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The CSRP2BP histone acetyltransferase drives smooth muscle gene expression

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ABSTRACT

The expression of nearly all smooth muscle genes are controlled by serum response factor binding sites in their promoter regions. However, SRF alone is not sufficient for regulating smooth muscle cell development. It associates with other cardiovascular specific cofactors to regulate smooth muscle gene expression. Previously, we showed that the transcription co-factor CRP2 was a regulator of smooth muscle gene expression. Here, we report that CSRP2BP, a coactivator for CRP2, is a histone acetyltransferase and a driver of smooth muscle gene expression. CSRP2BP directly interacted with SRF, CRP2 and myocardin. CSRP2BP synergistically activated smooth muscle gene promoters in an SRF-dependent manner. A combination of SRF, GATA6 and CRP2 required CSRP2BP for robust smooth muscle gene promoter activity. Knockdown of *Csrp2bp* in smooth muscle cells resulted in reduced smooth muscle gene expression. We conclude that the CSRP2BP histone acetyltransferase is a coactivator for CRP2 that works synergistically with SRF and myocardin to regulate smooth muscle gene expression.

INTRODUCTION

Smooth muscle cells (SMCs) express a unique array of contractile and structural gene isoforms, which distinguish them from striated muscle cells. Smooth muscle genes are expressed in immature striated muscle during embryonic development and are also reactivated in pathologic hypertrophy illustrating the very parallel nature in which smooth and striated muscle gene expression is regulated (1–6). Serum response factor (SRF) regulates expression of smooth muscle genes such as alpha smooth muscle actin (αSMA), smooth muscle myosin heavy chain, smooth muscle calponin h1, SM22alpha (SM22α) and smooth muscle actin gamma (SMAγ) (7–9). Evolutionarily conserved serum response elements (SREs), which contain CC $(A/T)_6$GG nucleotide sequences and serve as SRF binding sites, can be found within 2 kb upstream of many smooth muscle gene transcription start sites (10–12). Previous studies have established that these *cis*-acting SREs are critical for the temporal and spatial expression of SMC genes (13).

Although SRF is indispensable for muscle gene expression, SRF alone is not sufficient to drive expression of

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these genes. SRF is a weak trans-activator and its expression is not restricted to muscle. During the embryonic development, SRF is widely expressed in mesoderm-derived tissues, including skeletal and cardiac muscles. Thus, SRF associates with muscle-specific coregulators to drive muscle gene expression (14–16). Myocardin, which is expressed in striated and smooth muscle, is an important SRF coactivator. Myocardin physically interacts with SRF and together induce strong activation of SRF target genes (17,18). Because myocardin is expressed in both striated and smooth muscle, there must exist other SRF-interacting factors that provide specificity for smooth muscle gene regulation. Among these factors is a LIM-domain only protein, cystein and serine rich protein 2 (CRP2), which is a key bridging factor to bring other coactivators, including GATA6 to SRF and augment smooth muscle gene expression (14,19). Both SRF/Myocardin complexes and SRF/GATA6/CRP2 complexes are sufficient to drive smooth muscle gene expression.

Histone lysine acetylation is strongly correlated with transcriptionally active euchromatin. Coactivator complexes typically contain histone acetyltransferase (HAT) activity, whereas corepressor complexes typically contain histone deacetylase (HDAC) activity (20,21). Several HDACs, especially class II HDACs, are enriched in cardiac, skeletal and smooth muscles, where they play essential roles in tissue-specific transcriptional regulation (22,23). Myocardin associates with both HATs and HDACs to modulate its activity (24). Surprisingly, unlike HDACs, previously studied HATs (i.e. CBP and p300) have broad expression in most tissues (25,26).

Here, we report that the HAT CSRP2BP interacts with SRF and two of its coactivators, CRP2 and myocardin. CSRP2BP (also known as ATAC2) is a component of the ATAC chromatin remodeling complex (27,28), CSRP2BP and GCN5 are the two subunits of the ATAC complex containing HAT activity. In Drosophila, CSRP2BP/ATAC2 was demonstrated to specifically acetylate histone H4 (29). It was showed that CSRP2BP play function in intestinal stem cell homeostasis (30). Here, we report that CSRP2BP strongly potentiates the expression of SRF smooth muscle target genes together with either Myocardin or CRP2. Thus, CSRP2BP adds smooth muscle gene specificity to SRF-driven gene activation.

MATERIALS AND METHODS

Expression vectors and transient cotransfection

CSRP2BP expression vectors were generated by cloning the mouse CSRP2BP full-length or different deletion fragments of cDNA in frame into the pcDNA3.1 or PCGN vector, for C-terminal V5 tag or N-terminal HA tag, respectively. CSRP2BP cDNA was cloned into pGEX4T2 to generate GST-fused CSRP2BP expression vector pGEX4T2-CSRP2BP. The PCGN-SRF, pCGN-GATA6, pCGN-CRP2, pGEX4T2-CRP2, pGEX4T2-SRF expression plasmids and luciferase reporter vectors as described (14). pCDNA3.1-myocardin plasmid was provided by Dr. Eric Olson (15). Fibroblast CV1 cells were co-transfected with 100 ng of luciferase reporter plasmid, 50–500 ng of the individual expression vectors using Fugene 6 (Roche). Luciferase assay was performed as described (14).

In vitro histone acetylation assay

HA tagged CSRP2BP protein expressed in transfected Hela cells was immunoprecipitated with HA antibody (Santa Cruz Biotech) from cell lysates and protein A/G plus beads (Santa Cruz Biotech). CSRP2BP was incubated for 30 min at 30°C with 10 μg of core histone proteins (Roche) and [^{3}H]-acetyl-CoA (Amersham) in 1X HAT buffer (Upstate Biotech). Histones were separated by NuPAGE electrophoresis (Invitrogen) and visualized by Coomassie blue staining. Acetylated histones were detected by autoradiography.

Immunoprecipitation assays

Antibody precipitation assays were performed as previously described (4,10). PAGE separated protein-antibody complexes were transferred to PVDF membranes and probed with antibodies as indicated and visualized with luminescent detection system (Pierce). Chromatin Immunoprecipitation (ChIP) assays were performed using ChIP Assay Kit (Upstate Biotech) following the protocol recommended by the manufacturer. Primers information for rat SMC gene promoters is available upon request.

GST pulldown assays

GST, GST-CRP2, GST-CSRP2BP and GST-SRF fusion proteins were expressed in *Escherichia coli* strain BL21 and purified with Glutathione Sepharose 4B (Amersham). Full-length CSRP2BP and deletion fragments were synthesized with TNT T7-coupled reticulocyte lysate system (Promega) and [^{35}]S-methionine. GST or GST fusion protein were incubated with lysates and then bound to Glutathione Sepharose 4B beads as described (14).

Primary human smooth muscle cells isolation and culture

Clinical discarded human umbilical cord arteries were collected from Department of Obstetrics and Gynecology of Affiliated Hospital of Hainan Medical University with an approval by the Ethics Committee of Affiliated Hospital of Hainan Medical University. Primary human vascular smooth muscle cells (hVSMCs) were isolated following previously published protocol (31). Briefly, 10 cm cord arteries were washed twice with 1x PBS and the Wharton's jelly that surrounds the arteries were removed by scissors. Arteries were cut into 1 mm pieces followed by subsequent enzymatic digestion with type I collagenase (Gibco). Digestion media were collected and centrifuged. The cell suspension was incubated at 37°C and 5% of CO_2 with DMEM-F12 supplied with 10% of FBS. The medium was half-changed every 3 days. In 1–2 weeks, cells grew to confluence and were passaged. VSMCs were identified by positive-staining with anti-alpha smooth muscle actin antibody (Abcam, ab21027). The third and fourth passage cells were used for experiments.

