The Biochemical Interplay between MCP1 and ATR

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Abstract
The maintenance of genome integrity requires and is regulated by two PIKKs [PI3K (phosphoinositide 3-kinase)-related kinases], the ATM (ataxia-telangiectasia mutated) and the ATR (ATM- and Rad3-related). ATR is the major coordinator of the response to DNA damage agents that interfere with progression of the replication fork and inhibit DNA synthesis. Since our previous work showed that MCP1 was associated with the early replication stages of DNA replication, we questioned whether MCP1 was required for the ATR pathway. Biochemical and immunological approaches revealed an association of MCP1 with ATR in undamaged and damaged cells. In damaged cells, the ATR-MCP1 interaction was primarily exhibited by the 31kDa form of MCP1. In undamaged cells, both 31kDa and 33kDa MCP1 isoforms interacted with ATR. The immunoprecipitation and immunofluorescence assays suggest that MCP1 may be involved in ATR pathway, namely by its association with ATR, under physiological conditions and after DNA damage.

Keywords**:** ATR, MCP1, DNA damage, cell cycle

INTRODUCTION

In eukaryotic cells, genomic integrity is maintained by cell cycle checkpoints that are mainly mediated by two phosphoinositide 3-kinase like protein-kinases (PIKKs): the ataxia telangiectasia mutated (ATM), and the ATM- and Rad3-related (ATR) **[1, 2]**. A third PIKK, DNA-PK, is a DNA dependent protein kinase also associated with the DNA damage response after double-stranded breaks (DSBs). ATM is mainly activated by DSBs, of endogenous or exogenous sources, while single stranded DNA (ssDNA) accumulation is the first step to activate ATR. Once produced, ssDNA is covered by replication protein A (RPA) **[1, 3]**, and the full activation of ATR at the sites of DNA damage or stalled replication forks requires the ATR-ATRIP complex kinase, the Rad17-RCF complex that recruits the 9-1-1-complex, and the DNA topoisomerase II binding protein 1 (TOPBP1) **[3-7]**. Following recruitment to chromatin, ATR phosphorylates one of its targets, the checkpoint kinase 1 (Chk1), which dissociates from chromatin and blocks cell cycle progression by modulating the function of cyclin-dependent kinases (CDKs), activating proteins involved in DNA repair, stabilizing the replicating forks, and regulating replication origins [**8-10**]. Importantly, ATR is essential for cell survival **[11]**. The proteins involved in the ATR signaling and its regulators are numerous but the pathways that lead to their function in maintaining replication fork integrity and affecting other cellular pathways are poorly understood **[7, 9, 12-15]**.

Recently, we determined that Metaphase Chromosome Protein 1 (MCP1) was associated with several components of the pre-replication complex **[16]** and was required for the early steps of DNA replication **[17]**. In this context, we wanted to determine if MCP1 was also involved in the checkpoint mediated by ATR that responds to DNA damage specifically during S-phase. We investigated whether MCP1 is associated to ATR pathway, by determining whether these two proteins interact, using co-immunoprecipitation and reverse co-immunoprecipitation assays. In addition, we analyzed the immunofluorescence patterns of MCP1 and ATR in mitotically growing cells and in cells exposed to DNA damage agents.

MATERIAL AND METHODS

Cell lines

Human HeLa (CCL-93021013 cells from ECACC), a human cervix epithelial carcinoma cell line, MO59K (ATCC-CRL2365), a human glioblastoma cell line, and K562 (ECACC-89121407 cells), a human myelogenous leukemia cell line (chromosome number 2n=46) were grown as formerly described **[16]**.

