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Transcriptional Activation of Human *Matrix Metalloproteinase-9* Gene Expression by Multiple Coactivators

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Summary

Matrix metalloproteinase-9 (MMP-9), a proteolytic enzyme for matrix proteins, chemokines and cytokines, is a major target in cancer and autoimmune diseases since it is aberrantly upregulated. To control MMP-9 expression in pathological conditions, it is necessary to understand the regulatory mechanisms of MMP-9 expression. *MMP-9* gene expression is regulated primarily at the transcriptional level. In this study, we investigated the role of multiple coactivators in regulating MMP-9 transcription. We demonstrate that multiple transcriptional coactivators are involved in *MMP-9* promoter activation, including CBP/p300, PCAF, CARM1 and GRIP1. Furthermore, enhancement of *MMP-9* promoter activity requires the histone acetyltransferase activity of PCAF but not that of CBP/p300, and the methyltransferase activity of CARM1. More importantly, these coactivators are not only able to activate *MMP-9* promoter activity independently, but also function in a synergistic manner. Significant synergy was observed among CARM1, p300 and GRIP1, which is dependent on the interaction of p300 and CARM1 with the AD1 and AD2 domains of GRIP1, respectively. This suggests the formation of a ternary coactivator complex on the *MMP-9* promoter. Chromatin immunoprecipitation assays demonstrate that these coactivators associate with the endogenous *MMP-9* promoter, and that siRNA knockdown of expression of these coactivators reduces endogenous MMP-9 expression. Taken together, these studies demonstrate a new level of transcriptional regulation of MMP-9 expression by the cooperative action of coactivators.

Keywords

CARM1; p300; GRIP1; MMP-9; transcription

Introduction

Matrix metalloproteinase-9 (MMP-9), a member of a group of 23 structurally conserved human proteolytic enzymes, plays important roles in normal physiological processes such as reproduction, inflammation and wound healing.^{1,2} However, elevated levels of MMP-9 are detected in multiple human cancers such as breast, colon, brain and lung cancer,³ and inflammatory diseases such as multiple sclerosis and rheumatoid arthritis.¹ Inhibition of

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MMP-9 expression by interferon- β in multiple sclerosis⁴ or by RNAi targeting in brain tumors⁵ has been shown to be beneficial. To better control the expression of MMP-9, it is a prerequisite to understand MMP-9 regulation under both physiological and pathological conditions. Expression of MMP-9 is regulated primarily at the transcriptional level, which is tightly and specifically regulated.^{1,6,7} Multiple signaling pathways induce *MMP-9* gene transcription by activating sequence specific transcription factors, such as AP-1, NF- κ B, Sp1 and Ets1, which subsequently bind to *cis*-elements on the *MMP-9* promoter.^{1,6,7} This promotes the further recruitment of chromatin remodeling complexes, coactivators and general transcriptional machinery to induce MMP-9 expression.⁷ However, the identity and functions of coactivators involved in MMP-9 expression are still not clear. Thus, in this study, the role of three classes of coactivators in MMP-9 expression was determined.

The cAMP-response-element binding protein (CREB)-binding protein (CBP) and p300 were originally identified as proteins that bind to CREB and adenoviral E1A, respectively.⁸ They are homologous proteins and mainly function as transcriptional coactivators with intrinsic histone acetyltransferase (HAT) activity. CBP and p300 are key regulators of RNA polymerase II-mediated transcription, functioning to link sequence specific transcriptional activators to the general transcriptional machinery, thereby stabilizing the pre-initiation complex.⁸ They also function as a scaffold for the assembly of multi-protein complexes. Furthermore, as HATs, they acetylate not only four histone tails to relax chromatin structure,^{9,10} but also a number of non-histone proteins such as p53 and NF- κ B p65 to increase their DNA-binding ability.^{11,12} By interacting with a variety of transcriptional factors, CBP and p300 regulate expression of a large number of genes.¹³ The p300/CBP associated factor (PCAF) was originally identified as a homolog to the yeast histone acetylase GCN5, and binds to the CH3 domain of CBP/p300.¹⁴ PCAF also has intrinsic HAT activity, with a histone H3 preference.¹⁴

Protein arginine methyltransferases (PRMTs) include eleven enzymes with conserved catalytic motifs that catalyze mono- or di-methylation of arginine residues in proteins to regulate gene expression, muscle differentiation and tumorigenesis.¹⁵ Coactivator-associated arginine methyltransferase 1 (CARM1), also known as PRMT4, and PRMT1 are two PRMTs that play important roles in transcriptional activation by methylating histones and non-histone substrates.^{15,16} CARM1 specifically methylates histone 3 (H3) at N-terminal arginine 2, 17, 26 and some sites in the C-terminus,^{15,17,18} while PRMT1 methylates H4 at arginine 3.¹⁹ Also, CARM1 can methylate CBP/p300 in the KIX domain to promote nuclear hormone transcription.²⁰ Methylation of CBP at arginine 714, 742 and 768 has also been identified to be critical for hormone and CIITA-induced gene activation.^{21,22} PRMT1 is able to methylate STAT-1 at arginine 31 to protect it from the binding of its inhibitor PIAS1, and therefore activate interferon α/β -induced transcription.²³

