 30 min reoxygenated after 7 h hypoxic incubation. (B) Phosphorylation of immunoprecipitated Cdc7 is susceptible for inhibition of Cdk2 by rottlerin *in vivo*. Chromatin bound Cdc7 was immunoprecipitated with anti-Cdc7 antibody from salt eluted chromatin bound proteins, hypoxia and reoxygenation. Immunoprecipitation and following kinase assay were performed as described. The kinase reaction was stopped by boiling in protein sample buffer and the proteins were separated by SDS/PAGE (12% polyacrylamide). After blotting, the membrane was autoradiographed. Rottlerin (20 mM) was added 3 h before reoxygenation where indicated. H, 7 h hypoxic incubation after medium renewal; 30', 30 min reoxygenated after 7 h hypoxic incubation.

decreased both in the presence and in the absence of rottlerin. The amount of Cdc7 under different incubation conditions and *in vivo* phosphorylation after reoxygenation were not affected by rottlerin treatment. Subsequently, chromatin bound Cdc7 was immunoprecipitated using anti-Cdc7 antibody and kinase assay was performed. Fig. 6B shows that *in vivo* administration of rottlerin caused a significant reduction of *in vitro* phosphorylation of immunoprecipitated Cdc7 from hypoxic and reoxygenated cells. The *in vitro* phosphorylation of immunoprecipitated Cdc7 is susceptible to inhibition of Cdk2 by *in vivo* administration of rottlerin.

Phosphorylation of Cdc7 by Cdk2 is reported to be not essential for replicon initiation triggered by reoxygenation. In order to ascertain the role of phosphorylation of Cdc7 by Cdk2 on synchronous wave of replicon initiation after reoxygenation of hypoxic T24 cells, alkaline sedimentation analyses of the length distribution of pulse labeled daughter strand DNA was carried out. On the basis of numerous prior studies (Probst *et al.*, 1984; Probst *et al.*, 1988; Riedinger *et*