Real-time PCR analysis

Total RNA was isolated using RNAeasy mini kit (Qiagen) following manufacturer's protocol. Reverse transcription (RT) assays used SuperScript Supermix (Invitrogen) with random hexamers. Quantitative real-time PCR used Universal Library Probe (Roche) and FastStart Taqman probe Master Mix (Roche) in a HTS7900 realtime PCR machine (Applied Biosystems). Primer and probe sequences are available upon request. Semi-quantitative PCRs were performed using Platinum Taq (Invitrogen) and the amplification products were detected on agarose electrophoresis.

siRNA

The duplex rat CSRP2BP siRNAs were synthesized by Ambion. The sequences of the two siRNA specifically against rat CSRP2BP were 5′-AGUAUUGUCAGCCCUUACA-3′ (si1757) and 5′-GUGGGAAGUCCUGUUUAUU-3′ (si548). The scramble siRNAs for them are 5′-CGUCGAACCUAUACAAUUA-3′ (sc1757) and 5′-UAUGAUGUGGCUCUAGUGU-3′ (sc548). siRNAs were transfected into A7r5 cells using Lipofectamine2000 reagent (Invitrogen). Scramble siRNAs were used as negative control. siRNA transfection efficiency was determined using BLOCK-IT fluorescent siRNA (Invitrogen). Two Human CSRP2BP Stealth siRNAs and a negative control, si126184, si126186, siNC (Thermo Fisher HSS126184, HSS126186 and 12935300, respectively) were used for knockdown experiments with hVSMCs.

Scratch-wound assays

Scratch-wound assays were established by using ibidi Culture Insert Two Wells (ibidi) following manufacturer's protocol. After siRNA knockdown of CSRP2BP, Scratch assays were performed on both A7r5 and human primary cultured smooth muscle cells. Cell migration was captured at an hourly interval. Data were analyzed by using previously published methods (32).

RESULTS

CSRP2BP localizes to the nucleus and acetylates histones H3 and H4

Drosophila CSRP2BP/ATAC2 is a subunit of the ATAC chromatin remodeling complex and was shown to exhibit HAT activity specific for histone H4 (29). We first determined the subcellular localization of mouse CSRP2BP by expressing the full-length V5 epitope-tagged protein in CV1 cells and performing immunofluorescence using an antibody against V5 (Figure 1A). We observed very high enrichment of CSRP2BP in the nucleus consistent with the notion that it is a chromatin modifying enzyme. We also performed immunostaining against CSRP2BP on the primary cultured hVSMCs. As shown in Figure 1B, the endogenous CSRP2BP was expressed in both nucleus and cytoplasm of hVSMCs, with stronger expression in nucleus. To test the substrate specificity of CSRP2BP, we first tested the ability of the full-length protein to acetylate purified individual core histones (Figure 1C). We observed acetylation of all core histones with histone H3 receiving the highest level. Next, we tested the HAT activity of full-length and serial deletion mutants of CSRP2BP (Figure 1D). In this assay, HA epitope-tagged CSRP2BP proteins were expressed in Hela cells and then isolated by immunoprecipitation using an antibody against HA. Core histones were incubated with recombinant CSRP2BP and ^{3}H acetyl-CoA and separated by electrophoresis. Full-length (1–779 aa) CSRP2BP caused strong acetylation of histones H3 and H4. The three mutants lacking the C-terminal HAT domain (1–663 aa, 1–710 aa, 314–663 aa) exhibited little to no acetyltransferase activity, whereas the two mutants that retained the C-terminal domain (314–779 aa, 664–779 aa) acetylated H3, H4 and to a lesser extent H2A and/or H2B. Finally, we generated three CSRP2BP mutants with single amino acid substitutions to conserved residues within the HAT domain (R708A, R707A and G710A) and assayed their HAT activity in a reaction with purified core histones (Figure 1E). All three single amino acid substitutions resulted in reduced acetyltransferase activity.

CSRP2BP stimulated smooth muscle gene expression

We next asked if CSRP2BP was necessary for smooth muscle gene expression in the rat aortic smooth muscle cell line A7r5. Two siRNAs (si548 and si1757) made against rat *CSRP2BP* mRNA were transfected into the A7r5 cells. Both siRNAs gave more than 75% knockdown of *CSRP2BP* mRNA as measured by quantitative RT-PCR assays. Quantitative RT-PCR using Taqman probes specific for *calponin* and *smooth muscle myosin heavy chain* showed that their transcript levels in si548 and si1757 transfected cells were down regulated by ∼40% to 70% compared to untransfected control cells, while the scramble control (sc548 and sc1757) transfected cells had unchanged or slightly elevated expression level of these genes. Decreased smooth muscle gene expression following substantial reduction of CSRP2BP support the idea that CSRP2BP is required for optimal expression of smooth muscle genes (Figure 2A). We performed similar knockdown experiments on cultured hVSMCs, and observed decreased expression of smooth muscle genes at both protein and mRNA levels (Figure 2B and C), consistent with the findings from A7r5 cells. Next, we asked if CSRP2BP was required for smooth muscle cell contractile function. We performed Scratch-wound assays on CSRP2BP knockdown A7r5 and primary cultured hVSMCs. Both knockdown of CSRP2BP either A7r5 rat smooth muscle cells or hVSMCs significantly decreased the rate of cell migration into scratch-wounds compared to the control groups (Figure 2D and E). Collectively, these findings demonstrate that CSRP2BP is required for the normal contractility of SMCs.

CSRP2BP and cofactors powerfully activated SMC marker gene reporters

CRP2 was shown to work as a potent co-activator for SMC genes expression, as a bridging factor connecting SRF with GATA6 (14). Thus, we hypothesized that CSRP2BP functions as a coactivator in this complex. To test this hypothesis, we assayed the effect of CSRP2BP on transactivation of