Cell synchronization, drugs, centrifugal elutriation and flow cytometry

HeLa, and MO59K cells were plated at $1x10^6$/ml were synchronized G_1/S with 5μg/ml aphidicolin (APH) and 2.5mM hydroxyurea (HU) for 18 hours, or by double thymidine block (DTB) and fixed according to the methods previously used **[16]**. HeLa,

K562 or MO59K cells were treated with 1μM camptothecin (CPT), a specific inhibitor of topoisomerase I (top I), for 3 hours. Human K562 cells at different phases of the cell cycle were enriched by centrifugal elutriation as previously described **[16]**. Briefly, cells were equilibrated in the chamber (Beckman Coulter, Avanti J-20 centrifuge and JE-5.0 rotor with a 40-ml chamber) for 10 minutes with a constant flow of 20 ml/min for effective separation and fractions (50 ml) were collected at increasing flow rates (ranging from 24 to 50 ml/min). Cells from each fraction were normalized prior to preparation of nuclear extracts, labeled with 50 μM BrdU, and fixed with ice-cold ethanol 70% overnight. DNA from $1x10^6$ cells/ml was denatured; BrdU was labeled with mouse anti-BrdU-FITC (0.5 μg/ml) antibody (Becton Dickinson & Co), and re-suspended in DNA staining solution [0.5 mg/ml RNase (Quiagen, USA), 50 μg/ml propidium iodide (Sigma Chemical Co) in PBS]. DNA content of the cells was analyzed by FACS (FACSCalibur flow cytometer, Becton Dickin-son, Mountain View, CA) with excitation of 488 nm to distribute the cells through the cell cycle. Data were acquired in a list mode data file, gated to 25,000 events in cell cycle, using the CellQuest Pro software, version 4.0.2 (Becton Dickinson). Results represent three assays performed in duplicated

Immunoprecipitation and western blotting

HeLa, and MO59K, nuclei preparations were prepared as earlier described **[16, 17, 18]** and chromatin digestion was performed treating nuclear extracts with *micrococcal nuclease* 40μg/ml, for 30 minutes on ice. Nuclei preparations from HeLa and K562 as well as total HeLa cell extracts were prepared as described **[16]**. Immunoprecipitation experiments and western blotting were performed according to previously published methods **[16, 19]**.

Immunofluorescence

Indirect immunofluorescence was performed according to the methods described **[16, 19]**. Cells were fixed in 3.7% PFA in HPEM buffer at RT for 10 minutes. DNA visualization was performed using 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in mounting media (Biomeda Corp., CA). All preparations were observed in an Olympus IX 70 microscope using 63x and 100x objectives. Images were processed with Adobe Photoshop 7.0 (Adobe) software.

Antibodies

Primary antibodies: mouse monoclonal anti-MCP1 antibody, diluted 1:3000 for western blot and 1:1000 for immunofluorescence, was prepared and affinity immunopurified as formerly described **[17]**. Affinity purified goat polyclonal antibody, diluted 1:100 (Santa Cruz Biotechnology, Inc.): anti-human ATR (C-19). Polyclonal rabbit antibody: anti-human β-tubulin (H-23 h5), diluted 1:500 (Santa Cruz Biotechnology, Inc.). Secondary antibodies: donkey horseradish peroxidase labeled antibodies, diluted 1:3000 (Santa Cruz Biotechnology, Inc.): anti-goat, anti-rabbit, and anti-mouse. Affinity purified sheep Cy3 conjugated antibody (Jackson Laboratories), diluted 1:300: anti-mouse. Alexa Fluor488 donkey antibodies, diluted 1:200 (Molecular Probes): anti-goat. Mouse IgG_1 (0.5 μg/ml) anti-BrdU FITC conjugated antibody (Becton Dickinson) for FACS.

RESULTS

The prevention of S-phase progression after MCP1 depletion, the MCP1's interaction with several components of the replication complex, and its localization at early replication foci **[16, 17]** led us to inquire if MCP1 was a potential protein involved in the ATR response, after DNA damage. The association between MCP1 and ATR proteins was analyzed by biochemical and immunological assays using three cell lines, a human cervix epithelial carcinoma (HeLa), a human glioblastoma (MO59K), and a human Caucasian chronic myelogenous leukemia (K562). We used these cell lines to check whether the interaction patterns were the same in all the cell lines and not a cell line-specific effect.

