Requirement for Multiple Domains of the Protein Arginine Methyltransferase CARM1 in Its Transcriptional Coactivator Function*

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The p160 coactivator complex plays a critical role in transcriptional activation by nuclear receptors and possibly other classes of DNA-binding transcriptional activators. The complex contains at least one of three p160 coactivators (SRC-1, GRIP1/TIF2, or pCIP/RAC3/ACTR/AIB1/TRAM1), a histone acetyltransferase such as CBP or p300, and the histone methyltransferase CARM1 (coactivator-associated arginine methyltransferase 1). Methylation of histone $H_3$ and possibly other proteins in the transcription initiation complex by CARM1 occurs along with acetylation of histones and other proteins by CBP and p300 to help remodel chromatin structure and recruit RNA polymerase II. Here we show that other domains of CARM1 are required for the coactivator function of CARM1 in addition to the methyltransferase activity. The methyltransferase GRIP1, binding, and homooligomerization activities all reside in the central region of CARM1, which is highly conserved among the entire protein arginine methyltransferase family. In addition to this conserved domain, the unique N- and C-terminal regions of CARM1 were also required for enhancement of transcriptional activation by nuclear receptors. While the N-terminal region has no known activity at present, the C-terminal part of CARM1 contains an autonomous activation domain, suggesting that it interacts with other proteins that help to mediate CARM1 coactivator function.

Activation of transcription by DNA-binding transcriptional activator proteins is mediated by coactivators, which locally remodel chromatin structure and recruit RNA polymerase II and its transcription initiation complex to the promoter. Members of the nuclear receptor (NR) family of transcriptional activator proteins, which include the receptors for steroid and thyroid hormones, retinoids, and vitamin D, as well as so-called orphan receptors (1–3), recruit several different complexes of coactivator proteins to their target gene promoters (4–8).

One coactivator complex, which plays a central role in mediating transcriptional activation includes at least one of the three related 160-kDa proteins commonly referred to as p160 coactivators (SRC-1, GRIP1/TIF2, and pCIP/RAC3/ACTR/AIB1/TRAM1). The p160 coactivators bind directly and in a ligand-dependent manner to the C-terminal AF2 activation domains of NRs through three LXXLL motifs (where L is a leucine and X, any amino acid) located in the central part of the p160 polypeptide chain. The C-terminal region of the p160 coactivators can also interact with the N-terminal AF1 activation domains of some NRs (9–11). The p160 coactivators contribute to transcriptional activation by bringing other associated coactivator proteins with them to the promoter. The p160 coactivator complex includes either of the two related proteins p300 and CBP, which bind to the AD1 activation domain of p160 coactivators (12–14) and function as coactivators for many DNA-binding transcriptional activators, including NRs (15). Recent studies have confirmed hormone-dependent recruitment of p160 coactivators, CBP, and p300 to promoters activated by NRs (16–19). CBP and p300 contribute to chromatin remodeling by acetylating histones, and also acetylate other components of the transcription initiation complex (16, 20–22). CBP and p300 can also bind directly to basal transcription factors and may thereby help to assemble the transcription initiation complex (12). Thus multiple domains of CBP and p300 apparently contribute to chromatin remodeling and recruitment/activation of RNA polymerase II.

The activation domain AD2, located at the C terminus of p160 factors, binds CARM1, which belongs to a family of previously identified arginine-specific protein methyltransferases (PRMTs) (23). CARM1 enhances nuclear receptor function in a p160-dependent manner in transient transfection assays. CARM1, p300/CBP, and a p160 coactivator can also form a ternary complex which functions synergistically to enhance NR function and requires the methyltransferase activity of CARM1 to do so (24, 25). CARM1 methylates histone H3 at Arg-17 and Arg-26 in vitro (26), and chromatin immunoprecipitation studies indicate that CARM1 is specifically recruited to steroid hormone-regulated promoters in vivo in response to the hormone and methylates histone H3 as part of the transcription initiation process (27, 28).