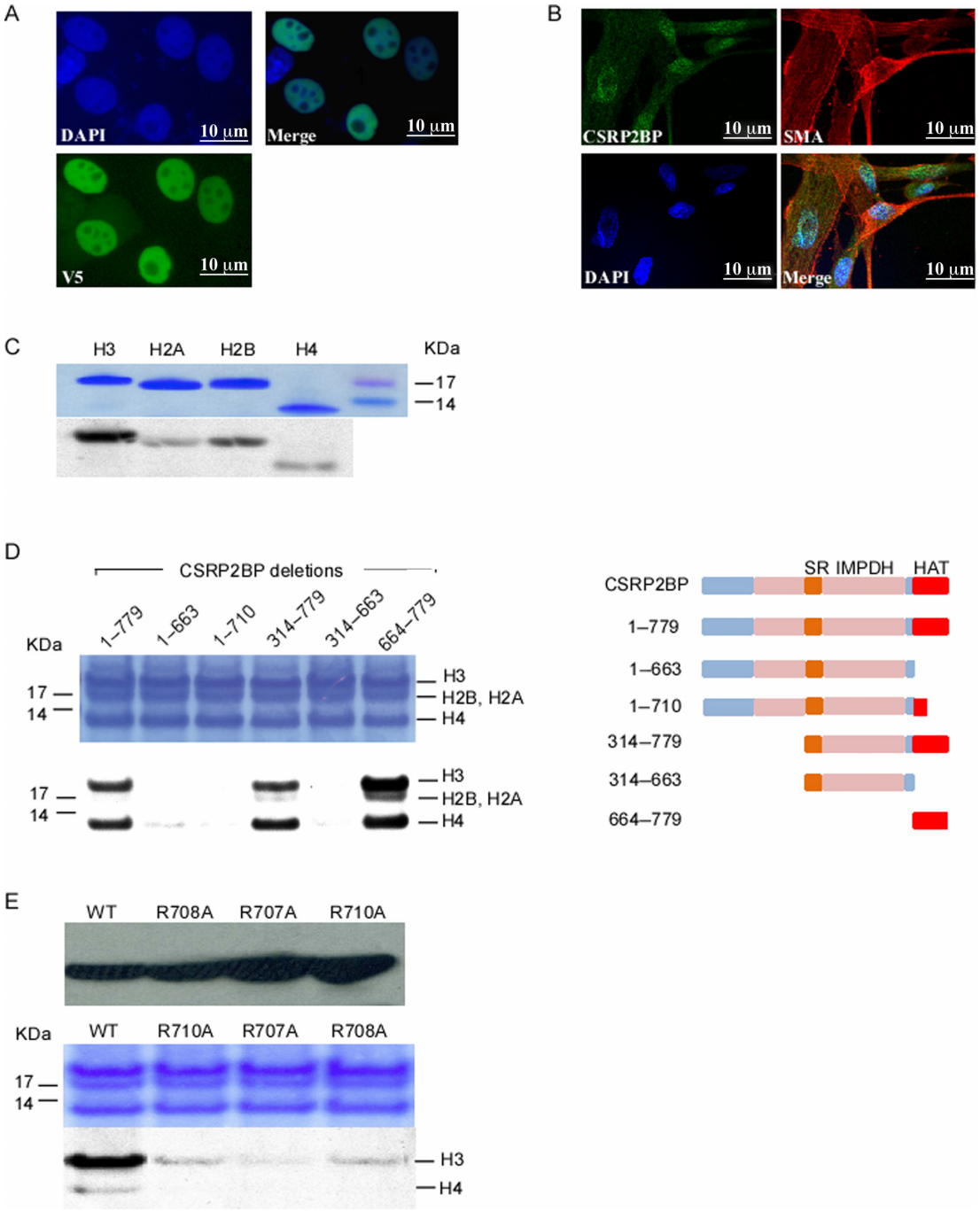

Figure 1. CSRP2BP was localized in the nucleus and acetylated histones H3 and H4. (**A**) Exogenous CSRP2BP was localized in nucleus of CV1 cells. The localization of V5-tagged CSRP2BP expressed in CV1 cells was revealed by fluorescent immuno-staining using monoclonal anti-V5 antibody (left, top). Nuclei were shown by DAPI staining (left, bottom). The merged picture (right) showed CSRP2BP was rich in the cell nuclei. (**B**) Endogenous CSRP2BP expression of human vascular smooth muscle cells (hVSMC) was enriched in nucleus. The immunofluorescence staining of anti-CSRP2BP/anti-SMA/DAPI on the primary cultured hVSMCs revealed that endogenous CSRP2BP was present in both nuclear and cytoplasm, but was more enriched in nucleus. (**C**) CSRP2BP strongly acetylated histone H3, secondarily H4 and H2B. To assessed the acetyltransferase activity of CSRP2BP, purified individual histones were incubated with cellular expressed HA-tagged full-length CSRP2BP in a reaction containing [3H] labeled–acetyl CoA. Preferential acetylation of H3 and weaker activity with H4 and H2B were observed, following PAGE and autoradiography on X ray film. The Coomassie stained gel showed the individual histones and molecular weight markers. (**D**) Deletions in the catalytic HAT domain blocked its acetyltrasferase activity. HA-tagged CSRP2BP deletions were expressed in Hela cells and immunoprecipitated with HA antibody. Core histones were incubated with recombinant CSRP2BP and ^{3}H acetyl-CoA and separated by electrophoresis. The top panel showed Coomassie stained core histones. The bottom panel showed X-ray film autoradiography of the radio-labeled histones. CSRP2BP deletion constructs were indicated above each lane. Full-length CSRP2BP acetylated H3 and secondarily H4. The CSRP2BP mutant protein lacking the C-terminal HAT domain (1-663, 1–710, 314–663) exhibited little to no acetyltransferase activity, whereas the two mutants that retained the C-terminal domain (314-779, 664–779) were sufficient for acetylating core histones *in vitro*. (**E**) Single amino acid mutations in the catalytic HAT domain blocked its acetyltrasferase activity. Western blot showed the expression of CSRP2BP point mutants G710A, R707A and R708A compared to wt CSRP2BP. Point mutations in the catalytic HAT domain blocked its acetyltransferase activity.