The p160 steroid receptor coactivator (SRC) family includes three members, SRC-1/NCoA-1 (hereafter referred to as SRC-1), SRC-2/GRIP1/NCoA-2/TIF2 (hereafter referred to as GRIP1) and SRC-3/p/CIP/RAC3/ACTR/AIB1/TRAM-1 (hereafter referred to as ACTR).²⁴ They are proteins with approximately 40% homology and share similar domain structures.²⁴ The C-terminus of p160 has two transactivation domains, AD1 and AD2, through which activating signals are transmitted to other coactivators and the general transcriptional machinery. CBP/p300 and PCAF interact with the AD1 domain,^{25–27} and CARM1 and PRMT1 interact with the AD2 domain.^{17,28} In nuclear receptor-dependent transactivation, the p160 proteins are recruited to promoters through direct interactions with nuclear receptors, and then they recruit additional secondary coactivators such as CBP, p300, CARM1 and PRMT1 to form multiple protein complexes that activate gene transcription.¹⁶

Studies have shown that the above three major classes of coactivators play important roles in nuclear receptor-mediated gene transcription.^{16,17,29–33} In addition, they are involved in

regulation of some NF- κ B-mediated genes such as E-selectin, IP-10, IL-8 and MIP-2.^{34–40} Our previous studies demonstrated that *MMP-9* gene expression depends on binding of the sequence specific activators AP-1 and NF- κ B to their corresponding *cis*-elements on the *MMP-9* promoter. In this study, we have determined that CBP/p300, PCAF, CARM1 and GRIP1 are all involved in *MMP-9* gene expression, likely by functioning as a transcriptional complex. Furthermore, reduction of cellular concentrations of p300, CARM1 and GRIP1 by siRNA knockdown diminishes expression of the endogenous *MMP-9* gene, validating the importance of these coactivators in *MMP-9* expression.

Results

CBP and p300 function as coactivators for *MMP-9* promoter activity

Since CBP and p300 are present at the *MMP-9* promoter and they function as transcriptional coactivators for multiple transcription factors,⁸ their roles in *MMP-9* expression were evaluated using a *MMP-9* promoter luciferase assay. Increasing amounts of wild type CBP (Fig. 1A) significantly enhanced phorbol 12-myristate 13-acetate (PMA)-induced *MMP-9* promoter activity in HeLa cells in a dose-dependent manner (Fig. 1B), indicating that CBP functions as a coactivator for *MMP-9* expression. Similar results were observed for p300 (Fig. 1C). To determine if the HAT activity of CBP and p300 are required for their ability to activate *MMP-9* promoter transcription, two HAT-deficient mutant constructs, CBP WY and p300 WY (Fig. 1A),⁴¹ were expressed and *MMP-9* promoter activity determined. The CBP WY and p300 WY constructs enhanced PMA-induced reporter activity in a comparable manner as the wild type construct (Figs. 1B and 1C). The third CH3 domain of CBP/p300 interacts with multiple transcription factors such as PCAF, TFIIB, RNA helicase A, Ets1, Jun B and c-fos.¹³ A construct expressing a mutant form of p300 lacking the CH3 domain (p300 Δ CH3) (Fig. 1A),⁴² was tested to assess its effect on *MMP-9* activation. This construct slightly increased *MMP-9* promoter activity compared to empty vector transfected cells at 0.2 μ g. No further effect was observed with increasing amounts of the p300 Δ CH3 construct (Fig. 1C). Therefore, these data suggest that CBP and p300 function as coactivators for *MMP-9* promoter activation, that their HAT activity is not necessary, and that the CH3 domain is required for optimal activation of *MMP-9* promoter activity. The expression levels of CBP and CBP WY, and of p300, p300 WY and p300 Δ CH3 are comparable as demonstrated by reverse transcription-PCR (RT-PCR) (Fig. 1D).

PCAF is a coactivator for *MMP-9* promoter activation and its HAT activity is critical

Since the HAT activities of CBP and p300 were not required for *MMP-9* promoter activation, the role of PCAF, another HAT, in *MMP-9* promoter activity was evaluated. Increasing amounts of PCAF (Fig. 2A) enhanced both basal and PMA-inducible *MMP-9* reporter activity (Fig. 2B). HeLa cells were transfected with two HAT-deficient mutants of PCAF, PCAF Δ HAT1 and PCAF Δ HAT2 (Fig. 2A),⁴³ together with *MMP-9*-luc. PCAF Δ HAT1 was only able to enhance *MMP-9* promoter activity at the highest concentration (0.6 μ g) (Fig. 2B), while PCAF Δ HAT2 increased *MMP-9* promoter activity at all three concentrations tested (Fig. 2B). However, the degree of activation by PCAF Δ HAT1 and PCAF Δ HAT2 was significantly lower than that of wild type PCAF. These differences are not due to different expression levels of the proteins (Fig. 2C).

CARM1 and GRIP1 augment *MMP-9* promoter activity and the methyltransferase activity of CARM1 is required for this response

MMP-9 is a NF- κ B regulated gene¹ and previous studies have shown that the arginine methyltransferase CARM1 is a coactivator for expression of a subset of NF- κ B regulated genes.³⁴ CARM1 is recruited to the *MMP-9* promoter upon activation,⁷ although its function is unknown. CARM1 was expressed in HeLa cells and *MMP-9* promoter activity determined

(Fig. 3A). At the highest concentration of expression plasmid tested (0.6 μ g) CARM1 enhanced PMA-induced *MMP-9* promoter activity (Fig. 3B). CARM1E267Q, an enzymatic activity deficient construct (Fig. 3A),³⁰ had no effect on *MMP-9* promoter activity at all concentrations tested (Fig. 3B), indicating that CARM1 methyltransferase activity is necessary for *MMP-9* reporter activity. Immunoblotting analysis demonstrated comparable expression levels of CARM1 and CARM1E267Q (Fig. 3C).