Determination of the interaction between MCP1 and ATR was performed in cells synchronized by centrifugal elutriation, a method that allowed the separation of cells at different interphase stages without activation of checkpoint responses **[20]**. The cell cycle profile of K562 elutriated at G_1, early-S, middle-S, late-S and G_2 phases, was evaluated by flow cytometry and was previously reported **[16]**. We also analyzed the MCP1-ATR interaction in cell lines synchronized at early S-phase with hydroxyurea (HU), aphidicolin (APH), double thymidine block (DTB), and after addition of camptothecin (CPT). Exposure to these agents leads to the activation of ATR checkpoint: hydroxyurea stalls replication forks **[21]**; aphidicolin prevents DNA polymerase α and δ activities **[21-23]**; double thymidine block (DTB) halts elongation leading to the accumulation of cells at very start of S-phase **[21]**; and camptothecin specifically inhibits topoisomerase I **[24-26]**.

Nuclei isolated from HeLa cells were collected at early S-phase (ES), by DTB, once thymidine prevents elongation of replication fork and cells are stopped at beginning of S-phase (G_1/S transition). For DTB, cells were incubated thymidine for 20 hours, and then washed twice in phosphate buffer saline (PBS); thymidine was add for more 16 hours and cells were washed twice in PBS; replication restarted in a synchronized manner and cells will remain relatively synchronous for 1-2 cell divisions **[27]**. These nuclei preparations were immunoprecipitated with anti-MCP1 antibody and the 300kDa ATR protein was detected with anti-ATR antibody by immunoblotting (Fig. 1-A). Western blot (WB) with anti-ATR

antibody using nuclear extracts from HeLa cells, showed that ATR protein was detected before the IP (Fig. 1-A). By western blot (WB), both MCP1 forms were recognized with the anti-MCP1 antibody, in HeLa nuclear extracts (Fig. 1-B). The association of ATR with both MCP1 isoforms was also detected when HeLa nuclear extracts were reversely co-immunoprecipitated with anti-ATR antibody followed by immunoblotting with anti-MCP1 antibody, (Fig. 1-B). In HeLa cells synchronized at early S-phase with 2,5mM hydroxyurea (HU), with 5µg/ml aphidicolin (APH) for 18 hours, and treated with 1µM camptothecin (CPT), only the 31kDa form of MCP1 was co-immunoprecipitated by anti-ATR antibody. So, after treatment of the cells with drugs that leads to ATR activation, the 31kDa MCP1 form was the isoform associated to ATR, after DNA damage (Fig. 1-B). Western blot (WB) with anti-MCP1 antibody, in K562 nuclear extracts non-synchronized (N/S), showed both MCP1 forms (Fig. 1-C). The same association, with both forms of MCP1, was also obtained when the immunoprecipitation was performed with anti-ATR antibody followed by immunoblotting with anti-MCP1 antibody, in K562 nuclear preparations synchronized by elutriation at G_1, S and G_2 phases (Fig. 1-C). As a control, K562 nuclear extracts synchronized at the same interphase stages were immunoprecipitated with a mouse immunoglobulin (mIgG) (Fig. 1-C). Extracts of K562 cells elutriated at G_1, S and G_2 phases and treated with CPT, showed the interaction of the 31kDa MCP1 form with ATR, when the immunoprecipitation was done with anti-ATR antibody, followed by immunoblotting with anti-MCP1 antibody (Fig. 1-D). Western blot (WB) with anti-MCP1 antibody, in K562 non-synchronized cells (N/S), detected the two MCP1 forms (Fig. 1-D). But, in K562 cells treated with CPT, the interaction of ATR was only detected with the 31kDa form of MCP1, after immunoprecipitation with anti-ATR antibody and western blot with anti-MCP1 antibody (Fig. 1-D). Moreover, in K562 cell extracts at early-S (ES), middle-S (MS) and late- S (LS), and those treated with CPT, the ATR protein was detected by reverse co-immunoprecipitation with anti-MCP1 antibody, followed immunoblotting with anti-ATR antibody (Fig. 1-E). The ATR protein was not detected when immunoprecipitation was performed with mIgG followed bywestern blot with ant-ATR antibody (Fig. 1-E). As a loading control, a western blot of HeLa total cell extracts non-synchronized (N/S) and synchronized at early S-phase (ES), at middle S-phase (MS) and at late-S/G_2 phase (LS/G_2) was done with anti-β-tubulin antibody (Fig. 1-F).

