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MRG15 activates the *cdc2* promoter via histone acetylation in human cells

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Abstract

Chromatin remodeling is required for transcriptional activation and repression. MRG15 (MORF4L1), a chromatin modulator, is a highly conserved protein and is present in complexes containing histone acetyltransferases (HATs) as well as histone deacetylases (HDACs). Loss of expression of MRG15 in mice and *Drosophila* results in embryonic lethality and fibroblast and neural stem/progenitor cells cultured from *Mrg15* null mouse embryos exhibit marked proliferative defects when compared with wild type cells. To determine the role of MRG15 in cell cycle progression we performed chromatin immunoprecipitation with an antibody to MRG15 on normal human fibroblasts as they entered the cell cycle from a quiescent state, and analyzed various cell cycle gene promoters. The results demonstrated a 3-fold increase in MRG15 occupancy at the *cdc2* promoter during S phase of the cell cycle and a concomitant increase in acetylated histone H4. H4 lysine 12 was acetylated at 24 hours post serum stimulation while there was no change in acetylation of lysine 16. HDAC1 and 2 were decreased at this promoter during cell cycle progression. Over-expression of MRG15 in HeLa cells activated a *cdc2* promoter-reporter construct in a dose dependent manner, whereas knockdown of MRG15 resulted in decreased promoter activity. In order to implicate HAT activity, we treated cells with the HAT inhibitor anacardic acid and determined that HAT inhibition results in loss of expression of *cdc2* mRNA. Further, chromatin immunoprecipitation with Tip60 localizes the protein to the same 110 bp stretch of the *cdc2* promoter pulled down by MRG15. Additionally, we determined that co-transfection of MRG15 with the known associated HAT Tip60 had a cooperative effect in activating the *cdc2* promoter. These results suggest that MRG15 is acting in a HAT complex involving Tip60 to modify chromatin via acetylation of histone H4 at the *cdc2* promoter to activate transcription.

Keywords

MRG15; Tip60; *cdc2*; normal human fibroblasts

Introduction

In studies to identify senescence related genes modified in immortal cells, the Mortality Factor on chromosome 4 (MORF4) gene was cloned as a genomic fragment that caused a subset of immortalized cell lines to enter senescence following transfection [1]. A related member of this gene family, MRG15 (*MORF4* related gene on chromosome 15), is

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ubiquitously expressed in cells and tissues, is highly conserved from yeast to mammals [2, 3] and is essential for chromatin remodeling during transcriptional regulation and DNA repair [4, 5]. The *Mrg15* knockout is embryonic lethal in mice and *Drosophila* [6, 7] and analyses of *Mrg15* null versus wild type derived cells have demonstrated deficits in proliferation of mouse embryonic fibroblasts as well as neural stem/progenitor cells [8].

MRG15 is a unique protein in that it is present in both histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes. MRG15 and its homologues associate with the HAT Tip60 complex in mammalian cells [9–11], the dTip60 complex in *Drosophila* [7], and the NuA4 complex in budding yeast [12]. In mammals MRG15 is also found in the mSin3A HDAC complex [13]. MRG15 contains a chromodomain that has been shown to have an affinity for H3K36me3 in yeast [14] as well as mammals [15], and in budding yeast this interaction appears to be necessary for recruitment of the RPD3 HDAC complex to transcribed genes to prevent improper transcription initiation [16].