The p160 family of coactivators has been shown to activate NF- κ B-mediated transcription,^{36,38,39} and GRIP1 is required for the coactivator function of CARM1 and p300 for estrogen receptor-mediated transcription.³¹ Thus, the role of GRIP1 in activation of *MMP-9* promoter transcription was tested. GRIP1 enhanced both basal and PMA-inducible *MMP-9* promoter activity (Fig. 3D). The expression of GRIP1 was confirmed by immunoblotting analysis (Fig. 3E).

Synergistic activation of *MMP-9* promoter transcription by three classes of coactivators

Thus far, our data illustrate that members of three classes of coactivators, HATs, PRMTs and p160 proteins, function as coactivators for *MMP-9* promoter activation. Whether cooperative interactions occur among these coactivators was next determined. Cells were transfected with 0.2 μ g of the expression constructs for CARM1, p300 or GRIP1 alone or in various combinations. When the individual expression constructs were transfected alone, they had only a modest effect on *MMP-9* reporter activity or no effect (Fig. 4, bars 3–8). Coexpression of CARM1 and p300 had an additive effect on activation of the *MMP-9* reporter (Fig. 4, bars 15 & 16). Synergistic coactivation of the *MMP-9* promoter was observed when CARM1 was coexpressed with GRIP1 (Fig. 4, bars 17 & 18), or when p300 was expressed with GRIP1 (Fig. 4, bars 25 & 26). Remarkably, when these three coactivators were expressed together, both basal and PMA-induced *MMP-9* reporter activity was strikingly increased to 110-fold and 764-fold, respectively (Fig. 4, bars 33 & 34).

GRIP1 showed strong synergistic effects with CARM1 and p300 (Fig. 4, bars 33 & 34), however, in the absence of GRIP1, CARM1 and p300 had only an additive effect (Fig. 4, bars 15 & 16). These results suggest that GRIP1 is necessary for mediating synergy between these three coactivators. Studies have shown that CARM1, p300 and GRIP1 can form a ternary coactivator complex through the binding of CBP/p300 to the AD1 domain and other unidentified regions of GRIP1, and binding of CARM1 to the AD2 domain of GRIP1 (Fig. 4).³⁰ Therefore, three GRIP1 deletion constructs, GRIP1 Δ AD1,³¹ GRIP1 Δ AD2²⁷ and GRIP1 Δ AD1+ Δ AD2³¹ were tested. When the individual deletion constructs were transfected into HeLa cells, they had minor or no effect on *MMP-9* reporter activity (Fig. 4, bars 9–14). Coexpression of CARM1 with GRIP1 Δ AD1 (Fig. 4, bars 19 & 20) showed the same degree of synergy as wild type GRIP1 (Fig. 4, bars 17 & 18). However, when GRIP1 Δ AD2 or GRIP1 Δ AD1+ Δ AD2 were tested, no additive or synergistic effect with CARM1 was observed (Fig. 4, bars 21–24). These data indicate that the cooperation between CARM1 and GRIP1 depends on the AD2 domain of GRIP1. p300 did not synergize with GRIP1 Δ AD1 to activate *MMP-9* reporter activity (Fig. 4, bars 27 & 28), and less synergy was observed with GRIP1 Δ AD2 (Fig. 4, bars 29 & 30), compared to wild type GRIP1 (Fig. 4, bars 25 & 26). GRIP1 Δ AD1+ Δ AD2 completely lost its ability to synergize with p300 (Fig. 4, bars 31 & 32). Collectively, these data indicate that the AD1 domain of GRIP1 is required to mediate the synergistic effect with p300.

Next, we examined the role of the GRIP1 AD1 and AD2 domains in the substantial synergy among CARM1, p300 and GRIP1. When GRIP1 Δ AD1 was coexpressed with p300 and CARM1, basal and PMA-inducible *MMP-9* promoter activity dropped to 29-fold and 188-fold, respectively (Fig. 4, bars 35 & 36), a substantial reduction compared to the effect of wild type GRIP1 (Fig. 4, bars 33 & 34). Deletion of the AD2 domain had a similar effect (Fig. 4, bars

37 & 38). Deletion of both AD1 and AD2 domains significantly diminished the synergistic effect among the three coactivators (Fig. 4, bars 39 & 40). Our data support the formation of a ternary coactivator complex at the *MMP-9* promoter upon overexpression of the coactivators in which p300 and CARM1 bind the AD1 and AD2 domains of GRIP1, respectively.

Multiple coactivators associate with the *MMP-9* promoter and siRNA knockdown of p300, CARM1 or GRIP1 inhibit *MMP-9* expression

The *MMP-9* reporter assays demonstrated that multiple coactivators are involved in *MMP-9* promoter activation. To confirm their roles in expression of the endogenous *MMP-9* gene, chromatin immunoprecipitation (ChIP) assays were performed. As demonstrated previously, ⁷ in the basal state, there were low levels of CBP, p300 and CARM1 associated with the *MMP-9* promoter, which were enhanced upon PMA treatment (Fig. 5A). PCAF and GRIP1 were associated with the *MMP-9* promoter in the basal state, and the presence of PCAF and GRIP1 was modestly enhanced by PMA treatment (Fig. 5A). To determine the functional importance of p300, CARM1 and GRIP1 in expression of endogenous *MMP-9*, their levels were individually or combinatorially inhibited by siRNA duplexes. As shown by RT-PCR, p300 siRNA duplexes caused a reduction of p300 mRNA levels (Fig. 5B, p300, lane 4). In cells transfected with siRNAs to both p300 and GRIP1 or p300 and CARM1, the mRNA level of p300 was also reduced (Fig. 5B, p300, lanes 5 and 6), although to a lesser degree compared to cells transfected only with p300 siRNA. Similarly, CARM1 or GRIP1 siRNA alone transfected cells showed a reduction of mRNA levels of CARM1 and GRIP1, respectively (Fig. 5B, CARM1, lane 4, & GRIP1, lane 4). Under coactivator knockdown conditions, expression of PMA-induced *MMP-9* expression was evaluated (Fig. 5C). *MMP-9* mRNA levels were reduced to ~55% in CARM1 or p300 alone knockdown cells compared to mock transfected cells (Fig. 5C). Knockdown of GRIP1 led to a greater reduction (64%) of *MMP-9* mRNA expression (Fig. 5C). When GRIP1 was inhibited with either CARM1 or p300, *MMP-9* mRNA levels were reduced by ~52% (Fig. 5C). *MMP-9* mRNA levels were reduced by ~34% in cells with siRNAs targeting both CARM1 and p300 (Fig. 5C). Thus, *MMP-9* expression is not further reduced by knockdown of two different coactivators.