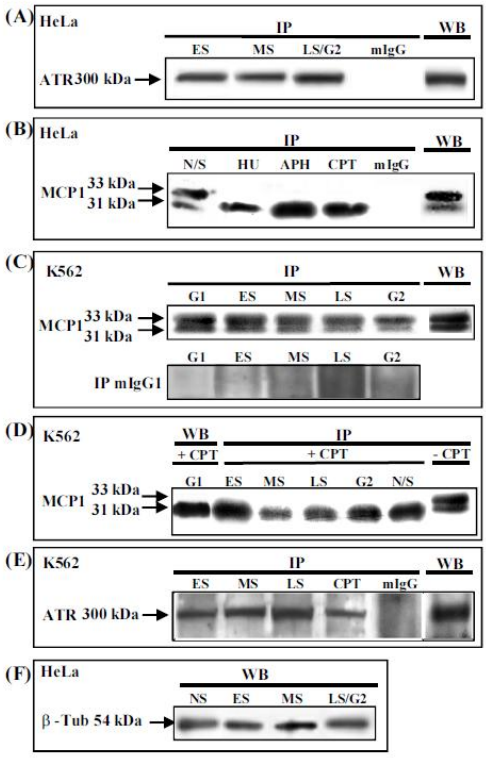

***FIG I*: (A)** Immunoprecipitation (IP) with anti-MCP1 antibody, followed by western blot with anti-ATR antibody detected the ATR protein, in nuclear extracts from HeLa cells, which were synchronized at early S-phase (ES), at middle S-phase (MS) and at late-S/G_2 phase (LS/G_2); western blot (WB) with anti-ATR antibody of nuclear extracts from HeLa cells, the ATR protein was detected before the IP. **(B)** The 31kDa form of MCP1 was immunoprecipitated by reverse co-immunoprecipitation (IP) with anti-ATR antibody, followed by western blot with anti-MCP1 antibody, of HeLa nuclear extracts synchronized at early S-phase with 2,5mM hydroxyurea (HU), 5mg/ml aphidicolin (APH) for 18 hours, and treated with 1mM camptothecin (CPT) for 3 hours; in non-synchronized cells (N/S), the two MCP1 forms were co-immunoprecipitated; the 31kDa and 33kDa MCP1 isoforms were detected by western blot (WB) with anti-MCP1 antibody, in nuclear extracts before the IP. **(C)** K562 nuclear extracts, obtained after synchronization at G_1, early-S (ES), middle-S (MS), late-S (LS) and G_2 phases, by centrifugal elutriation, were immunoprecipitated with anti-ATR antibody, followed western blot with anti-MCP1 antibody, and showed that the 33kDa and 31kDa forms of MCP1 were co-immunoprecipitated with ATR; western blot (WB) of nuclear extracts from K562 cells with anti-MCP1 antibody showing both MCP1 forms before the IP. Control experiments were done with a mIgG, using K562 nuclear extracts elutriated at different interphase stages. **(D)** K562 nuclear extracts, treated with CPT after cell synchronization at G_1, early-S (ES), middle-S (MS), and late-S (LS), and G_2 phases, were immunoprecipitated with anti-ATR antibody followed by western blot with anti-MCP1 antibody, and only the 31kDa form of MCP1 was co-immunoprecipitated with ATR protein, after checkpoint activation; immunoprecipitation with anti-ATR antibody followed by western blot with anti-MCP1 antibody, in K562 cells not synchronized (N/S) and not treated with CPT, detected the two MCP1 forms. **(E)** K562 nuclear extracts synchronized by centrifugal elutriation at early-S (ES), middle-S (MS), late-S (LS), and treated with 1mM camptothecin (CPT) for 3 hours, were reversely co-immunoprecipitated (IP) with anti-MCP1 antibody followed immunoblotting with anti-ATR antibody, showed the presence of ATR protein; western blot (WB) with anti-ATR antibody, of nuclear extracts from K562 cells, that recognized the ATR protein, before IP. **(F)** As a loading control, we performed a western blot of HeLa total cell extracts, non synchronized (N/S)

and synchronized at early S-phase (ES), at middle S-phase (MS) and at late-S/G_2 phase (LS/G_2), with the anti-β-tubulin antibody.