PRMTs are homodimeric or homo-oligomeric proteins...
and clarified by centrifugation before incubation overnight at 4 °C with monoclonal antibody (clone 3F10, Boehringer Mannheim) against the HA tag. Preblocked protein G-Sepharose (Amersham Biosciences) was then added for 2 h at 4 °C. Immunoprecipitates were recovered by rapid centrifugation, washed three times with NETN containing 0.1% Nonidet P-40 and resuspended in HMT Buffer (20 mm Tris-HCl, pH 8.0, 200 mm NaCl, 0.4 mm EDTA). In vitro histone methyltransferring assays—performed as follows: 3 μg of histone H3 (Boehringer Mannheim Biochemicals) was incubated with immunoprecipitated methyltransferases and 7 μM S-adenosyl-l-[methyl-3H]methionine (specific activity 14.7 Ci/mmol) in 30 μl of HMT buffer for 1 h at 30 °C. Reactions were stopped by addition of SDS loading buffer and analyzed by 15% SDS-PAGE and fluorography.


domains of the immunoprecipitated methyltransferases from the transfected cells (see above) were analyzed by SDS-PAGE on 12% gels. Immunoblotting was performed as described previously (38) with rat monoclonal antibody 3F10 against the HA epitope at 100 ng/ml as the primary antibody and horseradish peroxidase-conjugated anti-rat immunoglobulin G (Santa Cruz Biotechnology) at 180 ng/ml (1:2,500 dilution) as the secondary antibody.

## RESULTS

### Domains of CARM1 Involved in Coactivator Activity

To determine which parts of CARM1 are required for its coactivator function, N- and C-terminal truncations were made near the boundaries between the conserved central domain of CARM1 and its unique N-terminal (amino acids 1–150) and C-terminal (amino acids 480–608) regions (Fig. 1A). The coactivator activity of CARM1 and its mutants were tested with ER by transient transfection in CV-1 cells under two different conditions: with relatively high levels of transfected ER expression vector, where CARM1 cooperates with a p160 coactivator (23); and at very low levels of transfected ER expression vector, where CARM1 functions synergistically with a p160 coactivator and p300, such that all three of these coactivators must be co-expressed with ER to achieve efficient activation of an estrogen-dependent reporter gene (25). In both cases, the activity observed was shown previously to be completely dependent on the exposure of the cells to estradiol to activate ER.

At the higher level of ER vector (5 ng), GRIP1 expression enhanced reporter gene activation by ligand-bound ER, and co-expression of full-length CARM1 resulted in a further enhancement approximately in proportion to the amount of CARM1 expression vector used (Fig. 1B). However, mutants lacking the N-terminal part of CARM1 (mutant 121–608) or the C-terminal part (mutants 3–460, 530–580) had no effect on the reporter gene expression mediated by ER and GRIP1, suggesting that both ends of CARM1 contribute to its coactivator activity.

With a low concentration of ER expression vector (0.1 ng), p300 had no effect on the transcriptional activity observed with ER and GRIP1, and CARM1 (in the absence of p300) caused an enhancement of only 2-fold (Fig. 1C). However as previously described (25), expression of both CARM1 and p300 with GRIP1 resulted in a dramatic increase of ER-dependent reporter gene activity. Deletions of the unique N- or C-terminal part of CARM1 severely impaired the synergistic effect.

Thus the unique N- and C-terminal regions of CARM1 are required for coactivator activity with ER under both tested conditions. The expression level of each mutant was similar to intact in the various mutants. This line of experimentation also allowed us to assign specific functions of CARM1 to specific domains of the protein and thereby to explore the mechanisms...
by which the unique N- and C-terminal regions and the conserved central region of CARM1 contribute to the coactivator activity.

The GRIP1 Binding Domain of CARM1 Is Located in the Conserved Central Region—The lack of coactivator activity of the CARM1 mutants could be due to their inability to bind GRIP1. Binding between GRIP1 and CARM1 is required for CARM1 coactivator function (24), and the GRIP1 binding domain of CARM1 has not been mapped. Full-length CARM1 and its deletion mutants were translated in vitro in the presence of \[^{35}\text{S}\]methionine and incubated with either GST or GST-GRIP1 AD2 fusion proteins preloaded on glutathione-coupled beads. Bound proteins were eluted and analyzed by SDS-PAGE and fluorography. The input lanes represent 10% of each \[^{35}\text{S}\]labeled protein used in the binding assay. B, CV1 cells were transiently transfected with 250 ng of GK1 reporter plasmid, 125 ng of a pM vector encoding Gal4DBD (left lane) or Gal4DBD-GRIP1 fusion protein (all other lanes), and varying concentrations (100, 200, and 400 ng) of vectors encoding full-length CARM1 or deletion mutants as indicated. The luciferase activities shown are from a single experiment which is representative of three independent experiments.