To further explore the role of GRIP1 in endogenous *MMP-9* expression, the effect of GRIP1 knockdown in recruitment of transcription factors to the *MMP-9* promoter was determined. *MMP-9* is a NF- κ B regulated gene, therefore the recruitment of NF- κ B p65 was evaluated first. p65 was recruited to the *MMP-9* promoter upon PMA treatment (Fig. 5D, lanes 2–4), which was reduced in GRIP1 siRNA treated cells from 2 to 6 h (Fig. 5D, lanes 6–8). Similarly, the recruitment of p300 was also reduced in GRIP1 siRNA treated cells (Fig. 5D, compare lanes 2–4 and lanes 6–8). The association of GRIP1 with the *MMP-9* promoter was reduced at basal (compare lane 1 and lane 5) and at 6 h post-PMA stimulation (compare lane 4 and lane 8). However, the remaining limited amounts of GRIP1 in the cells were recruited to the *MMP-9* promoter at 2 and 4 h (Fig. 5D). These data suggest the important role of GRIP1 in the association of p65 and p300 with the endogenous *MMP-9* promoter. Together, these data demonstrate that p300, GRIP1 and CARM1 play a vital role in endogenous *MMP-9* gene expression, and that the expression levels of *MMP-9* are tightly regulated by the cellular concentrations of the coactivators.

Discussion

In this study, we investigated the coactivators involved in human *MMP-9* gene expression, and found that members of three classes of coactivators are essential for *MMP-9* expression, including CBP/p300, PCAF, CARM1 and GRIP1. These three classes of coactivators can induce *MMP-9* promoter activity in independent, additive and synergistic manners. ChIP assays demonstrate their association with the endogenous *MMP-9* promoter, and siRNA

knockdown of the coactivators reduced expression of the endogenous *MMP-9* gene. These data confirm the importance of coactivators in endogenous *MMP-9* gene expression.

We have previously shown that PMA is a potent inducer of *MMP-9*,⁷ and thus utilized it for our studies. Upon PMA stimulation, CBP and p300 were recruited to the *MMP-9* promoter (Fig. 5A), and overexpression of CBP and p300 significantly increased PMA-induced *MMP-9* promoter activity (Fig. 1B and 1C). This effect was independent of the HAT activity of either CBP or p300, but required the p300 CH3 domain (Fig. 1B and 1C). Besides functioning as HATs to activate gene transcription, CBP and p300 also provide a platform for formation of multiple protein complexes and bridge interactions between transcription factors and transcriptional machinery such as the RNA polymerase II complex,⁸ which may explain their coactivator roles in *MMP-9* expression. Further studies indicated the CH3 domain interacting protein, PCAF, enhanced *MMP-9* promoter activity (Fig. 2B), and its HAT activity was required for maximal induction (Fig. 2B). Also, PCAF was recruited to the *MMP-9* promoter upon PMA treatment (Fig. 5A). These data suggest that PCAF may be the HAT for activation of the *MMP-9* promoter. A transcription factor specific requirement of distinct coactivator components and HAT activity has been previously documented. The HAT activity of PCAF, but not CBP, was required for p65-mediated E-selectin reporter activation.³⁶ In nuclear receptor-mediated transcription, the HAT of PCAF but not of CBP/p300 was required.^{29,30} On the other hand, for CREB regulated genes, the HAT of CBP but not of PCAF was essential.²⁹

It has been shown that expression of a subset of NF- κ B regulated genes, such as MCP-1, IP-10, MIP-2 and G-CSF, is impaired in CARM1 knockout mouse embryo fibroblasts.³⁴ Knockdown of CARM1 by RNAi reduced the expression of NF- κ B regulated genes in 293T cells such as IL-8, IP-10 and TNF- α .³⁷ However, the requirement for CARM1 is gene-specific since the expression of other NF- κ B regulated genes such as IL-6, I κ B α and COX-2 was not affected by CARM1 knockout.³⁴ We found that *MMP-9* promoter activity was enhanced when CARM1 was overexpressed (Fig. 3B), and that CARM1 was recruited to the *MMP-9* promoter upon PMA treatment (Fig. 5A). Furthermore, knockdown of CARM1 expression substantially reduced the expression of endogenous *MMP-9* mRNA (Fig. 5B and 5C). These data demonstrate that CARM1 acts as a coactivator for *MMP-9* expression and is required for *MMP-9* expression. This differs from what was observed for p65-mediated TNF- α activation, in which CARM1 was able to activate *TNF- α* promoter activity in 293T cells only in the presence of CBP/p300.³⁷ This difference may be due to different endogenous cellular concentrations of CARM1. That is, 293 cells may already have enough CARM1, so overexpression alone would not show any effect. However, it is also possible that the coactivators have gene-specific effects for different NF- κ B regulated genes. CARM1 functions mainly through methylating histones or non-histone proteins involved in transcription.¹⁶ In several NF- κ B regulated gene promoters, CARM1 recruitment upon TNF- α treatment is correlated with methylation of histone H3 at arginine 17,^{34,37} which is linked to gene activation.⁴⁴ We previously demonstrated that H3 arginine 17 methylation occurs coincident with CARM1 recruitment at the *MMP-9* promoter.⁷ When the methyltransferase activity of CARM1 was eliminated, CARM1 lost its ability to activate *MMP-9* reporter activity (Fig. 3B; CARM1E267Q). Thus, methylation of histones by CARM1 may contribute to its coactivator function for *MMP-9* expression.