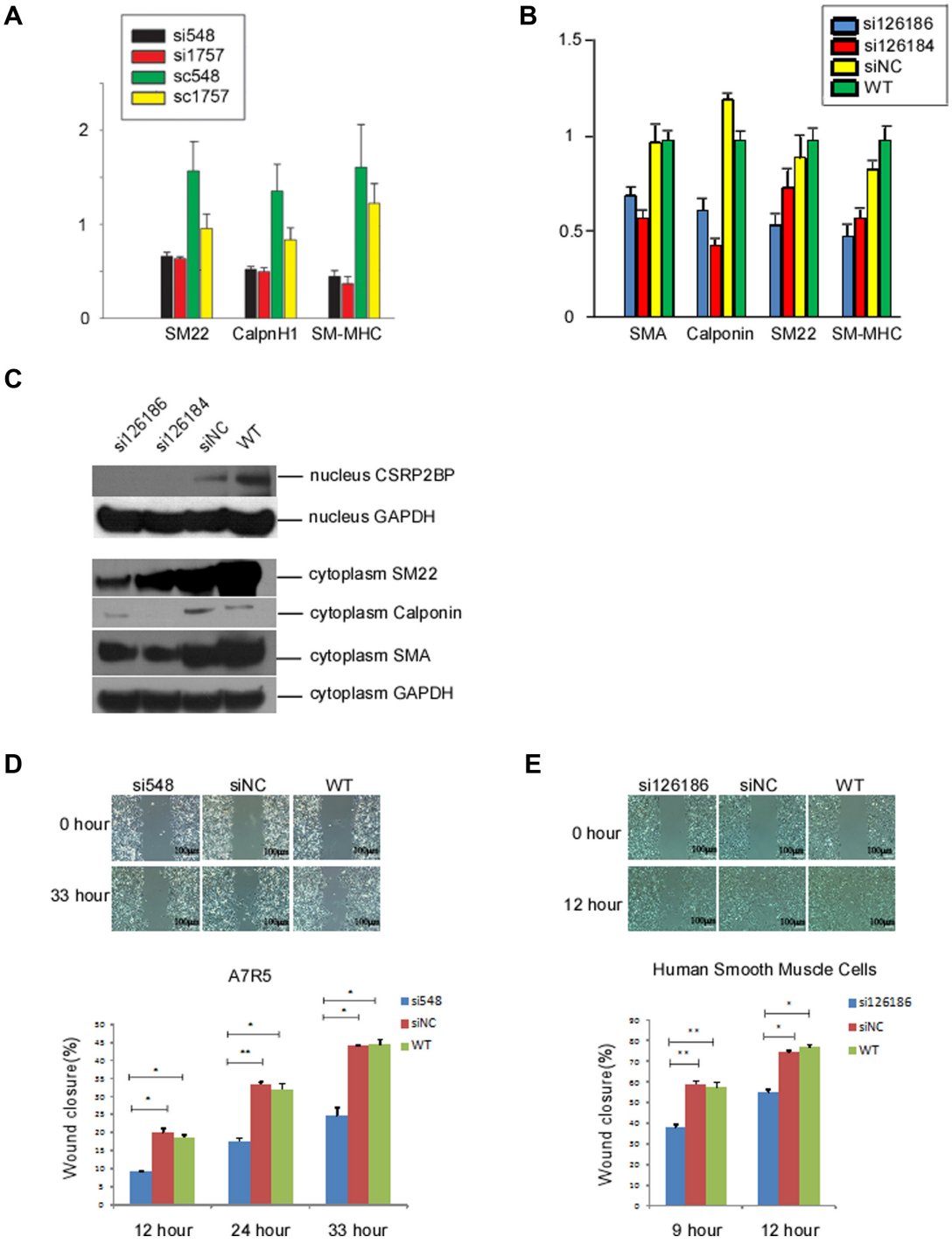

Figure 2. CSRP2BP stimulated smooth muscle gene expression. (**A**) siRNA knockdown of CSRP2BP down-regulated the rat smooth muscle genes activity. Two siRNAs targeting rat CSRP2BP (si548 and si1757) and two scramble controls (sc548 and sc1757) were tested in the rat vascular smooth muscle A7r5 cells. The mRNA levels of SM22α, calponin and SM-MHC were compared between knockdown and control groups by quantitative RT-PCR. Gene expression in untransfected cells was normalized to GAPDH and was taken as 1. $n = 3$. (**B**) siRNA knockdown of CSRP2BP down-regulated human smooth muscle genes activity. Two siRNAs targeting human CSRP2BP (si126186 and si126184) and a siRNA control (siNC) were tested in the human vascular smooth muscle cells. The mRNA levels of SMA, SM22α, calponin and SM-MHC were compared between knockdown and control groups by quantitative RT-PCR. Gene expression in untransfected cells was normalized to GAPDH and was taken as 1. $n = 3$. (**C**) The protein levels of SMA, SM22α and calponin were compared between CSRP2BP knockdown and control groups in the human vascular smooth muscle cells by Western blot. (**D**) Scratch-wound assays were performed in A7r5 cells. Representative phase contrast images were shown 33 h post scratching. Knockdown of CSRP2BP significantly decreased the rate of A7r5 cell migration into scratch-wounds compared to the control group (representative data at 12 h, 24 h and 33 h were shown). $n = 4$.*, $P < 0.05$, **, $P < 0.01$. (**E**) Scratch-wound assays were performed on the primary cultured hVSMCs. Representative phase contrast images were shown 12 h post scratching. Knockdown of CSRP2BP significantly decreased the rate of hVSMCs cell migration into scratch-wounds compared to the control group (representative data at 9 h and 12 h were shown). Knockdown of CSRP2BP significantly decreased the rate of cell migration into scratch-wounds compared to the control groups. $n = 4$, *, $P < 0.05$, **, $P < 0.01$.

the SMA promoter by SRF, GATA6 and CRP2. CSRP2BP strongly activated the SMA promoter luciferase reporter activity in a dose-dependent manner, eliciting ∼1200-fold activation at the highest CSRP2BP dosage (Figure 3A). Next, we tested the effect of CSRP2BP on SRF/GATA6/CRP2-mediated activation of other luciferase reporters under the control of smooth muscle gene promoter/enhancers. SRF alone, CSRP2BP alone or SRF + CSRP2BP had no effect on promoter activity. SRF + GATA6 + CRP2 stimulated a minor increase in activity for SMA-luc and SM22-luc. The combination of SRF + GATA6 + CRP2 + CSRP2BP resulted in strong upregulation of transcription from all the smooth muscle gene promoters tested, including SMA, SM22α, calponin and SMGA (Figure 3B).

We next tested whether SRF's DNA-binding activity or transactivation domain is required for SRF/GATA6/CRP2/CSRP2BP synergistic activation of the SMA promoter. Strong activation of SMA-luc was only observed when expression plasmids for all four factors were co-transfected. SMA-luc activity was strongly attenuated when a SRF DNA-binding domain mutant (SRFpm) or a C-terminal transactivation domain mutant (SRFΔC) (residues 1–266 aa) were substituted (Figure 3C). The SMA promoter contains two SRF binding sites (CArG boxes). Mutation of either SRF binding sites completely eliminated trans-activation by SRF/CRP2/GATA6/CSRP2BP (Figure 3D). These data support the idea that SRF plays a central role to direct assembly of a transactivation complex containing CSRP2BP on smooth muscle gene promoters.

CSRP2BP bound avidly with SRF, CRP2 but not GATA6

Our work thus far supports the idea that CSRP2BP is a coactivator for a complex of transcription factors, including SRF, CRP2 and GATA6. We next wished to determine which of these factors interact with CSRP2BP. We performed co-immunoprecipitation assays using extracts from Hela cells after expression of epitope-tagged proteins. V5-CSRP2BP co-immunoprecipitated with HA-SRF (Figure 4A). V5-Myocardin co-immunoprecipitated with HA-CSRP2BP (Figure 4B). V5-CSRP2BP co-immunoprecipitated with HA-CRP2 (Figure 4C). However, V5-CSRP2BP did not co-immunoprecipitate with myc-GATA6 (Figure 4D).

We next mapped the region (s) of CSRP2BP capable of interaction with CRP2 or SRF. GST-fused full-length SRF or CRP2 proteins were tested for interaction with [35S]methionine-labeled CSRP2BP protein fragments in GST pull-down assays. Full-length CSRP2BP associated with both SRF and CRP2. A CRP2 interaction domain was mapped to the region of 543–663 aa. This same region associated with SRF. SRF also associated to a lesser extent with the CSRP2BP C-terminal HAT domain (664–779 aa) (Figure 4E). Thus, the residues of CSRP2BP between amino acids 543 and 663, just prior to the HAT domain, served as the primary interaction domain for both SRF and CRP2.

We also mapped the region of CRP2 capable of interacting with CSRP2BP. CRP2 is a 193 amino acid protein composed of two tandem LIM domains. GST-fused full-length CSRP2BP proteins were tested for interaction with [^{35}S]methionine-labeled CRP2 protein fragments in GST pull-down assays. The results of these assays identified a CSRP2BP interaction domain within the N-terminal LIM domain of CRP2 (1–112 aa) (Figure 4F).

We next tested which region (s) of the CSRP2BP protein was able to coactivate for SRF, GATA6 and CRP2 using the SMA-luc reporter. Fragments lacking the HAT domain (1–710 aa, 1–663 aa, 314–663 aa and 1–313 aa) greatly attenuated transcription. Deletion of the SRF/CRP2 interaction domain (Δ545–663) also attenuated transcription but not quite to the extent of constructs lacking the HAT domain. The HAT domain alone (664–779 aa) did not coactivate transcription. Interestingly, one CSRP2BP mutant that lacks a substantial portion of the N-terminal region, but contained the interaction domain and the HAT domain (314–779 aa), showed robust coactivation comparable to that of wild-type CSRP2BP (Figure 4G).

Finally, we tested the effects of three different CSRP2BP HAT domain single amino acid substitution mutants (G710A, R707A and R708A) on coactivation of the SMA promoter by SRF, CRP2 and GATA6. Expression of wild-type CSRP2BP potently increased transcription compared to that of SRF/CRP2/GATA6 alone. Compared to SRF/CRP2/GATA6 alone, all three CSRP2BP HAT domain mutants modestly increased transcription, but were not capable of the strong coactivation seen for wild-type CSRP2BP (Figure 4H). Collectively, these data support the idea that CSRP2BP serves as a coactivator for the SRF/CRP2/GATA6 complex primarily through its histone acetylation activity.

Mutual coactivation and interaction shared between CSRP2BP and myocardin

We next tested whether CSRP2BP could cooperate with SRF and myocardin to drive transcription from smooth muscle gene promoters. Myocardin is a well-known coactivator for SRF. SRF alone or SRF plus myocardin did not promote transcription of the SMA-luc reporter; however, the combination of SRF, myocardin and CSRP2BP led to a strong induction of SMA promoter activity. Without myocardin, expression of SRF and CSRP2BP was not sufficient to drive SMA promoter activity. Likewise, in the absence of SRF, expression of myocardin and CSRP2BP was not sufficient to drive SMA promoter activity (Figure 5A). These results were similar for other smooth muscle gene promoters, including SM22, calponin and SMGA. Neither SRF alone nor SRF plus myocardin was able to drive expression of these promoters. Only the combination of SRF, myocardin and CSRP2BP led to robust promoter activity (Figure 5B).