The Unique CARM1 Ends Are Not Required for the Methyltransferase Activity—Full-length CARM1 and its deletion mutants were expressed in COS7 cells by transient transfection, isolated by immunoprecipitation, and incubated with histone H3 in the presence of \[^{3}\text{H}\]AdoMet; methylated histone H3 was detected by SDS-PAGE and fluorography (Fig. 3). The mutant lacking the N-terminal part of CARM1 (amino acids 1–120) or the C-terminal region (amino acids 501–608) still methylated histone H3 efficiently, showing that these unique domains were not required for the enzymatic activity of CARM1, at least in vitro. The lower activity of CARM1-(121–460) AD2 fusion protein (consisting of the GRIP1 C-terminal amino acids 1122–1462), which were preloaded on glutathione-Sepharose beads. None of the CARM1 proteins bound to GST, but full-length CARM1 and several CARM1 fragments bound GST-GRIP1 AD2 (Fig. 2A). Deletion of the N terminus (amino acids 1–120) or the C terminus (amino acids 501–608 or 581–608) of CARM1 did not impair binding to GRIP1 AD2, showing that these domains are not required for the \textit{in vitro} interaction. The CARM1 mutant lacking amino acids 461–608 was still retained by GST-GRIP1 AD2 but to a lesser extent. However, the CARM1 C-terminal region alone was not able to bind GRIP1 AD2. A mammalian two-hybrid assay confirmed these results (Fig. 2B). These results localized the GRIP1 binding domain of CARM1 within the central conserved domain (amino acids 121–460) and demonstrated that the unique N- and C-terminal regions of CARM1 are neither necessary nor sufficient for the interaction with GRIP1.
608) was due to its lower expression level (Fig. 1A). However, the mutant lacking amino acids 461–608, which includes a small portion of the central conserved domain, was inactive. The unique C-terminal region (amino acids 461–608) by itself did not exhibit any enzymatic activity. Thus, the methyltransferase activity resides within amino acids 121–500.

The Conserved Central Part of CARM1 Also Contains Its Homo-oligomerization Domain—To localize the homo-oligomerization domain, we tested the ability of the CARM1 deletion mutants to interact with wild type CARM1. All mutants lacking N- and C-terminal amino acids were retained by the bead-bound GST-CARM1 fusion protein, although CARM1-(3–460) bound very weakly compared with wild type CARM1 (Fig. 4A); thus the unique ends of CARM1 are not required for its homo-oligomerization in vitro. Furthermore, by itself the C-terminal fragment (amino acids 461–608) did not bind to GST-CARM1. Mammalian two-hybrid assays produced very similar results in vivo (Fig. 4B). We, therefore, located the CARM1 homo-oligomerization domain in the conserved central part of the protein along with the methyltransferase and GRIP1 binding activities.

The Unique C-terminal Region of CARM1 Is a Transcriptional Activation Domain—Since the unique N- and C-terminal regions of CARM1 were important for its coactivator function but played no role in its methyltransferase, GRIP1-binding, or homo-oligomerization activities, we tested whether these unique regions might contain an autonomous activation function. CARM1 mutants fused to the Gal4 DBD were tested for their ability to activate expression of a reporter gene controlled by Gal4 response elements (Fig. 5). Fusion of full-length CARM1 to the Gal4 DBD enhanced the reporter gene expression, indicating the presence of an autonomous transactivation domain somewhere in the CARM1 protein. A mutant deficient in methyltransferase activity (C1 VLD) (23) fused to Gal4 DBD also increased the transcription driven by Gal4 response elements. This suggests that the methyltransferase activity of CARM1 is not necessary for the observed transcriptional activation activity. A mutant constituted only by the N-terminal part of CARM1 (amino acids 3–126) had little or no ability to increase the reporter gene expression. The mutant 121–608 was almost as effective as full-length CARM1, thus showing that the N-terminal part was not involved in the activity. Mutants lacking the C-terminal part of CARM1 (mutants 3–500; 3–580) were also active but to a lesser extent than full-length CARM1. Moreover, deletion of residues 461–608 totally abolished the autonomous transactivation activity of CARM1. Finally, the CARM1 fragments 461–608 and 501–608 exhibited activity ten times that of wild type CARM1, indicating that CARM1 contains a strong autonomous activation domain in its unique C-terminal region (Fig. 5, right panel).