The p160 family protein GRIP1 alone had only a modest effect on *MMP-9* promoter activation (Fig. 3D), and its association with the endogenous *MMP-9* promoter was not modulated by PMA treatment (Fig. 5A). This suggests that it is not an effective coactivator by itself, and may need other proteins to function. SRC-1, another p160 family member, interacts with p50³⁹, c-Jun and c-fos.⁴⁵ We found an *in vivo* association of p50 with GRIP1 in unstimulated cells by coimmunoprecipitation (data not shown), and low levels of p50 are present on the *MMP-9*

promoter in unstimulated cells.⁷ This may explain the constitutive association of GRIP1 with the *MMP-9* promoter (Fig. 5A). More importantly, knockdown of GRIP1 in HeLa cells had a substantial effect on *MMP-9* expression (Fig. 5B and 5C), demonstrating that it is required for *MMP-9* expression. Synergy by various combinations of coactivators has been shown for nuclear receptor function, for example, between CARM1 and GRIP1,¹⁷ CARM1 and PRMT1,²⁸ CARM1 and p300,³¹ and among CBP/p300, CARM1 and GRIP1.³⁰ p65-activated HIV LTR luciferase activity is also synergistically enhanced by p300, CARM1 and GRIP1.³⁴ Synergy between CBP and SRC-1 or PCAF has been demonstrated for p65-activated *E-selectin* promoter activity.³⁶ In our study, we found that the combination of CARM1 plus p300 had an additive effect on *MMP-9* promoter transcription (Fig. 4, bars 15 & 16). GRIP1 significantly enhanced the ability of either CARM1 or p300 to activate *MMP-9* promoter activity (Fig. 4, bars 17 & 18 and 25 & 26). The most pronounced synergistic effect was observed when all three coactivators were simultaneously expressed (Fig. 4, bars 33 & 34). More importantly, the studies performed with the GRIP1 mutants clearly demonstrated the critical role of GRIP1 in mediating the formation of a ternary coactivator complex with p300 and CARM1 through its AD1 and AD2 domains for *MMP-9* reporter activity (Fig. 4). Furthermore, knockdown of GRIP1 by siRNA had a greater effect on *MMP-9* mRNA expression than knockdown of CARM1 or p300 (Fig. 5C). However, when GRIP1 was reduced together with either p300 or CARM1, their effects on *MMP-9* mRNA expression were less than GRIP1 knockdown alone. This may be due to less efficient knockdown of the coactivators with siRNA duplexes targeting different proteins (Figs. 5B & C). The siRNA duplexes may interfere or compete with each other for the RNAi machinery. In addition, the ChIP assays performed in cells with GRIP1 knockdown demonstrated that recruitment of p65, p300 and GRIP1 to the endogenous *MMP-9* promoter were reduced (Fig. 5D). Together, these data suggest the important role of GRIP1 in promoting endogenous *MMP-9* gene expression.

MMP-9 transcription is a complex tightly regulated process⁷ and this study illustrates additional complexity with respect to the involvement of coactivators. *MMP-9* promoter assays, siRNA studies and ChIP assays suggest activation of *MMP-9* gene expression by the synergistic action of multiple coactivators. In the proposed model shown in Figure 6, upon induction of *MMP-9* gene expression by PMA, sequence specific transcription factors such as AP-1 and NF- κ B are activated and recruited to the *MMP-9* promoter.⁷ They can further recruit the chromatin remodeling complex component Brg-1 to relax chromatin and coactivators such as CBP/p300 and CARM1.⁷ CBP/p300 associated PCAF is also recruited to the *MMP-9* promoter. At the promoter, CBP/p300 and CARM1 likely bind to the AD1 and AD2 domains, respectively, of GRIP1 to form a ternary complex. The ternary complex positions the coactivators in proximal distance to promote their functions in a synergistic way. One way may be that PCAF and CARM1 collaborate to modify histones to promote the further relaxation of *MMP-9* chromatin, allowing the binding of transcriptional factors and providing docking sites for other transcriptional coactivators. It has been demonstrated previously that acetylation of H3 lysine 18 and 23 by CBP/p300 promotes the recruitment of CARM1 and methylation of arginine 17 on the *pS2* promoter following estrogen stimulation.⁴⁶ On the other hand, modification of coactivators can also contribute to their synergy. For example, methylation of CBP/p300 by CARM1 plays an important role in GRIP1- and estrogen-induced gene activation.²¹ Furthermore, CARM1 interacts with the chromatin remodeling complex component Brg-1 and coassembles at estrogen receptor regulated gene promoters, so they can enhance each others functions.⁴⁷ Similar events may occur on the *MMP-9* promoter, thereby contributing to the synergistic effects among the coactivators. Our studies suggest that these coactivators may be potential targets in controlling aberrant *MMP-9* expression in disease states. It has been shown that some coactivators are overexpressed in cancers. For example, CARM1 is overexpressed in prostate carcinoma⁴⁸ and breast cancer.⁴⁹ ACTR, another p160 protein, is amplified and overexpressed in breast and ovarian cancers.⁵⁰ *MMP-9* is also upregulated in breast, ovarian and prostate cancers,³ so its upregulation may result from the

overexpression of these coactivators. Reduction of coactivator expression in these cancers may lead to less MMP-9 expression, and therefore control tumor growth, angiogenesis and invasion.