We next investigated the role of SRF in assembling a complex with myocardin and CSRP2BP to drive smooth muscle gene transcription. In combination with myocardin and CSRP2BP, wild-type SRF produced strong activation of the SMA-luc reporter. An SRF mutant lacking the C-terminal transactivation domain (SRFΔC) was unable to drive transcription from the SMA-luc reporter even in the presence of myocardin and CSRP2BP. Modest promoter activity was observed when an SRF DNA-binding domain mutant (SRFpm) was substituted (Figure 5C).

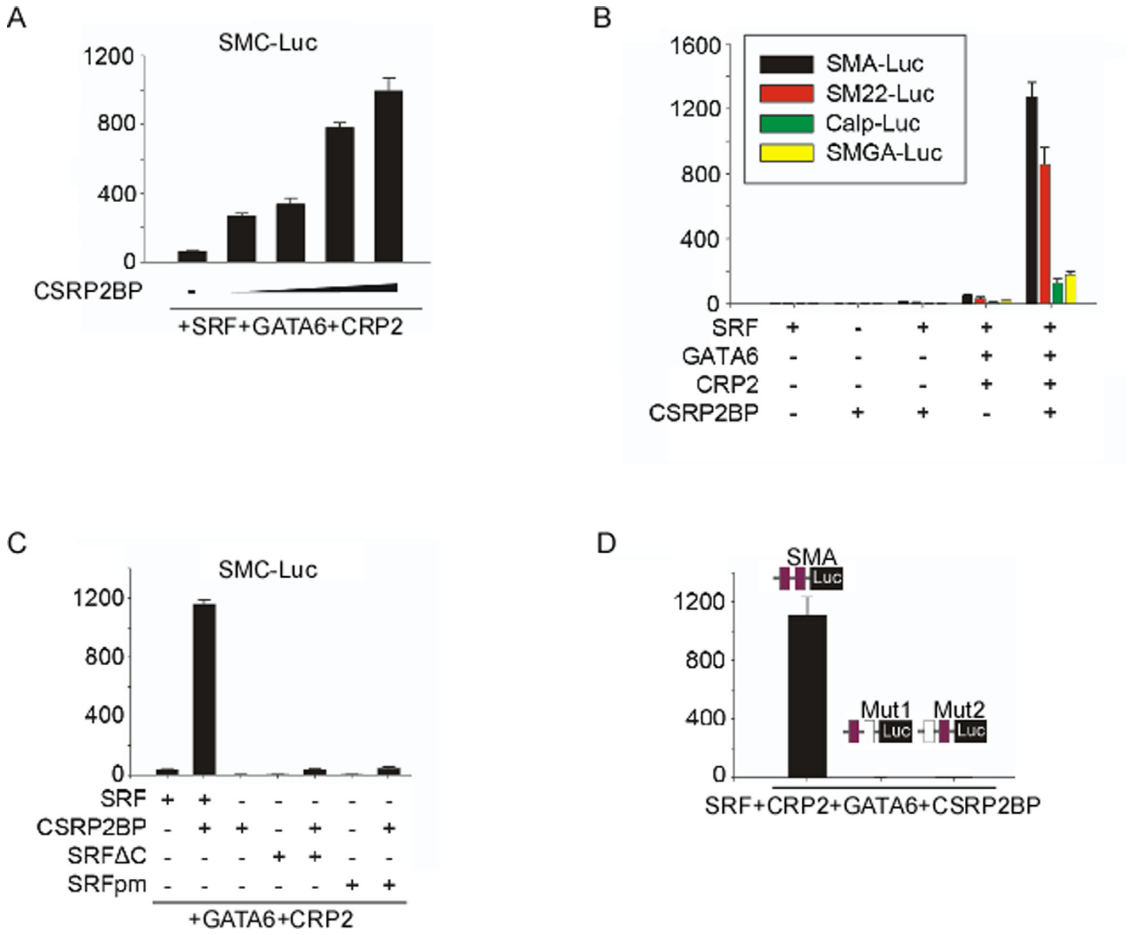

Figure 3. CSRP2BP and cofactors powerfully activated smooth muscle cell (SMC) marker gene reporters. (**A**) CV-1 cells were co-transfected with SMA-LUC with combinations of SRF, GATA6 and CRP2 expression vectors. $n = 3$. (**B**) CV-1 cells were co-transfected with luciferase reporters linked to either SMA, SM22α or calponin promoters with combinations of SRF, GATA6, CRP2 and CSRP2BP expression vectors as indicated in the lower panel. $n = 3$. (**C**) CV-1 cells were transfected with a SMA-LUC and combination of expression vectors as before with the addition of two SRF mutant proteins SRFΔC and SRFpm. $n = 3$. (**D**) CV-1 cells were transfected with SMA-LUC or the SMA-LUC promoter with one of the two CArG boxes mutated as indicated above the and the combinations of cofactor expression vectors. $n = 3$.

The SMA promoter contains two CArG boxes, which are SRF-binding elements. We next tested whether these sites are necessary for activation of the SMA-luc reporter by the combination of SRF, myocardin and CSRP2BP. As demonstrated earlier, expression of SRF, myocardin and CSRP2BP led to robust activation of the SMA-luc reporter. Mutation of either CArG box (mut1 or mut2) reduced reporter activity to basal levels (Figure 5D). Lack of promoter activity upon mutation of either CArG box or in the presence of SRFpm, strongly suggest that SRF DNA-binding is a prerequisite for assembly of a complex including CSRP2BP that drives smooth muscle gene expression.

We next tested if CSRP2BP directly interacts with myocardin. GST pull-down assays were performed with GST-myocardin (full length) and *in vitro* translated [35S]methionine-labeled deletion mutants of CSRP2BP. The results of these assays revealed two sites on CSRP2BP capable of interacting with myocardin. Similar to the sites capable of interaction with SRF, both the C-terminal HAT domain (664–779 aa) and an adjacent region (543–663 aa) associated with myocardin (Figure 5E).

In a complementary experiment, we mapped the region (s) of myocardin capable of interaction with full-length CSRP2BP. GST pull-down assays were performed with GST-CSRP2BP and *in vitro* translated [35S]methionine-labeled deletion mutants of myocardin. A single CSRP2BP interaction site was identified within the C-terminal transcription activation domain of myocardin (Figure 5F). We next examined the importance of functional interaction between CSRP2BP and myocardin in the reporter activity assays. Fragments lacking the HAT domain (1–710 aa) greatly attenuated the activation of the target reporter. Deletion of both the HAT domain of CSRP2BP and the interaction domain of myocardin (1–542 aa) dramatically attenuated the synergy. Also, deletion of the physical interaction domain between CSRP2BP and myocardin and between CSRP2BP and SRF (Δ545–663) significantly decreased the functional cooperation between them but not to the levels observed from deletion of the HAT domain, although the HAT domain alone (664–779 aa) did not activate the promoter tested. Interestingly, one CSRP2BP mutant that lacks a substantial portion of the N-terminal region, but