The C-terminal activation domain of CARM1 may contribute to coactivator function through protein-protein interactions with some important component of the transcription machinery. If so, overexpression of the isolated C-terminal domain might inhibit the coactivator function of full-length CARM1 by competing with CARM1 for the interaction with this transcription machinery component. CARM1-C (amino acids 461–608) strongly inhibited the coactivator effect of CARM1 on the hormone-dependent, ER-mediated activation of reporter gene expression (Fig. 6A, upper panel). CARM1-C had little or no effect on the basal ER activity observed in the absence of hormone. The specificity of the inhibitory effect was also demonstrated by the fact that CARM1-C had no effect on the expression of a RSV promoter-driven β-galactosidase reporter gene, which was tested in a parallel experiment (lower panel). CARM1-C also inhibited the autonomous transactivation activity of full-length CARM1 fused to Gal4 DBD (Fig. 6B, left panel). In contrast full-length CARM1, used as a positive control, enhanced the activity of the Gal4-CARM1 fusion protein, presumably through homo-oligomerization. In a mammalian two-hybrid assay, the interaction of Gal4-CARM1 with VP16-CARM1 was only slightly inhibited by CARM1-C (Fig. 6B, right panel), consistent with our previous finding that CARM1-C cannot bind to full-length CARM1 (Fig. 4). Thus, the negative effect of CARM1-C on the autonomous activation activity of CARM1 is not caused by a disruption of the homo-oligomer but could rather be because of a competition of CARM1-C with CARM1 for the interaction with another transcription factor that binds CARM1-C.
DISCUSSION

Multiple Functions of the Conserved Central Region of CARM1—CARM1 belongs to the PRMT family of arginine-specific protein methyltransferases, which share a conserved core region of about 330 amino acids that contains the methyltransferase activity. X-ray crystallography of mammalian PRMT3 and yeast Rmt1/Hmt1 demonstrated that the conserved region forms two separate structural domains that combine to form the active enzyme (29, 39) (Fig. 7). The N-terminal part, which is the most highly conserved in primary amino acid sequence among family members, is composed of mixed $\alpha$-helices and $\beta$-strands. The C-terminal part of the core forms an elongated 9-stranded $\beta$-barrel structure. Homodimerization is apparently required to form an active enzyme. The dimer interface is formed by reciprocal contact between the $\alpha$-helical region of one monomer and a tri-helical arm extending from the surface of the $\beta$-barrel structure of the other monomer. The AdoMet binding pocket is formed by the $\alpha$-$\beta$ region. The arginine residue of the protein substrate binds in an acidic pocket containing two glutamate residues, which interact directly with the two terminal amino groups of the arginine side chain. The portions of the protein substrate surrounding the target arginine residue are predicted to fit in a groove between the $\alpha$-$\beta$ region and the $\beta$-barrel structure (29, 39).
As expected from the three-dimensional structure and the high degree of conservation among PRMT members, our studies located the methyltransferase and homo-oligomerization activities of CARM1 approximately within the conserved region (amino acids 150–480 of CARM1) (Figs. 3, 4, and 7). A C-terminal deletion to amino acid 460, which removed the last β-strand of the β-barrel structure, eliminated the methyltransferase activity and thus demonstrated that the entire conserved barrel structure is required for methyltransferase activity. The C-terminal β-strand may contribute to structural integrity of the entire domain or could help to form the protein substrate-binding groove. The same deletion mutant retained partial-to-full homo-oligomerization and GRIP1 binding activity (Figs. 2 and 4), indicating that structural integrity was not completely disrupted. Our finding that the central conserved region of CARM1 also contained the GRIP1 binding activity (Figs. 2 and 7) is consistent with previous findings that multiple members of the PRMT family can bind to the C-terminal region of GRIP1 (25, 36). The GRIP1 binding activity of CARM1 is undoubtedly required for the coactivator function of CARM1, since we previously showed that the presence of GRIP1 and its C-terminal CARM1-binding region are required for the coactivator function of CARM1 (23, 24).

While CARM1 shares homology with the PRMT family throughout the methyltransferase domain, CARM1 has a unique set of protein substrates, including histone H3 and p300/CPB (23, 40). In addition, CARM1 is the only PRMT member tested to date which can cooperate synergistically with p300, CBP, or p/CAF to enhance transcriptional activity by NRs (25). Previous studies have shown that the coactivator function of CARM1 depends on its methyltransferase activity (23, 25). Moreover, chromatin immunoprecipitation assays demonstrated that steroid hormones stimulate recruitment of CARM1 and methylation of histone H3 in a CARM1-specific manner at promoters of stably integrated, steroid hormone-responsive genes (27, 28). Thus, the unique transcriptional coactivator function of CARM1 is at least partly due to its unique methyltransferase substrate specificity.