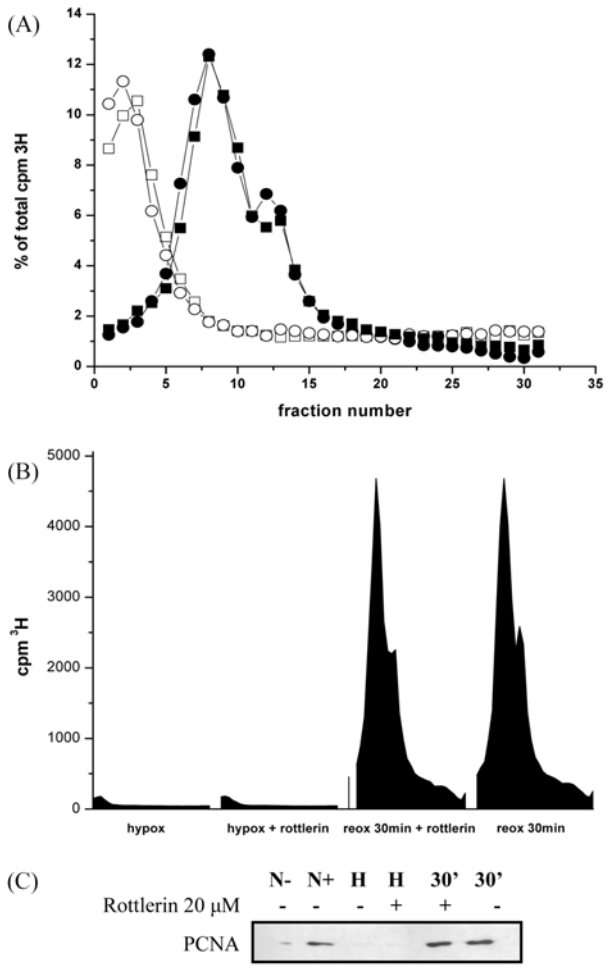

Fig. 7. Inhibition of phosphorylation of Cdc7 by Cdk2 does not affect the replicon initiation triggered by reoxygenation. (A) Alkaline sedimentation patterns of pulse-labelled T24 DNA after lysis on top of the gradients. Eight minutes before the end of the respective incubation conditions, nascent daughter strand DNA chains were pulse-labelled with 7 µCi [^{3}H]dThd · mL$^-$. Rottlerin (20 µM) was added 3 h before reoxygenation. □, 7 h Hypoxic incubation after medium renewal; ○, 7 h Hypoxic incubation after medium renewal in presence of rottlerin; ■, 30 min reoxygenated after 7 h hypoxic incubation in presence of rottlerin; ●, 30 min reoxygenated after 7 h hypoxic incubation. (B) Comparison of the gradient profiles of hypoxic and 30 min reoxygenated T24 cells. Profiles are depicted consecutively in total c.p.m. Rottlerin (20 mM) was added 3 h before reoxygenation. Hypoxia, 1968 c.p.m.; Hypoxia with rottlerin treatment, 2011 c.p.m.; Reoxygenated 30 min with rottlerin treatment, 35864 c.p.m.; Reoxygenated 30 min, 38353 c.p.m. Each profile consists of 31 fractions. (C) Western blot and subsequent immunological detection of chromatin bound PCNA. Rottlerin (20 µM) was added 3 h before reoxygenation where indicated. N–, 7 h normoxic incubation without medium renewal; N+, 7 h normoxic incubation after medium renewal; H, 7 h hypoxic incubation after medium renewal; 30', 30 min reoxygenation after 7 h hypoxic incubation.

al., 1992; Gekeler *et al.*, 1993; Riedinger *et al.*, 2002), 8 min ^{3}H-dThd pulses applied 30 min after reoxygenation is turned out to be most convenient for demonstrating initiation and succeeding elongation. The alkaline sedimentation profile (Fig. 7A and B) shows almost total absence of replicative activity in hypoxia both in the absence and in the presence of rottlerin. Interestingly, there is almost coincident prominent radioactivity peaks represent the initiation of DNA replication in 30 min after reoxygenated cultures both in the presence and in the absence of rottlerin. All the 2 profiles from reoxygenation and reoxygenation with rottlerin treatment are identical. To further address this point, chromatin bound PCNA was separated on a 8% SDS-Polyacrylamide gel and immuno-probed for PCNA. An exceptional low signal of PCNA under normoxic without medium renewal represents the G1 arrest (Fig. 7C). A very low amount of PCNA in both rottlerin treated and non treated hypoxic cultures indicates the hypoxic replicon arrest. Convincingly, an essential increase of chromatin bound PCNA was seen after reoxygenation both in the presence and in the absence of rottlerin as well as in normoxic after medium renewal. This high amount of chromatin associated PCNA represents the initiation of DNA replication and successive elongation.

Discussion

In response to several hours hypoxia, cells undergo reversible replicon arrest (situated very close before actual initiation) and which enters into synchronous wave of initiation by elevating the oxygen level (Probst *et al.*, 1984; Probst *et al.*, 1988; Probst *et al.*, 1989; Riedinger *et al.*, 2002; van Betteraey-Nikoleit *et al.*, 2003). This oxygen dependent regulation of DNA replication is an important property of proliferating mammalian cells, serving basal function, such as protection against metabolic catastrophes during embryonic development or wound healing and in tumour growth (Probst *et al.*, 1999; Choi *et al.*, 2003a). Human T24 bladder carcinoma cells permits a selective accumulation of a defined of replicon (i.e. scheduled to be activated as first of the S-phase) at a "hypoxic pre-initiation state" in response to hypoxia, in absence of other activated replicons (van Betteraey-Nikoleit *et al.*, 2003). This hypoxic pre-initiation state appears very similar to the pre-replicative complex described by Bell and Dutta (Bell and Dutta, 2002; van Betteraey-Nikoleit *et al.*, 2003). The suspicion is, hypoxia interferes with the switching process of pre-replicative complex to initiation of replication (van Betteraey-Nikoleit *et al.*, 2003). In eukaryotes, Cdk2 and Cdc7 kinases are known to promote the switching process of pre-replicative complex to active DNA replication (Kelly and Brown, 2000; Bell and Dutta, 2002). Cdk2 is thought to phosphorylate diverse factors including pRB, transcription factor and some components of pre-replicative complex such as MCM and Cdc6 (Ohtani *et al.*, 1998; Angus *et al.*, 2002). The second kinase, Cdc7 phosphorylates MCM2, a component of MCM2-7 complex at pre-replicative complex. Binding of MCM2 subunit to its complex functions as an inhibitor for the