Materials and Methods

Reagents and antibodies

PMA was purchased from Calbiochem (San Diego, CA, USA). Polyclonal rabbit antibodies against CBP, p300, GRIP1, pCAF and normal IgG control were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse antibodies against actin and HA tag, and polyclonal rabbit antibody against FLAG tag were purchased from Sigma (St. Louis, MO, USA). Polyclonal rabbit antibody against p65 was purchased from Abcam (Cambridge, MA, USA). The secondary peroxidase-conjugated antibodies and ECL reagents were from Pierce (Rockford, IL, USA). Protein A/G agarose/Salmon sperm DNA for the ChIP assays, and the CARM1 antibody were from Upstate Cell Signaling Solution (Charlottesville, VA, USA). The Fugene 6 transfection reagent was purchased from Roche (Basel, Switzerland).

Cell lines and plasmids

HeLa cells were passaged as previously described.⁵¹ The luciferase reporter plasmid for MMP-9 (MMP-9-Luc) containing 670 bp of the human *MMP-9* promoter was obtained from Dr. D. Boyd, MD Anderson Cancer Center, Houston, TX, USA.⁵² pcDNA3 was from Invitrogen (Carlsbad, CA, USA). pCMV-CBP-FLAG was a generous gift of Dr. M. G. Rosenfeld, University of California, San Diego, CA, USA.²⁹ pCMV β -HA-p300 was purchased from Upstate Cell Signaling Solution. pCMV β -p300 Δ CH3-HA was a gift from Dr. D. Livingston, Dana-Farber Cancer Institute, Boston, MA, USA.⁴² pcDNA3-CBPWY-HA and pCMV β -p300WY-HA were from Dr. R. Eckner, New Jersey Medical School, Newark, NJ, USA.⁴¹ pCX-FLAG-PCAF was obtained from Dr. Y. Nakatani at the National Institutes of Health, Bethesda, MD, USA.¹⁴ pCX-FLAG-PCAF Δ HAT1 and pCX-FLAG-PCAF Δ HAT2 were from Dr. D. Liao at the University of Florida, Gainesville, FL, USA.⁴³ The pSG5.HA vector was obtained from Dr. M. Stallcup, University of Southern California, Los Angeles, CA, USA. CARM1,¹⁷ CARM1E267Q,³⁰ GRIP1,¹⁷ GRIP1 Δ AD1,³¹ GRIP1 Δ AD2²⁷ and GRIP1 Δ AD1+ Δ AD2³¹ were expressed from the pSG5.HA vector and were a generous gift from Dr. M. Stallcup.

Transient transfection and luciferase assay

HeLa cells (1.8×10^5) in six-well culture dishes were transiently transfected with 0.2 μ g of MMP-9-Luc and increasing amounts of coactivator expression constructs (0, 0.2, 0.4, 0.6 μ g) using the Fugene 6 reagent following manufacturer's instructions. Total DNA was normalized by addition of the empty vector pcDNA3 or pSG5.HA. After recovery, cells were incubated in serum-free media or in the presence of PMA (50 ng/ml) for 16 h and whole cell extracts were used for luciferase assay and normalized to total protein as described before.⁵¹ The luciferase activity of the untreated and no coactivator overexpressed sample was arbitrarily set at 1 for calculation of fold induction upon PMA treatment.

Coactivator overexpression as detected by RT-PCR and immunoblotting

HeLa cells were transfected with CBP or p300 overexpression constructs for 48 h and then total cellular RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, California, USA), and one μ g of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) with oligo (dT)₁₅ primer according the manufacturer's protocol. cDNAs were used as a template for the semi-quantitative PCR reaction. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. GAPDH was amplified using the primers: forward 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and

reverse 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.⁵³ p300 was amplified using the primers: forward 5'-GTATGATCCGTGGCAGTGTG-3' and reverse 5'-CCCTATGCTTGGGGGAGTAT-3'. CBP was amplified using the primers: forward 5'-ACCCAGGCCTCCTCAATAGT-3' and reverse 5'-TTGCTTGCTCTCGTCTCTGA-3'.

Immunoblotting was performed as previously described.⁵⁴ HeLa cells were transfected with PCAF, CARM1 and GRIP1 overexpression constructs for 48 h and then cells were lysed in 1X RIPA buffer. Cell lysates were separated on 8% SDS-PAGE gel and transferred to nitrocellulose membranes. After blocking the membranes with 5% non-fat milk, the membranes were incubated with specific primary antibodies at 4°C overnight. After three washings, membranes were incubated with secondary antibodies at room temperature for 45 minutes. Membranes were washed three times again and ECL reagents used to detect the proteins.