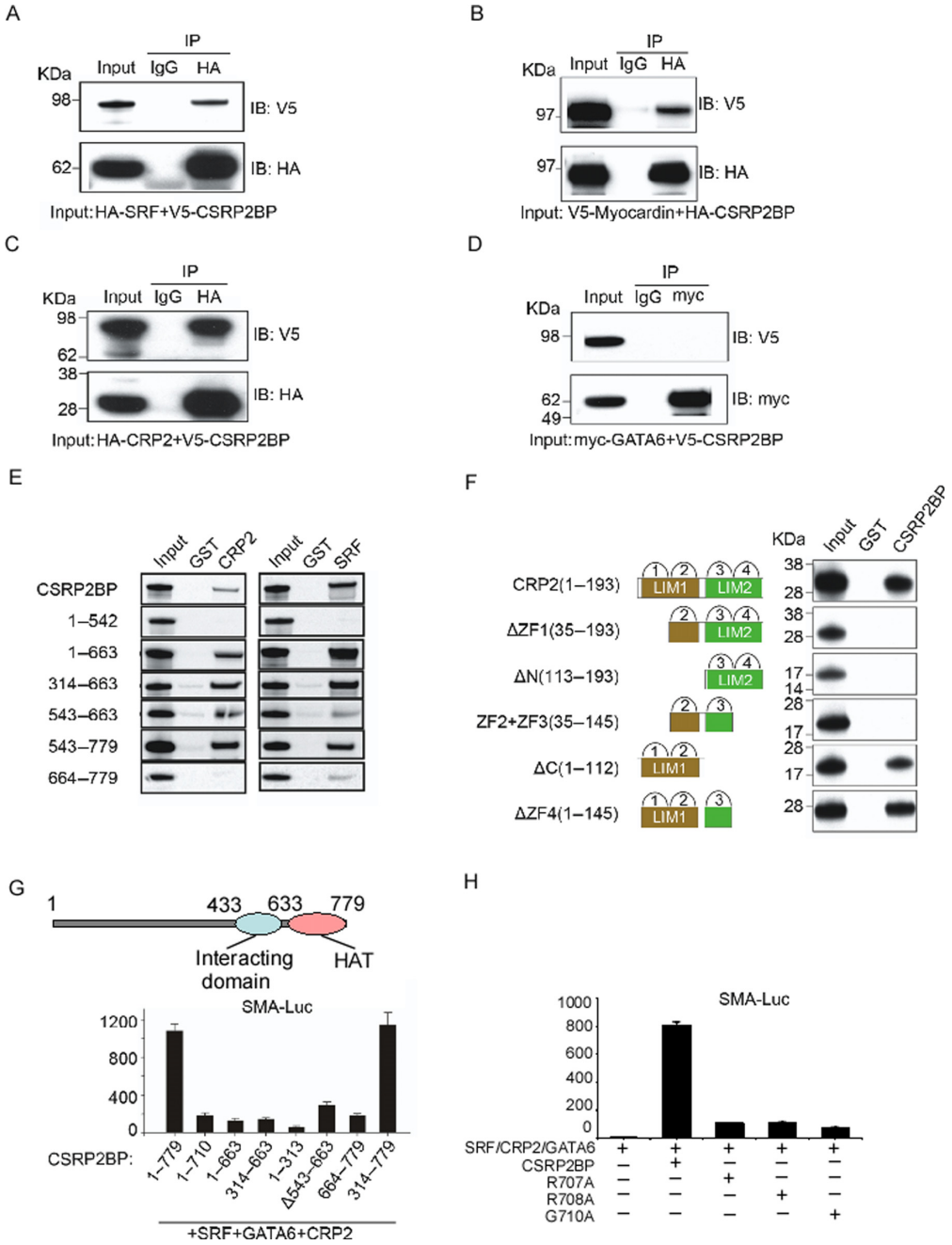

Figure 4. CSRP2BP bound avidly with SRF, CRP2 but not GATA6. (**A**, **C** and **D**) CSRP2BP co-immunoprecipitated with SRF and CRP2 but not with GATA6. The lysate from HeLa cells cotransfected with plasmid vectors expressing V5-tagged CSRP2BP and HA-tagged SRF, V5-tagged CSRP2BP and HA-tagged CRP2 or V5-tagged CSRP2BP and myc-tagged GATA6 were immunoprecipitated with anti-HA antibody or control normal IgG. Input lanes were loaded with the 5% of the lysate used for immunoprecipitation. Blots were stained with anti-V5 antibody, anti-HA antibody or anti-c-myc antibody as indicated. (**B**) Myocardin was coprecipitated with CSRP2BP. The interaction between CSRP2BP and myocardin was tested by coimmunoprecipitation assays using co-expressed HA-tagged CSRP2BP and V5-tagged myocardin. V5-myocardin was coprecipitated with HA-CSRP2BP. (**E**) Mapping of the interaction domains in CSRP2BP for association with CRP2 and SRF. Various [^{35}S]-labeled *in vitro* translated CSRP2BP deletion mutants, as indicated on the left panel, were incubated with either GST-CRP2, GST-SRF or control GST proteins purified from *E. coli* in the GST pull-down assays. The right panel shows the detection of CSRP2BP deletion mutants by autoradiography. (**F**) Mapping of interaction domains in CRP2 for CSRP2BP. *In vitro* translated [^{35}S labeled CRP2 deletion mutants, as indicated on the left panel, were incubated with either GST-CSRP2BP or control GST proteins purified from *E. coli* in the GST pull-down assays. The right panel showed detection of CRP2 deletion mutants by autoradiography. (**G**) Both HAT domain and the interaction domain were required for co-activation transcription. CV-1 cells were transfected with a luciferase reporter linked to the SMA promoter and SRF, GATA6 and CRP2 expression vectors. Plasmid vectors expressing CSRP2BP deletions were co-transfected with the SM promoter luciferase reporters. Disruption of the HAT domain (residues 1–710 aa, 1–663 aa, 314–663 aa and 1–313 aa) or deletion of the interaction domain (residues 664–779 aa and Δ543-663 aa) did not coactivate transcription. One CSRP2BP mutant, which lacked a substantial portion of the N-terminal region (residues 314–779 aa), but contained the interaction domain and the HAT domain, still showed robust activation comparable to the wild type CSRP2BP. $n = 3$. (**H**) Single amino acid mutations in the catalytic HAT domain blocked the co-activation transcription. CSRP2BP mutants (G710A, R707A and R708A) were co-transfected with the SM promoter luciferase reporters. Compared to the wild type CSRP2BP, all of the HAT mutants did not coactivate transcription. $n = 3$.