In this study we examined the role of MRG15 in cell cycle progression in normal human fibroblasts. We had found that reduction of MRG15 protein levels, using siRNA, in young fibroblasts caused a small but consistent decrease (10–15%) in the percentage of cells undergoing DNA synthesis, as measured by BrdU uptake, and conversely over-expression in pre-senescent fibroblasts resulted in an increase (10–12%) in the number of cells synthesizing DNA. We therefore initiated a chromatin immunoprecipitation analysis of cell cycle regulatory gene promoters in serum-stimulated quiescent fibroblasts. This revealed a marked increase in MRG15 occupancy of the *cdc2* promoter at 24 hours post stimulation, a time at which active transcription of *cdc2* was occurring and also when the maximum number of this semi-synchronized cell population were in S phase [17, 18]. A concomitant increase in acetylated histone H4, involving lysine 12 but not 16, was observed as well as a decrease in HDAC1 and 2 occupancy at this promoter. Analysis of the *cdc2* promoter activity, using a promoter-reporter construct, demonstrated that MRG15 over-expression increased activity whereas knockdown of MRG15 levels resulted in reduced activity of this promoter. Because of known interaction with the Tip60 HAT complex, we further examined the *cdc2* promoter for Tip60 occupancy and detected it with primers overlapping the *cdc2* promoter region MRG15 was found to occupy. Additionally, cotransfection with Tip60 and MRG15 cooperatively activated the *cdc2* promoter while HAT inhibition with anacardic acid blocked the production of *cdc2* mRNA in treated cells following serum stimulation. Our data suggest that MRG15 accumulates at specific cell cycle gene promoters in proximity to HAT machinery, and recruits the necessary chromatin remodeling machinery to acetylate histone H4, specifically at H4K12, and allow the initiation of transcription.

Materials and methods

Cell Culture

HCA2 (foreskin-derived fibroblasts) were subcultured in minimal essential medium (MEM) with Hanks' salts (HMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone) as previously described [17]. Quiescent cells were obtained by culturing confluent young cells in medium supplemented with 1% FBS, for at least one week. The addition of 10% FBS caused the cells to re-enter the cell cycle and harvesting at 0, 6, 12, and 24 hours post serum stimulation allowed the analysis of changes at various stages of the cell cycle.

HeLa cells were maintained in HMEM supplemented with 10% FetalClone III serum (HyClone).

For experiments involving cell culture dishes the medium used was MEM with Earle's salts (EMEM) supplemented with 10% serum and cells were incubated in a 37°C 5% CO₂ incubator.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as described [19–21]. Briefly, chromatin of quiescent cells and cells at different times following serum-stimulation was crosslinked with 1% formaldehyde and cells harvested in cold SDS buffer with protease inhibitors. Following sonication protein concentration was determined and lysate containing 1 mg total protein was precleared with 25 µl of salmon sperm/protein A/G agarose beads (Upstate Biotech) or Dynabeads (Invitrogen). A 25 µl sample of the lysate (2.5% total input) was reserved as a total control while proceeding with the immunoprecipitation of the remaining sample. The sample was incubated overnight at 4°C with primary antibody (2 µg). The following day the protein-DNA complexes were coupled to salmon sperm/protein A/G agarose beads or Dynabeads, pelleted, and washed [19]. Input controls and washed complex beads were resuspended in 1% SDS, 0.1M NaHCO₃ and incubated overnight at 65°C to elute the protein-DNA complexes from the beads and reverse crosslinks. The following day the samples were incubated in a proteinase K solution at 37°C for 2 hours and the DNA was isolated by phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation. Pelleted DNA was resuspended in 300 µl of H₂O. Controls included a no antibody negative control, use of an irrelevant antibody (anti-HA), and amplification with primers to another region of the promoter being examined in addition to the acetylcholine receptor gene. Each ChIP was performed in triplicate and repeated at least twice, again in triplicate, to confirm reproducibility.

Antibodies used were: αMRG15, full length [22], αHDAC1 (Santa Cruz Biotechnology, Catalog sc-7872), αHDAC2 (Santa Cruz Biotechnology, Catalog sc-7899), αAcH4 (Upstate, Catalog #06-598), αH4 pan (Upstate, Catalog #05-858), αH4K12 (Abcam, Catalog ab1761), αH4K16 (Upstate, Catalog #070329), αHA (Santa Cruz Biotechnology, Catalog sc-805), αTip60 (Santa Cruz Biotechnology, Catalog sc-5727).