SiRNA knockdown and real-time quantitative PCR

HeLa cells (1×10^5) in 12-well culture dishes were transiently transfected with 50 nM SMART pool on-target plus siRNA duplexes for p300, CARM1, or GRIP1 alone or in combination using the DharmaFECT reagent 1 according to the manufacturer's protocol (Dharmacon, Chicago, IL, USA). 48 h after transfection, the media was changed to serum free media overnight. Cells were then untreated or treated with PMA (50 ng/ml) for 8 h. Total cellular RNA was extracted, and one μ g of total RNA was used to perform reverse transcription. The levels of p300, CARM1, GRIP1 and GAPDH were determined by semi-quantitative PCR. GAPDH and p300 were amplified using the primers as described above. CARM1 was amplified with primers: forward 5'-GCCACAACAACCTGATTCCT-3' and reverse 5'-TGTTCCAGCAGATGACAAGC-3', and GRIP1 was amplified with primers: forward 5'-GCAGCTGCCAACATAGATGA-3' and reverse 5'-CAAATCAAGCAGGACTGCAA-3'. The level of MMP-9 mRNA was determined by real-time quantitative PCR using the SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) in the Applied Biosystems 7500 real-time PCR system according to the manufacturer's instructions. MMP-9 was amplified with primer forward 5'-GAACCAATCTCACCGACAGG-3' and reverse 5'-GCCACCCGAGTGTAACCATA-3' and normalized to GAPDH levels. The mRNA level of untransfected PMA-treated sample was arbitrarily set as 100% for calculation of relative mRNA levels in other conditions.

ChIP assay

ChIP assays were performed as previously described.^{7,51} Cells were cross-linked and the nuclei were extracted and sonicated. After preclearing, the soluble chromatin was immunoprecipitated with 4 μ g of appropriate antibodies and protein A/G beads were added to bind the precipitates. After several washings, elutes were heated to reverse the cross-linking. DNA fragments were purified and analyzed by semi-quantitative PCR using the following primers for human MMP-9: forward 5'-GAC CAA GGG ATG GGG GAT C-3' and reverse 5'-CTT GAC AGG CAA GTG CTG AC-3'.

Statistical analysis

Data are presented as mean $\pm$ standard error (S.E.), and the Student's *t*-test was used to determine statistical difference. P values of ≤ 0.05 were considered to be statistically significant.

Abbreviations

MMP, matrix metalloproteinase
CREB, cAMP-response-element binding protein
CBP, CREB-binding protein

CH, cysteine-histidine
 HAT, histone acetyltransferase
 PCAF, p300/CBP associated factor
 PRMT, protein arginine methyltransferase
 CARM1, coactivator-associated arginine methyltransferase 1
 H3, histone 3
 SRC, steroid receptor coactivator
 PMA, phorbol 12-myristate 13-acetate
 MMP-9-luc, luciferase reporter plasmid for MMP-9
 ChIP, chromatin immunoprecipitation
 RT-PCR, reverse transcription-PCR
 S.E., standard error.

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Fig. 1.

CBP and p300 function as coactivators for *MMP-9* promoter activity. (A) Schematic diagram of CBP/p300 and mutant constructs. CH: cysteine-histidine-rich domains; KIX: CREB-binding domain; Bromo: bromodomain; HAT: histone acetyltransferase activity domain; Q-rich: glutamine-rich region; W: tryptophan; Y: tyrosine; A: alanine; S: serine. (B) HeLa cells were transiently transfected with the *MMP-9*-Luc reporter construct and 0 to 0.6 μ g of pCMV-CBP, pcDNA3-CBPWY or pcDNA3 to normalize the total DNA transfected, and *MMP-9* promoter activity determined as described in Materials and Methods. The results are the mean $\pm$ S.E. of at least three independent experiments. (C) HeLa cells were transiently transfected with the *MMP-9*-Luc reporter construct and 0 to 0.6 μ g of pCMV β -p300, pCMV β -p300WY or pCMV β -p300 Δ CH3, and *MMP-9* promoter activity determined as described in Materials and Methods. The results are the mean $\pm$ S.E. of at least three independent experiments. For (B) & (C), * $p \leq 0.05$ compared to pcDNA3 only transfected PMA treated samples; # $p \leq 0.05$ between the two linked samples; NS, not significant between the two linked samples. (D) HeLa cells were transiently transfected with 0.6 μ g of pcDNA3, pCMV-CBP, pcDNA3-CBPWY, pCMV β -p300, pCMV β -p300WY or pCMV β -p300 Δ CH3 for 48 h, and total RNA was then isolated and subjected to RT-PCR analysis with primers for CBP or p300. GAPDH mRNA levels were utilized as a control.

Fig. 2. PCAF enhances *MMP-9* promoter activity and its HAT activity is necessary for optimal induction. (A) Schematic diagram of PCAF and deletion constructs. HAT: histone acetyltransferase activity domain; Bromo: bromodomain. (B) HeLa cells were transiently transfected with the *MMP-9*-Luc reporter construct and 0 to 0.6 μg of pCX-PCAF, pCX-PCAF ΔHAT1 or pCX-PCAF ΔHAT2, and *MMP-9* promoter activity determined as described in Materials and Methods. The results are the mean ± S.E. of at least three independent experiments. * $p \leq 0.05$ compared to pcDNA3 only transfected PMA treated samples; # $p \leq 0.05$ between the two linked samples; NS, not significant compared to pcDNA3 only transfected PMA treated samples. (C) HeLa cells were transiently transfected with 0.4 μg of pcDNA3, pCX-FLAG-PCAF, pCX-FLAG-PCAF ΔHAT1 or pCX-FLAG-PCAF ΔHAT2 for 48 h, and whole cell lysates isolated and subjected to immunoblotting analysis. Actin was utilized as a loading control.