helicase activity of MCM4/6/7 (Lee and Hurwitz, 2000; Ishimi *et al.*, 2001). Cdc7 mediated phosphorylation of MCM2 is thought to release of MCM2 from its complex and thereby activates the helicase activity of MCM4/6/7 followed initiation (Yan *et al.*, 1993; Chong *et al.*, 1995; Todorov *et al.*, 1995; Ishimi *et al.*, 2001).

This report demonstrated that the high level of MCM2 associates with cellular chromatin when T24 cells arrested in G1 are restimulated by feeding under hypoxic condition and which represents the hypoxic pre-initiation state (van Betteraey-Nikoleit *et al.*, 2003). In contrast, cytosolic MCM2 was barely found under hypoxia. The reoxygenation after several hours of hypoxia induced a gradual decrease of nucleosolic as well as of chromatin bound MCM2 and an increase in cytosol. This data indicates the dissociation of MCM2 from chromatin (Todorov *et al.*, 1995). It has been shown that during the activation of pre-RC for initiation, MCM2 dissociates from chromatin and transports from the nucleus to the cytoplasm through its phosphorylation (Todorov *et al.*, 1995; Fujita *et al.*, 1996; Kimura *et al.*, 1996; Ishimi *et al.*, 2001). However, *In vivo* phosphorylation of chromatin bound MCM2 after reoxygenation is confirmed by removal of its lower band through the phosphatase digestion. The western blot and immunoprecipitation results show that MCM2 is not completely dissociated from chromatin after 1 h reoxygenation and the very low amount of chromatin bound and nucleosolic MCM2 after 1 h reoxygenation may support the study that apart from the initiation, MCM2 is essential for the elongation (Labib *et al.*, 2000).

On the other hand, numerous studies have suggested that MCM2 is a target for Cdc7 kinase (Lei *et al.*, 1997; Brown and Kelly, 1998; Masai *et al.*, 2000; Ishimi *et al.*, 2001). In T24 cells, chromatin bound Cdc7 expressed as one major band at 67 kDa, a lower minor band, and an upper minor band. In contrast to MCM2, the phosphatase digestion suggests that upper minor band represents the phosphorylation of chromatin bound Cdc7. This phosphorylation band of chromatin bound Cdc7 is absent under hypoxia and increased significantly during replicon initiation triggered by O_2 recovery. The chromatin bound Cdc7 is virtually absent in early G1-phase under starvation, binds to chromatin in late G1phase under hypoxia, increases slightly during succeeding S-phase under normoxia after medium renewal and after reoxygenation. Unlike MCM2, reoxygenation causes no dissociation of Cdc7 from chromatin (Oshiro *et al.*, 1999; Sato *et al.*, 2003). However, the kinase activity of Cdc7 is regulated by its regulatory subunit and kinase activity reaching a peak at G1 through S-phase (Sclafani *et al.*, 1988; Masai and Arai, 2000; Ogino *et al.*, 2001; Yanow *et al.*, 2003; Yamashita *et al.*, 2005). Although the level of chromatin bound MCM2 decreased after reoxygenation and the level chromatin bound Cdc7 did not vary significantly under different incubation conditions, the coimmunoprecipitation among Cdc7 and MCM2 is virtually absent in hypoxic replicon arrest and increased significantly during initiation after subsequent reoxygenation. This increase of coimmunoprecipitation of MCM2 and Cdc7 indicating the evidence of increase of both proteins for interaction after reoxygenation. Together, the absent of coimmunoprecipitation and absent of phosphorylation of Cdc7 in hypoxic T24 cells suggest that unphosphorylated Cdc7 under hypoxia is not capable of coimmunoprecipitation with MCM2.