Quantitative real-time PCR was performed on a 384-well plate on an ABI Prism 7900HT. A master mix of 5 µl 2× SYBR green PCR master mix (Applied Biosystems), and 1 µl of 8 µM stock of both forward and reverse primers was prepared and to this 3 µl of the chromatin template was applied. Primers were designed around the E2F sites. The primer pairs were: AChR 5'-CCTTCATTGGGATCACCACG-3' and 5'-GGAGATGAGTACCAGCAGGTTG-3'; Cyclin A2 5'-GCGCTTTCATTGGTCCATTT-3' and 5'-GCGGCTGTTCTTGCACTTC-3'; Cyclin B1 5'-GGCAGCCGCAATGG-3' and 5'-CTCCCTCCTTATTGGCCTGTT-3'; cdc2 5'-GGGCTCTGATTGGCTGCTT-3' and 5'-AAGGGCCCCGGATTAC-3'; cdc2 (primer set 2 for Tip60) 5'-TTCCTCTTTCTTTCGCGCTCTA and 5'-TAGCTGGGCTCTGATTGGCT-3'; MCM4 5'-CCGAGCGAGGCCTACTTCT-3' and 5'-GGACAGTGCCGCTTCTTTCA-3'; and PCNA 5'-CTGGCTGCTGCGCA-3' and 5'-CACCACCCGCTTTGTGACT-3' [20, 21, 23]. Percent total values presented were calculated as described previously [19] and are an average of the percentage of the total input of three independent IPs as described previously.

RNA isolation and qPCR

RNA was isolated by Trizol (Invitrogen) according to manufacturer's instructions. Briefly, cells were harvested into ice cold Trizol. Following the addition of chloroform the lysate was separated by centrifugation and the aqueous phase collected. The RNA was precipitated with the addition of isopropanol and pelleted by centrifugation. RNA was then washed and resuspended in water.

Reverse transcriptase real-time PCR was performed as before [21], normalizing to RPLP0 as a relative control.

Transient transfections into HeLa cells

Cdc2 promoter-reporter construct cotransfection with wild type and mutant constructs of MRG15 and Tip60—HeLa cells were plated at 2.5×10^5 per well to 35 mm dishes in EMEM supplemented with 10% FetalClone III serum and transfected twenty-four hours later. A cdc2 promoter-luciferase reporter plasmid DNA, pSV2-cdc2pr, (a gift from Dr. C. K. Glass, University of California San Diego) that included the entire 3400 bp promoter region was used for these experiments [24]. 0.5 µg of pSV2-cdc2pr was transfected without or with pcDNA3.1-MRG15 at increasing concentrations (0.5 µg and 1 µg), or with mutant constructs in which the chromodomain (aa 26–62) or leucine zipper (aa 284–305) regions had been deleted (0.5 µg DNA). Cells were also co-transfected with the cdc2 promoter-reporter plasmid with 0.5 µg wild type pcDNA3.1-Tip60 alone, with 1 µg pcDNA3.1-MRG15 alone or with both Tip60 (0.5 µg) and MRG15 (0.5 µg and 1.0 µg) constructs. Total DNA for each transfection was equalized with appropriate empty plasmid (pcDNA3.1). Lipofectamine Plus was used for transfection as described previously [22, 25, 26]. Twenty-four hours later the cells were harvested and the luciferase assay performed. Luciferase activity was determined as previously described [22, 25]. Briefly cells were harvested in Reporter Lysis Buffer (Promega) following a freeze-thaw cycle. Cell lysate was then centrifuged for 10 minutes at 13200 rpm to isolate the soluble fraction, the supernatant collected and activity assayed by Luciferase Assay Kit (Promega). Concurrently protein concentrations were determined using the Pierce BCA assay. Luciferase activity was normalized against protein concentration and expressed as fold change versus promoter-reporter alone. Experiments were performed in triplicate and repeated at least three times to verify reproducibility.

Cdc2promoter-reporter construct cotransfection with siRNA against MRG15—

HeLa cells were cotransfected as above with 2 µg of pSV2-cdc2pr and various concentrations of siRNA directed against the Mrg15 sequence GCA CUC AGC UGC UCU ACA A (Dharmacon, Catalog number D-006379-01-0050). A siGENOME Non-Targeting siRNA #1 (Dharmacon, Catalog number D-001210-01-05) was used as control for non specific effects.