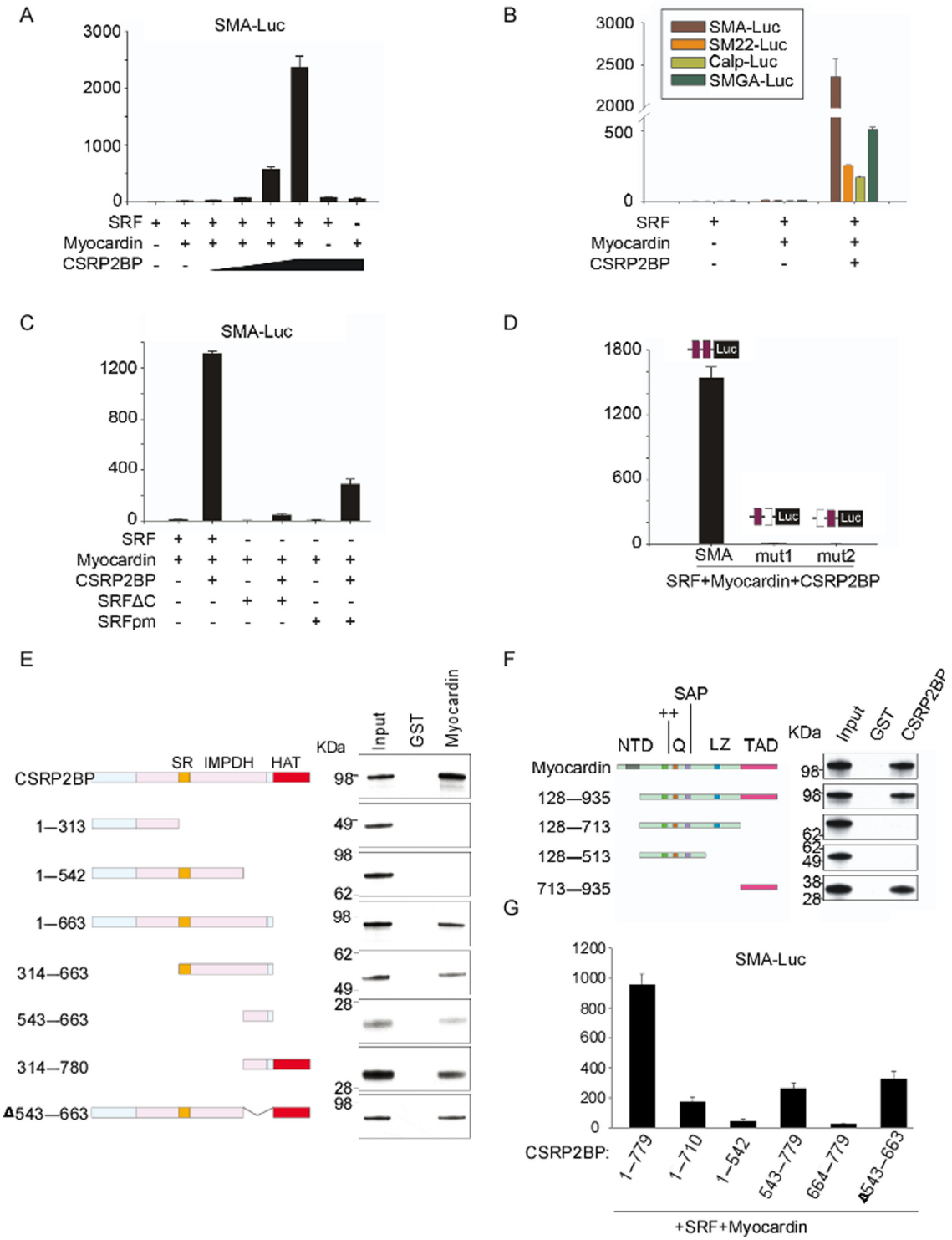

Figure 5. Mutual coactivation and interaction shared between CSRP2BP and myocardin. (**A**) CV-1 cells were co-transfected with a SMA-LUC and combinations of SRF and myocardin expression vectors. $n = 3$. (**B**) CV-1 cells were co-transfected with smooth muscle luciferase with combinations of expression vectors, as indicated under the chart. $n = 3$. (**C**) CV-1 cells were co-transfected with a luciferase reporter linked to the SMA promoter and expression vectors as before with the addition of two SRF mutant proteins SRFΔC and SRFpm. $n = 3$. (**D**) CV-1 cells were co-transfected with a SMA-LUC or the mutated CArG box SMA-LUC together with combinations of expression vectors. (**E**) Mapping the interaction domains of CSRP2BP for myocardin. [^{35}S]-labeled *in vitro* translated CSRP2BP deletion mutants were incubated with either GST-myocardin or control GST proteins purified from *E. coli* in the GST pull-down assays. Bound CSRP2BP deletion mutants were detected by autoradiography. $n = 3$. (**F**) Mapping the interaction domains of myocardin for CSRP2BP. [^{35}S]-labeled *in vitro* translated myocardin deletion mutants were incubated with either GST-CSRP2BP or control GST proteins purified from *E. coli* in the GST pull-down assays. Bound myocardin deletion mutants were detected by autoradiography. (**G**) Both HAT domain and the two interaction domains were required for the full activity of CSRP2BP. CV-1 cells were transfected with the SMA-Luc and expression vectors encoding SRF, myocardin and one of CSRP2BP deletion mutants as indicated. $n = 4$.

contained the interaction domain and the HAT domain (543–779 aa), only showed partial activity compared to the wild-type CSRP2BP (Figure 5G). Taken together, these findings suggest that both HAT domain and interaction domain of CSRP2BP are required for its full synergistic activity with SRF and myocardin.

CSRP2BP activated endogenous smooth muscle marker genes

Previous work from our laboratory showed that SRF, GATA6 and CRP2 coactivated smooth muscle marker gene expression in fibroblast 10T1/2 cells (14). Next, we tested whether co-transfection of CSRP2BP with these other factors further potentiated smooth muscle gene expression. mRNA levels of SMA, calponin, SM22α, SMGA and SM-MHC were elevated in 10T1/2 cells in the presence of co-transfected SRF, GATA4 and CRP2. Addition of a CSRP2BP expression plasmid further elevated expression of these endogenous smooth muscle genes, as shown by semi-quantitative RT-PCR assays (Figure 6A).

CSRP2BP/SM-HAT activated SMC gene expression by acetylating the chromatin histones in SMC gene promoters

Next, we explored whether CSRP2BP was recruited to the SM gene promoters by its DNA binding cofactors. We tested if CSRP2BP was recruited to smooth muscle gene promoters in A7r5 cells. HA-CSRP2BP was expressed by transient transfection. ChIP assays were performed using anti-SRF, anti-HA or control IgG antibodies. We found SRF and CSRP2BP to be associated with CArG elements in the promoter regions of the SMA, SM22, Calponin and SMGA genes (Figure 6B). Neither SRF or CSRP2BP were associated with DNA ∼1 kb away from the CArG boxes in the SMA and SM22α promoters, supporting the idea that SRF recruits CSRP2BP specifically to CArG boxes in smooth muscle gene promoters. Since CSRP2BP may act as a histone acetyltransferase, we next examined the histone acetylation on the regions surrounding SREs in the SMA promoter. ChIP assays with an anti-acetylated histone H4 antibody showed that overexpression of CSRP2BP in A7r5 cells strongly acetylated histones surrounding the SRE sites in the SMA promoter (Figure 6C), however, hyperacetylation of histones was not found in the 5′ flanking region ∼1 kb away from CArG elements. Therefore, we conclude that CSRP2BP only hyperacetylates the local histones surrounding the SREs.

DISCUSSION

Comparison with Drosophila CSRP2BP/ACTA2 data

Drosophila CSRP2BP was demonstrated to specifically acetylate histone H4 (29). We found that CSRP2BP acetylated both H3 and H4 (and perhaps H2A and H2B to a lesser extent). The difference between the activities of the two orthologs is likely due to specific amino acid sequence variations in the HAT domain. Furthermore, it is likely that other regions of the protein participate in histone binding and may affect substrate specificity.

CSRP2BP is an evolutionarily conserved HAT

CSRP2BP proteins were compared from a variety of vertebrate species (Supplementary Figure S1). There were over 88% positions with consensus sequences in all species. The carboxyl terminal region aligned to mouse CSRP2BP protein amino acids 668–779 has particularly high homology (over 98% consensus positions, 55% identity). *CSRP2BP* is a single copy gene in all the species examined. When genomic organization of *CSRP2BP* in different species was compared, the intron/exon organization was also highly conserved.

When the carboxyl terminal region CSRP2BP was subjected to Prosite scan, it was found to have great similarity to GNAT-type HAT domain. Other than the HAT domain, the protein pattern scan also revealed a conserved domain of about 450 amino acids that shared homology to the IMP dehydrogenase (IMPDH)/GMP reductase domain, which is also found in several other HAT-containing proteins, and a serine-rich region (Supplementary Figure S1). CSRP2BP does not contain a chromodomain and bromodomain or zinc finger domain, all of which are commonly found in GNAT and MYST family HATs (21,33). A hallmark of the HAT domain is the acetyl-CoA binding motif, which was compared between CSRP2BP and other HAT proteins (34). There is extensive homology between the CSRP2BP acetyl-CoA binding motif and 'motif A' in the HAT family, which forms CoA binding pocket according to the known crystal structure of the yeast HAT Esa1 (Supplementary Figure S2). In the CSRP2BP HAT domain, amino acid E668 corresponds to amino acid E157 in yGCN5, a key residue crucial for the catalytic activity.