Role of the Unique N- and C-terminal Regions of CARM1 in Its Coactivator Function—Deletion of the unique N- or C-terminal part of CARM1 totally abolished its coactivator function (Fig. 1), but had no effect on its ability to bind GRIP1 (Fig. 2), methylate histone H3 (Fig. 3), or form homo-oligomers (Fig. 4). Thus the unique N- and C-terminal regions must contribute to the coactivator function of CARM1 through a novel mechanism not involving any of these three activities. To date little is known about the functions of the unique N termini of PRMT family members; these unique N termini vary greatly in length as well as sequence (29). It has been proposed that the relatively short N terminus of mammalian PRMT1 and yeast Rmt1/Hmt1 may contribute to methyltransferase substrate specificity by interacting with regions of the substrate protein distinct from the sequence immediately surrounding the target arginine residue (29, 30). In addition, deletion of the relatively long unique N terminus of PRMT3 altered its substrate specificity in vitro (33). However, mutants of CARM1 lacking the unique N- or C-terminal regions appeared to be unaffected in their ability to methylate histone H3 (Fig. 3) and several other protein substrates that we tested.2 We also found that the N terminus of CARM1 did not contribute to the autonomous transcriptional activation activity of CARM1 (Fig. 5). Thus the mechanism by which the N-terminal region of CARM1 contributes to coactivator function remains unclear.

While the unique C-terminal part of CARM1 was not required for GRIP1 binding, methyltransferase, or homo-oligomerization activities, it contains a strong autonomous activation domain (Fig. 5), which presumably explains why this domain is necessary for the coactivator function of CARM1. CARM1 mutants lacking this domain were almost devoid of a transcriptional activation activity when fused to Gal4 DBD, indicating that the C terminus is responsible for most or all of the autonomous transactivation activity observed in full-length CARM1. The ineffective autonomous transcriptional activation activity associated with the mutants lacking the C-terminal region indicates that the methyltransferase activity per se is not sufficient for transcriptional activation (Fig. 5). PRMT1, which lacks a unique C-terminal domain and has a very short unique N-terminal domain, also exhibits no autonomous activation activity when fused to Gal4 DBD (36). Thus, although CARM1 methylates histone H3 and PRMT1 methylates histone H4, the simple recruitment of a histone methyltransferase activity to the promoter of a transient reporter gene is not sufficient to activate transcription. This is also consistent with our findings that the unique terminal domains of CARM1 are required in addition to the methyltransferase activity for the coactivator function of CARM1.

As a model for CARM1 function, we propose that CARM1 is recruited to the promoter through its interaction with the C-terminal domain of a p160 coactivator. The autonomous activation activity of the CARM1 C-terminal domain collaborates with the methyltransferase activity of the central domain and possibly an unknown activity in the unique N-terminal domain, to mediate the coactivator function of CARM1. The methyltransferase activity is responsible for the methylation of histone H3, which presumably contributes to chromatin remodeling. In addition, CARM1 may methylate other protein components of the transcription machinery. We propose that the autonomous activation activity of the C-terminal domain is due to its ability to interact with other proteins in the transcription machinery. For example, CARM1-C could interact with a component of the basal transcription machinery and thereby help to recruit RNA polymerase II; or CARM1-C could interact with another, currently unknown, coactivator and thereby recruit or maintain the additional coactivator in the complex with GRIP1, CARM1, and p300. The ability of the co-expressed C-terminal fragment of CARM1 to inhibit the coactivator function of full-length CARM1 (Fig. 6) supports our proposal that this region binds an important factor that contributes to the transcriptional activation process.

Thus, once bound to the promoter, CARM1 contributes to the transcriptional activation process through multiple downstream signaling mechanisms, i.e. through methylation of histones and possibly other proteins and through protein–protein interactions mediated by the unique C-terminal and possibly N-terminal domains. The use of multiple downstream signaling mechanisms by a single coactivator is not unique to CARM1. The coactivators p300, CBP, and p/CAF have multiple protein–protein interaction domains which also contribute to their coactivator function in collaboration with their histone acetyltransferase activities (12).

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