Forty-eight hours post-transfection cell lysate was prepared and 40 µg total protein was separated on a 10% SDS-PAGE gel. Following transfer to nitrocellulose MRG15 protein levels were detected by Western blot using a rabbit antibody against C-terminal peptide MRG15 purified in our lab. Equal amount of loading was confirmed by actin staining.

Luciferase activities and protein concentrations were obtained as described above. Experiments were performed in triplicate and repeated at least three times to verify reproducibility.

Inhibition of histone acetyltransferase activity by treatment with anacardic acid—Young, normal HCA2 fibroblasts were maintained in HMEM supplemented with 1% FBS for one week to ensure the cells had entered quiescence. HMEM supplemented with 10% serum and 50 µM anacardic acid or an equivalent volume of vehicle (DMSO) alone was then added to the cells. At appropriate time intervals cells were harvested, RNA isolated, and qPCR performed as described above.

Results

MRG15 occupancy at various gene promoters changes during cell cycle progression

Young (PD24), confluent HCA2 cells were serum-deprived for one week and chromatin immunoprecipitation (ChIP), using anti-MRG15 antibody, performed at 0, 6, 12, and 24 hours after serum stimulation, allowing us to determine occupancy of proteins at various cell cycle promoters as the synchronous population of cells progressed through one cell cycle. Because MRG15 not only associates with Rb, but also activates the E2F-responsive B-myb promoter through this association [22] we examined regions of the CCNA2, MCM4, PCNA, cdc2 and cyclin B promoters containing E2F-binding elements (EBEs). The results revealed the loss of MRG15 from the CCNA2 and PCNA promoters 6 hours after serum stimulation, and a marked increase in occupancy at the cdc2 promoter between 12 and 24 hours post serum stimulation (Fig. 1A).

Occupancy of other chromatin remodeling proteins at the cdc2 promoter during cell cycle progression

ChIP with relevant antibodies revealed an increase in acetylated histone H4 at the cdc2 promoter at 24 hours with loss of HDAC1 and 2 occupancy at 6–12 hours (Fig. 1B). Additional ChIP with relevant antibodies revealed increased acetylation of lysine 12 but not 16 in histone H4 24 hours post serum stimulation at the cdc2 promoter region (Fig. 1C). These results and the fact that cdc2 mRNA levels peaked between 12 and 24 hours post serum stimulation (Fig. 1D), suggested MRG15 might be involved with chromatin remodeling machinery to activate the cdc2 promoter.

MRG15 levels affect cdc2 promoter activity in HeLa cells

We co-transfected either pcDNA alone or increasing amounts of MRG15 with a cdc2 promoter-luciferase reporter construct into HeLa cells. Twenty four hours post-transfection luciferase activity was assayed. We found that promoter activation correlated with increasing levels of MRG15 (Fig. 2A). In contrast, when siRNA against MRG15 was co-transfected with the promoter-reporter construct, levels of MRG15 protein were reduced along with luciferase activity (Fig. 2B, C). Control siRNAs confirmed this was not due to non-specific effects of transfection of siRNA. Endogenous cdc2 mRNA levels were also decreased about 50% by MRG15 siRNA as determined by qPCR (data not shown).

The leucine zipper that is part of the MRG domain of MRG15 is required for activation of the cdc2 promoter

To determine the domain of the MRG15 protein that is required for cdc2 promoter activation we co-transfected the pSV2-cdc2pr plasmid with pcDNA3.1-MRG15 mutants with chromodomain or leucine zipper deletions. The MRG15 chromodomain deletion did not attenuate the ability of the expressed MRG15 protein to induce cdc2 promoter activity. However deletion of the MRG15 leucine zipper, that is included in the MRG domain, resulted in failure to activate the promoter at concentrations of both 0.5 and 1 µg (Fig. 2D, data not shown), indicating the importance of this region at the C-terminal end of MRG15 for activation of the cdc2 promoter. Expression of the transfected constructs was monitored by Western blotting (Fig. 2E).