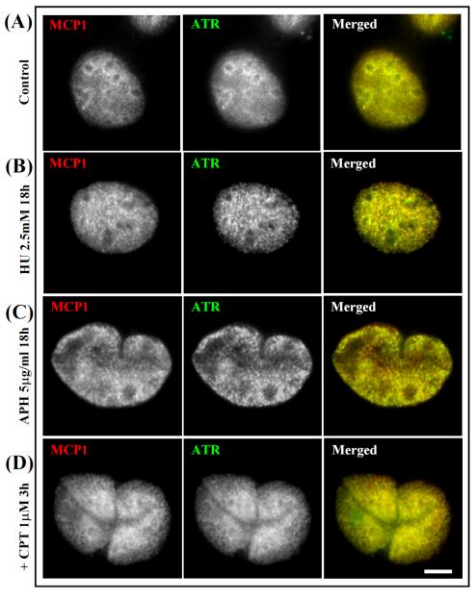

FIG II: Immunofluorescence of MCP1 (red) and ATR (green) proteins in human control MO59K cells **(A)**, and in MO59K cells treated with 2.5mM hydroxyurea **(B)**, with 5µg/ml aphidicolin for 18 hours **(C)**, and after treatment with 1µM camptothecin (CPT) for 3 hours **(D)**. Bar = 10µc.

The cellular localization of these proteins was analyzed, by immunofluorescence, in human MO59K cells. MCP1 showed a granular distribution that was co-localized with the weak ATR amount in the nucleus of MO59K untreated cells (Fig. 2A). It was demonstrated that ATR is located in the cytosol and perinuclear region, and it is recruited to chromatin over the unperturbed cell cycle, in particular during S-phase, where it forms nuclear foci **[8]**. The MCP1 and ATR were co-localized in nuclear foci after treatment with 2.5mM hydroxyurea (Fig. 2B), with 5µg/ml aphidicolin for 18 hours (Fig. 2C), and after treatment with 1µM camptothecin (CPT) for 3 hours (Fig. 2D), which are agents of genotoxic stress.

DISCUSSION

The process of DNA replication ensures accurate genome duplication in each cell cycle, and is halted in the presence of damage and chromatin stressing agents, through the DNA damage checkpoints. One of the safeguards of the genome is the ATR kinase that is mainly activated by single stranded DNA breaks. Junctions between single stranded DNA and double stranded DNA, ultraviolet light, replication stress induced by aphidicolin, hydroxyurea, camptothecin or anticancer drugs are also involved in the ATR activation **[1, 2, 5, 9, 14, 28, 29]**. Following ATR activation, the cell cycle is arrested, the activation of late replication origins is prevented, the stressed replication forks are stabilized and DNA repair is promoted **[2, 3, 7]**.

We previously reported that MCP1 interacts with several components of the replication machinery **[16]**, in addition to the requirement of MCP1 during the early events of DNA replication **[17]**. The 31kDa and 33kDa MCP1 forms, corresponding to the two different translation products from the same MCP1 mRNA, are phosphorylated during interphase and mitosis **[19, 30]**. Characterization of the two MCP1 forms by mass spectrometry (LC-MS/MS) revealed that these proteins have high levels of homology with histone H1 and its variants **[16]**.