CSRP2BP is a coactivator for smooth muscle transcription factors

CSRP2BP, first identified as a binding partner of CRP2, is an important regulator of smooth muscle gene expression (14,35). In combination with SRF, CRP2 and GATA6, CSRP2BP can synergistically activate SMC target gene promoters laden with multiple CArG boxes. This activation is SRF-dependent. In combination with SRF and myocardin, CSRP2BP can synergistically activate SMC target gene promoters laden with multiple CArG boxes. Knock-down of CSRP2BP in smooth muscle cells resulted in reduced levels of smooth muscle gene expression. *In vitro* pull-down assays mapped the interaction domains between CSRP2BP, SRF, CRP2 and myocardin. Therefore, CSRP2BP may be recruited to transcription factor complexes by SRF and one of its co-factors, either CRP2 or myocardin. Previously, the combination of SRF-CRP2-GATA6 increased the activity of the SMA reporter gene by over 1000-fold (19). In the present study, we chose to use suboptimal dosage of these transcription factors to reduce overall reporter activity, which allowed us to test CSRP2BP as a transactivator. This is a standard practice for identification of important co-factors (36,37).

Both structural prediction and experimental data suggest that the CSRP2BP is a HAT. Previously studied HATs are classified into two families: GNAT and MYST, depending on their structural similarity (38,39). Both families have a similar core acetyl-CoA binding motif. The HAT domain

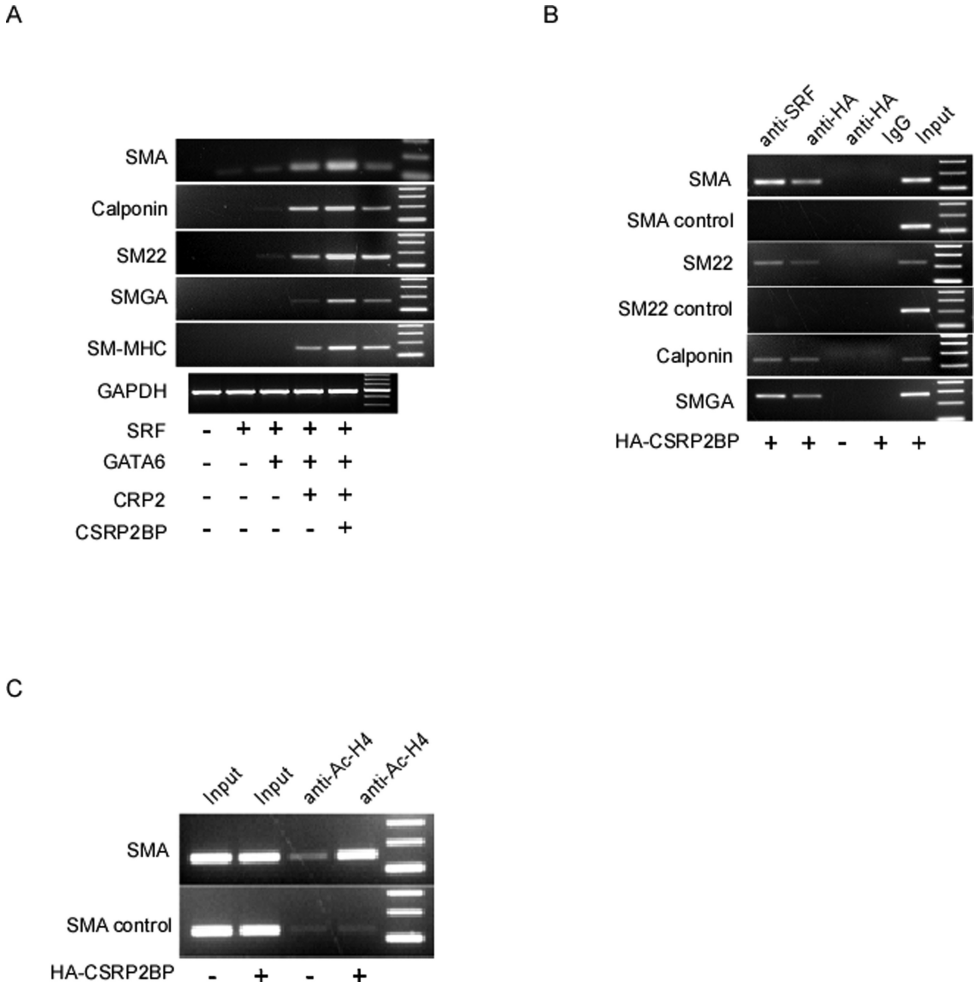

Figure 6. CSRP2BP activated endogenous smooth muscle marker genes. (**A**) Expression of the SRF/CRP2/GATA/CSRP2BP complex in fibroblasts strongly elevated smooth muscle genes transcription. Combinations of SRF, CRP2, GATA6 and CSRP2BP expression vectors were co-transfected into mouse 10T1/2 fibroblasts as indicated. Smooth muscle gene activity was assayed by semi-quantitative RT-PCR. Heart cDNA served as a positive control. GAPDH was used as a loading control. (**B**) CSRP2BP bound to smooth muscle gene promoters. HA-tagged CSRP2BP expression vector was transfected into A7r5 cells followed by chromatin immunoprecipitation (ChIP) assays with anti-HA antibody and control normal IgG. Following the removal of formaldehyde cross-links DNA was evaluated with PCR primers that overlapped CArG boxes of the smooth muscle gene promoters or control primers of sequences ∼1 kb upstream of the promoters. The results show that the CSRP2BP was recruited to smooth muscle gene promoters. SRF antibody was used as an additional control to show occupation of CArG boxes. (**C**) Overexpression of CSRP2BP hyperacetylatedthe histones in A7r5 cells. HA-CSRP2BP expression vectors were transiently transfected into A7r5 cells and the ChIP assays were performed with antibody against acetylated histone H4. The semi-quantitative PCR shows the relative level of SRE regions in the ChIP pull down with or without CSRP2BP overexpression.

in CSRP2BP contains a well-conserved acetyl-CoA binding motif. The lack of a chromodomain, bromodomain or zinc finger domain, which is commonly found in GNAT and MYST family members, supports the idea that CSRP2BP is a coactivator.

Smooth muscle genes are controlled by SRE sites in their promoter regions (14,40). SRF binds SREs and subsequently recruits coregulators to the promoters. LIM domain factor CRP2 and SAP domain factor myocardin both bind SRF and both SRF/GATA6/CRP2 and SRF/myocardin complexes activate smooth muscle gene expression (15,16,18). However, neither SRF nor any of its known promoters has exactly the same expression pattern as the SM gene expression pattern, suggesting there is no master regulator for SM genes activity. The current model for SM differentiation hypothesizes that the combinatorial existence of SRF and several of its cofactors in SM cells activates the SM genes.

CSRP2BP greatly promotes SM gene expression by SRF complex. CSR2BP is a coactivator and thus does not itself bind DNA. It must be recruited to chromatin via protein–protein interactions with transcription factors (like SRF) or other coregulators (like CRP2 and myocardin). In agreement, CSRP2BP forms complexes with SRF, CRP2 and myocardin. When CSRP2BP was tethered to DNA by fusion to a heterologous DNA-binding domain, it did not exhibit strong transcriptional activation; however, it synergistically increased SM promoter activity when in present in SRF complexes. CSRP2BP likely positively regulates SM gene transcription via its histone acetyltransferase activity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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