The concerted action of Cdk2 and Cdc7 is known to be required for phosphorylation of MCM2 (Masai *et al.*, 2000; Montagnoli *et al.*, 2006). It has also shown that Cdk2 phosphorylates Cdc7 and MCM2 *in vitro* (Kelly and Brown, 2000; Masai *et al.*, 2000). I found *in vitro* phosphorylation of immunoprecipitated Cdc7 in T24 cells. The autoradiograph shows that presence of more than one potential phosphorylation site on Cdc7 (Masai *et al.*, 2000). Furthermore, Cdk2 promotes the phosphorylation of Cdc7 (Jares and Blow, 2000; Masai *et al.*, 2000). This interpretation is supported by a strong coimmunoprecipitation between Cdk2 and Cdc7 in T24 cells. The mobility shift of Cdk2 on western blot suggests that administration of rottlerin 3 hours before stop or reoxygenation to hypoxic cells inhibits Cdk2 from hypoxic as well as from reoxygenation incubation conditions. The exact mechanism of rottlerin activity on Cdk2 under hypoxia is yet to be studied. However, i*n vitro* phosphorylation of Cdc7 is susceptible for *in vivo* administration of rottlerin. Although the *in vitro* phosphorylation of Cdc7 by Cdk2 is inhibited, no detectable changes on the behaviour of chromatin associated Cdc7 was found (Jares and Blow, 2000). The *in vivo* phosphorylation of Cdc7 in Cdk2 inhibited reoxygenated cells may give an idea that phosphorylation of Cdc7 is promoted not only by Cdk2 and also from some other sources, which are yet to be studied. Moreover, Inhibiting Cdk2 by rottlerin does not affect the changes of chromatin bound MCM2 in response to hypoxia and after reoxygenation. Consequently, these results strongly propose that phosphorylation of Cdc7 by Cdk2 has no detectable influence on changes of chromatin bound MCM2 after reoxygenation. This data supports the criterion that Cdk2 is not necessary for phosphorylation of MCM2 (Jares and Blow, 2000). Furthermore, alkaline sedimentation profile and the behaviour of proliferation marker PCNA in the chromatin fraction reveal that inhibiting Cdk2 and thereby inhibiting the phosphorylation of Cdc7 by administration of the rottlerin 3 h before reoxygenation to hypoxic cells has no effect on synchronous burst of DNA replication initiation occurring after reoxygenation. It has been shown that Cdk2 and phosphorylation of its possible targets Cdc6 and pRb are not necessary for releasing the burst of replicon initiations upon oxygen recovery (Stabenow *et al.*, 2005), but this criterion fails to deal with the phosphorylation of Cdc7 by Cdk2 and its effect in the initiation burst triggered by O_2 recovery. However, this report strongly supports the above mentioned criterion and suggests that the phosphorylation of Cdc7 by Cdk2 is not essential for the initiation burst released by reoxygenation.

In summary, dissociation and phosphorylation of chromatin

bound MCM2 are possibly essential for releasing the burst of initiations of hypoxically suppressed replicon upon reoxygenation. The *in vivo* phosphorylation of Cdc7 and changes of coimmunoprecipitation between Cdc7 and MCM2 provide the possibilities for the activation of Cdc7 kinase after reoxygenation. However, changes of MCM2 after reoxygenation are a Cdk2 independent manner and not compatible with concerted action of Cdk2 and Cdc7.

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