The HAT inhibitor anacardic acid prevents induction of cdc2 mRNA in serum stimulated HCA2 cells

We did an indirect test of the need for HAT activity to stimulate the cdc2 promoter. We quantitated cdc2 mRNA by RT-PCR at 12 and 24 hours post serum stimulation, in HCA2 cells untreated or treated with 50 µM anacardic acid, a HAT inhibitor [27], at the time of

serum stimulation. RNA levels increased in untreated but not treated cells (Fig. 3A), indicating lack of activation of the *cdc2* promoter in the absence of HAT activity.

Tip60 occupancy increases within the *cdc2* promoter at 24 hours post serum stimulation and acts synergistically with MRG15 to activate the *cdc2* promoter in HeLa cells

Since we observed activation of the *cdc2* promoter by MRG15, and because such activation is generally associated with HAT complexes and increased acetylation of H4, we tested the possibility that Tip60, a HAT with which MRG15 is known to associate, was acting with MRG15 to activate this promoter. ChIP analysis indicated that MRG15 and Tip60 localize to the same 110 bp region of the *cdc2* promoter (Fig. 3B). Further, either MRG15 or Tip60 cotransfected alone with the *cdc2* promoter-reporter plasmid could activate the *cdc2* promoter about 1.5–2 fold, but when co-transfected together the promoter activity increased to 3–4 times that of basal levels (Fig. 3C).

Discussion

The present study was initiated because of our previous results, which implicated a role for MRG15 in cell proliferation. These included the facts that both MRG15 null mouse embryonic fibroblasts [6] and neural stem/progenitor cells [8] exhibited proliferative defects when compared with wild type cells; MRG15 de-represses the B-myb promoter [22]; MRG15 knockout results in small embryos compared with wild type embryos [6]; and MRG15 upregulation results in increased BrdU labeling in pre-senescent normal human cells, and conversely a decrease in BrdU labeling in young cells following expression of siRNA against MRG15.

With new approaches we wished to explore the molecular mechanism(s) by which MRG15 positively affected cell proliferation. ChIP is a powerful technique and by combining this with analysis of the semi-synchronized population of serum stimulated normal cells with time after stimulation we were able to observe a marked increase in MRG15 at the *cdc2* promoter. The fact that this was accompanied by an increase in acetylated histone H4, involving lysine 12 but not lysine 16, and decrease in HDAC 1 and 2 at the promoter, as determined by ChIP, and the correlation with *cdc2* mRNA levels in the serum stimulated cells, suggested MRG15 was activating this promoter through association with a HAT complex. Increased activity of the *cdc2* promoter–luciferase reporter construct by co-transfection with increasing amounts of MRG15 and a decrease in the activity in the presence of siRNA to MRG15, provided further evidence for a role for MRG15 in activating the promoter.

MRG15 is known to be present in complexes involving the HATs hMOF or Tip 60 [4, 10]. We therefore attempted to determine which of these two HATs was involved in activating the *cdc2* promoter. Chromatin immunoprecipitation localized the HAT, Tip60, to 110 bp region of the *cdc2* promoter at which MRG15 was also present. In addition, when MRG15 and Tip60 were co-transfected, cooperative activation of this promoter was observed. Transfection of hMOF alone or together with MRG15 had no effect on the *cdc2* promoter. This is consistent with the result we obtained here in that the MRG15 mutant lacking the chromodomain did not affect *cdc2* promoter activity, but had been previously shown to disrupt the MRG15/hMOF interaction [4]. In this study, the loss of the leucine zipper region, that involves the MRG domain [28, 29], resulted in decreased activation of the *cdc2* promoter. Additionally, the Tip60 complex has been shown to target lysine 12 in histone H4 [30], along with other lysines, consistent with our results which show increased acetylation of H4K12 and not K16 24 hours after serum stimulation. Acetylation at H4K16 is known to occur through the action of hMOF [31]. Finally, the loss of production of *cdc2* RNA in the

presence of the HAT inhibitor anacardic acid lends further evidence for the involvement of a HAT.