Fig. 3. CARM1 and GRIP1 activate *MMP-9* promoter transcription and CARM1 methyltransferase activity is required. (A) Schematic diagram of CARM1 and CARM1E267Q. E: glutamic acid; Q: glutamine. (B) HeLa cells were transiently transfected with the *MMP-9*-Luc reporter construct and 0 to 0.6 μ g of pSG5-CARM1 or pSG5-CARM1E267Q, and *MMP-9* promoter activity determined as described in Materials and Methods. The results are the mean $\pm$ S.E. of at least three independent experiments. * $p \leq 0.05$ compared to pSG5 only transfected PMA treated samples; # $p \leq 0.05$ between the two linked samples; NS, not significant compared to pSG5 only transfected PMA treated samples. (C) HeLa cells were transiently transfected with 0.6 μ g of pSG5.HA, pSG5.HA-CARM1 or pSG5.HA-CARM1E267Q for 48 h and whole cell lysates isolated and subjected to immunoblotting analysis. Actin was utilized as a loading control. (D) HeLa cells were transiently transfected with the *MMP-9*-Luc reporter construct and 0 to 0.6 μ g of pSG5-GRIP1, and *MMP-9* promoter activity determined as described in Materials and Methods. The results are the mean $\pm$ S.E. of at least three independent experiments. * $p \leq 0.05$ compared to pSG5 only transfected PMA treated samples; NS, not significant compared to pSG5 only transfected PMA treated samples. (E) HeLa cells were transiently transfected with 0.6 μ g of pSG5.HA or pSG5.HA-GRIP1 for 48 h and whole cell lysates subjected to immunoblotting analysis. Actin was utilized as a loading control.

Fig. 4.

Synergy among p300, CARM1 and GRIP1 is dependent on the AD1 and AD2 domains of GRIP1. HeLa cells were transiently transfected with the MMP-9-Luc reporter construct and 0.2 μ g of various combinations of pSG5-CARM1, pCMV β -p300, pSG5-GRIP1, pSG5-GRIP1 Δ AD1, pSG5-GRIP1 Δ AD2 and pSG5-GRIP1 Δ AD1+ Δ AD2, and then *MMP-9* promoter activity determined as described in Materials and Methods. The results are the mean $\pm$ S.E. of at least three independent experiments. * $p \leq 0.05$ compared to empty vector only PMA treated samples (bar 2); † $p \leq 0.05$ compared to CARM1 or p300 construct alone PMA treated samples (bars 4 & 6); # $p \leq 0.05$ wild type GRIP1 transfected PMA treated samples compared to mutant GRIP1 transfected PMA treated samples. NS1, not significant compared to empty vector only PMA treated samples (bar 2); NS2, not significant between the two linked samples; NS3, not significant compared to corresponding single coactivator expressed PMA-treated samples (bars 4 & 6); NS4, not significant compared to CARM1 and p300 coexpressed PMA-treated samples (bar 16). Insert is a schematic diagram of GRIP1. bHLH/PAS: bHLH/Per/Ah receptor nuclear translocator (ARNT)/Sim domain involved in DNA binding and heterodimerization between proteins containing these motifs;²⁴ S/T: serine/threonine-rich regions; L1L2L3: three LXXLL (L, leucine; X, any amino acid) motifs for interaction with ligand-bound nuclear receptors;²⁴ AD1 and AD2: two intrinsic transcriptional activation domains. The AD1 domain is responsible for interaction with CBP and p300; The AD2 domain is responsible for interaction with CARM1 and PRMT1.^{17,24–28}

Fig. 5.

Knockdown of p300, CARM1 and GRIP1 reduces MMP-9 mRNA expression. (A) ChIP assays were performed with soluble chromatin from HeLa cells that were untreated or treated with PMA (50 ng/ml) for 2 to 6 h, using antibodies against CBP, p300, CARM1, pCAF and GRIP1. Polyclonal rabbit IgG is utilized as a negative control. Immunoprecipitated DNA fragments were amplified with MMP-9 ChIP primers. Input chromatin was subjected to PCR to control for variations in immunoprecipitation starting material. Representative of three independent experiments. (B) HeLa cells were left untransfected, mock transfected, or transfected with SMART pool siRNAs to p300, CARM1, GRIP1 or both. 48 h after transfection, cells were serum starved and then left untreated or treated with PMA for 8 h. Total RNA was then isolated and subjected to RT-PCR analysis with primers for p300, CARM1 and GRIP1 to evaluate level of the coactivators as described in Materials and Methods. GAPDH mRNA level was utilized as a control to normalize the variation between samples. Representative of two independent experiments. (C) The same cDNA samples obtained in B were used for real-time PCR with primers for MMP-9 and GAPDH to determine their mRNA levels. The mRNA level of untransfected PMA-treated sample was arbitrarily set as 100% for calculation of relative mRNA levels in other conditions. All reactions were performed in triplicate. The results are the mean $\pm$ standard deviation. * $p \leq 0.05$ compared to mock transfected PMA treated samples. (D) HeLa cells were left untransfected or transfected with siRNA to GRIP1, and 42 h after transfection, cells were treated with PMA (50 ng/ml) for 2 to 6 h. Soluble chromatin was

subjected to ChIP using antibodies against p65, p300 and GRIP1. Immunoprecipitated DNA fragments were amplified with MMP-9 ChIP primers by semi-quantitative PCR.

Fig. 6.

Proposed model for synergy among coactivators in *MMP-9* gene expression. The human *MMP-9* promoter is wrapped into a nucleosome structure.⁷ Activation of the *MMP-9* gene induces recruitment of transcription factors such as NF-κB, Sp1 and AP-1, which may further recruit multiple coactivators such as CBP/p300, PCAF and CARM1 to the *MMP-9* promoter.⁷ The tails of histones may be modified by those enzymes to further relax chromatin. Ac, acetylation; Me, methylation. The AD1 and AD2 domains of GRIP1 may bind to CBP/p300 and CARM1, respectively, to possibly promote the formation and stabilization of this multiple protein complex to synergistically induce transcription of the *MMP-9* gene.