In this work we analyzed whether MCP1 could be a component of the ATR pathway. For this, we used DNA damage agents, which activate the ATR kinase, in several human cell lines. In HeLa non-synchronized nuclear extracts, we detected an interaction between ATR and the two MCP1 forms. Also, in K562 cells that were synchronized by elutriation, at different stages of interphase, thus not subjected to replication stress, the two MCP1 forms interacted with ATR. Using these two approaches, which do not lead to activation of the checkpoint, both MCP1 forms were associated with the ATR throughout interphase, in K562 and HeLa cells. The association of ATR with chromatin at several stages of the cell cycle was previously reported in the absence of perceptible DNA damage and checkpoint activation **[8]**. However, after activation of ATR checkpoint, in the presence of agents that stalls replication or after a brief exposition to CPT, only the 31kDa form of MCP1 interacted with ATR, at several interphase stages. In fact, hydroxyurea and aphidilcolin are agents which induce replication stress, by stalling replication forks, and promote the enrichment of ATR on chromatin after activation of the cellular S-phase checkpoint **[8, 21-23, 31]**. Replication stalling promotes the translocation of ATR into the nucleus, where it forms nuclear foci, and its association with chromatin **[8, 31, 32]**. In HeLa cells treated with these agents, the interaction of MCP1 with ATR was done through the 31kDa form of MCP1. Camptothecin also induce replication stress by inhibiting topoisomerase I **[24-26]**. It was demonstrated that CPT induces S and G_2 phases arrest **[33]**. In K562, the interaction of MCP1 with ATR, after camptothecin treatment, was also made by the 31kDa MCP1 isoform. These results suggest that during cell cycle progression, under normal physiological conditions, both MCP1 forms are associated with ATR, while in the presence of genotoxic stress only the 31kDa MCP1 isoform is linked to ATR. Considering that nuclear preparations were treated with *micrococcal nuclease*, the interaction between MCP1 and ATR was not indirectly mediated by DNA/chromatin.

By immunofluorescence, the MCP1 protein was also co-localized with the low amount of ATR present in the nucleus of undamaged cells. We showed the co-localization, in the nuclear foci, of MCP1 with ATR, after treatment with hydroxyurea, aphidilcolin and

CPT. The accumulation of ATR into nuclear foci at sites of DNA damage was also demonstrated in response to ultraviolet (UV) light which also activates the ATR-dependent S-phase checkpoint **[34, 35]**. This activation is dependent of kinases, as the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK-ERK) **[31, 32]** or the ATR-Chk1 **[39, 40]** pathways; recruits proteins and phosphorylates substrates **[37-39]**; promotes the trafficking of several proteins to and from several organelles **[41, 42]**; and is coupled to several DNA repair pathways, namely the nucleotide excision repair (NER) pathway **[40, 41]**. Several reports suggest that checkpoint signaling through ATR is related to the process of DNA replication **[10, 43-45]**.

The interaction with the 31kDa MCP1 isoform was also demonstrated by reciprocal immunoprecipitation with antibodies against some members of the pre-RC components, namely Cdc6, ORC2, ORC4, MCM2, MCM3, MCM7, as well as Cdc45 and PCNA, but not with heterochromatic proteins, like HP1β **[16]**. MCM7 is a component of replication complex and is required for the formation of ATR-nuclear foci **[35]**. MCP1 is also associated with MCM7 **[16]** and these results suggest that MCP1, in addition to being a replication complex constituent, might be a sensor or an effector of the checkpoint in the S-phase. The nuclear localization of MCP1 and its preferential binding to the early origins sequences **[16, 29]** suggests that MCP1 beyond associating some proteins of replication factory might participate in complexes that contribute to chromatin remodeling, which is essential for replication, transcription or repair.

In conclusion, the two forms of MCP1 were associated with ATR, at different interphase stages in non-stressed cells. Following exposure to DNA damage agents, only the 31kDa form of MCP1 interacted with the activated ATR. This study adds MCP1 to the many potential substrates and signal transduction proteins involved in the DNA damage response **[7, 12, 13, 15, 46, 47]**, and reveals that MCP1 interacts with ATR, following DNA damage or in response to replication stress. Further experiments are required to clarify how MCP1 might affect the signaling pathway regulated by ATR.

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