The results presented here add to our knowledge of the action of MRG15 on various cell cycle genes. We had previously shown that MRG15 was in a complex with Rb and an adaptor protein PAM14 and was acting to de-repress the B-myb promoter by removing Rb/HDAC from the E2F site [22]. In the case of *cdc2*, MRG15 appears to be directly activating the promoter via histone H4 acetylation. It is tempting to speculate that since MRG15 is rapidly lost from the cyclin A and PCNA promoters after serum stimulation, that it is in a repressive HDAC-associated complex that must be removed to allow for activation of these genes. The data presented here demonstrate the complexity of action of chromatin modulators and the importance of histone modifications in many biological processes.

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Fig. 1.

(A) MRG15 occupancy of the cell cycle promoters MCM4, PCNA, Cyclin A2, Cyclin B1, and cdc2, as quiescent HCA2 cells are released into serum and progress through one cell cycle. Chromatin immunoprecipitation revealed that MRG15 occupancy of the cdc2 promoter increased most substantially between the 12 and 24 hour time points as the cells entered late S phase. Values shown are the average percent of total input of three independent immunoprecipitations and are representative of at least two separate experiments; (B) Chromatin immunoprecipitation also revealed an increase in H4 acetylation and decreases in both HDAC1 and HDAC2 at the same locus in the cdc2 promoter region with acetylation of lysine 12 of H4 exhibiting a very significant at 24 hours as compared to quiescent cells (C). The time period between 12 and 24 hours corresponds to the progression of the majority of this population of fibroblasts through S phase and to the time period when cdc2 mRNA expression shows the largest increase.

Fig. 2.

MRG15 exerts control over the *cdc2* promoter in HeLa cervical carcinoma cells. HeLa cells were cotransfected with a promoter-reporter construct containing the full length *cdc2* promoter followed by a luciferase reporter gene and (A) wild type MRG15, (B and C) siRNA directed against MRG15, (D and E) or MRG15 mutants lacking the chromodomain or leucine zipper domain. (A) Full-length MRG15 caused an increase in *cdc2* promoter-driven luciferase expression in a dose-dependent manner while (B) siRNA (100 nM) directed against MRG15 resulted in a statistically significant loss (p value 0.0026 as determined by ANOVA) of *cdc2* promoter-driven luciferase expression. Values expressed are relative luciferase units normalized to total protein. (D and E) Compared to transfection with MRG15, removal of the chromodomain (aa 26–62) caused little change in promoter firing whereas deletion of the leucine zipper (aa 284–305), which is part of the MRG domain, caused a decrease in induction of the promoter at both 0.5 and 1 μ g of DNA.

Fig.3.

MRG15 interacts with the HAT Tip60 to cooperatively modulate the *cdc2* promoter. (A) *cdc2* mRNA expression requires HAT activity. HCA2 fibroblasts were cultured in low serum (1%) conditions for one week until a semi-heterogeneous quiescent population was achieved. Cells were then either treated with 50uM anacardic acid, a HAT inhibitor, in DMSO or with DMSO alone in media supplemented with 10% FBS. Cells were harvested 12 and 24 hours post-stimulation and mRNA isolated. *cdc2* mRNA was quantitated by RT-PCR. Fibroblasts treated with vehicle alone showed a robust increase in *cdc2* mRNA compared to 0h while cells treated with the HAT inhibitor showed no increase; (B) Tip60 localizes to the *cdc2* promoter within 110 bp of MRG15. HCA2 fibroblasts were synchronized in low serum conditions for one week and then released to serum for 24 hours. Crosslinked cells were then immunoprecipitated with an antibody against Tip60. 24 hours post serum stimulation Tip60 was localized to a region within 110 bp of MRG15; (C) Tip60 and MRG15 act cooperatively at the *cdc2* promoter. HeLa cells were co-transfected with the *cdc2* promoter-reporter plasmid and empty vector, MRG15, Tip60, or MRG15 and Tip60 together. Cells were harvested 24 hours post-transfection and luciferase activity was assayed. Activity was normalized to total protein. Both MRG15 and Tip60 alone activated the promoter, but together had a cooperative effect.