Synergistic, p160 Coactivator-dependent Enhancement of Estrogen Receptor Function by CARM1 and p300*

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Members of the p160 coactivator family (steroid receptor coactivator-1 (SRC-1), glucocorticoid receptor interacting protein 1 (GRIP1), and activator of thyroid and retinoic acid receptors (ACTR)) mediate transcriptional activation by nuclear receptors. After being recruited to the promoter by nuclear receptors, the p160 coactivator transmits the activating signal via two C-terminal activation domains, AD1 and AD2. AD1 is a binding site for the related coactivators cAMP-response element binding protein binding protein (CBP) and p300, whereas AD2 binds to another coactivator, coactivator-associated arginine methyltransferase 1 (CARM1), a protein-arginine methyltransferase. The current study explored the cooperative functional and mechanistic relationships among GRIP1, CARM1, and p300 in transient transfection assays, where they enhanced the ability of the estrogen receptor (ER) to activate transcription of a reporter gene. The coactivator functions of p300 and CARM1 depended on the co-expression of GRIP1. Simultaneous co-expression of all three coactivators caused a synergistic enhancement of ER function. Deletion of the AD1 domain of GRIP1 abolished the ability of p300 to potentiate ER activity but had no effect on CARM1-mediated stimulation. In contrast, when the AD2 domain of GRIP1 was deleted, p300 still stimulated ER function through the mutant GRIP1, but CARM1 failed to do so. Thus, both binding of p300 to AD1 and binding of CARM1 to AD2 are required for their respective coactivator functions and for their synergy. Furthermore, CARM1 and p300 function independently through different activating domains of GRIP1, and their synergy suggests that they enhance transcription by different, complementary mechanisms.

Nuclear receptors (NRs)1 constitute a large superfamily of DNA binding transcriptional regulators that control the expression of specific target genes involved in many important cellular events such as differentiation and responses to extracellular stimuli (1–5). In addition to the receptors for steroid and thyroid hormones, retinoic acid, and vitamin D, the superfamily includes a large number of orphan receptors for which an activating ligand is either unnecessary or unknown. The great majority of NRs share the following three similarly organized functional domains: The DNA binding domain (DBD), which is the most highly conserved domain among the NR family members, is located in the central portion of the polypeptide chain. The hormone binding domain, located at the C-terminal end, also has many common sequence motifs among different NR family members. Besides binding hormone, the hormone binding domain also contains a very important activation function, AF-2, which transmits the activating signal from the DNA-bound NR to the transcription machinery. The N-terminal domains of NRs are not conserved in size or sequence, but many of them contain another activation function, AF-1. The overall transcriptional activation by the NR is because of the combined effects of AF-1 and AF-2; the relative importance of these two activation functions depends on the specific NR, cell type, and target gene promoter.

After binding to specific enhancer elements in the promoter region, NRs activate the gene by directing local remodeling of chromatin and recruiting the transcriptional initiation complex to the promoter. These events require participation of a number of cofactors called transcriptional coactivators, which interact with NRs directly or indirectly and help to transmit the activating signal to the transcription machinery by a variety of mechanisms (6–11). The focus of the current study is the p160 family of 160-kDa coactivators (SRC-1, GRIP1/transcriptional intermediary protein 2 (TIF2), and p300/CBP interacting protein/activator of thyroid and retinoic acid receptors (ACTR)/amplified in breast cancer 1 (AIB1)/receptor-associated coactivator 3 (RAC3)/thyroid receptor activator molecule 1 (TRAM1)) (7–9, 11). These coactivators bind directly to the DNA-bound NRs and apparently function by recruiting other coactivators to the promoter and possibly also by acetylation of histones or other proteins in the transcription machinery (12–14). Two separate domains of p160 coactivators can bind to the activation functions of the NRs. In the central region of their polypeptide chains the p160 coactivators contain three NR box or LXXLL motifs (where L stands for Leu and X can be any amino acid) that interact directly with the highly conserved AF-2 domains of NRs (15–18). The C-terminal region of the p160 coactivators can also interact with the AF-1 domain of some of the NRs (19–21).

After binding to the DNA-bound NR the p160 coactivator transmits the activation signal from the NR via at least two receptor coactivator-1; ER, estrogen receptor; PCR, polymerase chain reaction.
activation domains (AD) located near the C terminus (12, 18, 21). AD1, located approximately between amino acids 1040 and 1120 in GRIP1/transcriptional intermediary protein 2 (TIF2), binds CBP and p300, which serve as coactivators for many different classes of transcriptional activator proteins. The coactivator function of CBP and p300 probably involves a variety of mechanisms, because these proteins can bind to many transcriptional activators, other coactivators, and components of the basal transcription machinery; these properties have led to the proposal that CBP and p300 may serve as scaffolds or platforms for assembly of transcriptional coactivators and that the assembled complexes may be responsible for integration of signals from multiple regulatory pathways (8, 12, 22). CBP and p300 also contain a protein acetyltransferase activity that can acetylate histones and other proteins in the transcription machinery (23–27). The acetyltransferase activity was found to be essential for the coactivator function of CBP and p300 with some transcriptional activators (cAMP-response element binding protein and signal transducer and activator of transcription) but not with others (NRs) (26). Because CBP and p300 can bind directly to many NRs (22), their recruitment to NR-regulated promoters could be through this direct interaction, through binding to AD1 of p160 coactivators, or both.

The second activation domain (AD2) of p160 coactivators is located near the extreme C terminus (amino acids 1122–1462 of GRIP1) (18, 21). The mechanism of signaling by AD2 could involve a weak histone acetyltransferase activity found in this region (12, 13), but the importance of this acetyltransferase activity for coactivator function has not been established, and substrates that are efficiently acetylated have not been reported to date. Alternatively, signaling by AD2 could be accomplished by other transcriptional coactivators interacting with this domain. CARM1, which contains a histone methyltransferase activity, binds to the AD2 region of p160 coactivators and enhances transcriptional activation by NRs when it is co-expressed with a p160 coactivator (14). It is not yet known whether the coactivator function of CARM1 relies on the methyltransferase activity or other activation domains of CARM1.

The coactivator activities of CBP/p300 and CARMLN for NRs and their abilities to bind to known activation domains of p160 coactivators suggests that these three coactivators may work in concert to mediate transcriptional activation by NRs. Because the coactivator function of CARM1 for NRs relied on the co-expression of a p160 coactivator, and because CARM1 binds to the C-terminal AD2 region of p160 coactivators (14), we used specific AD1 and AD2 deletions in p160 coactivator GRIP1 to test whether the functional reliance of CARM1 on GRIP1 involved the binding of CARM1 to GRIP1 AD2. For these studies estrogen receptor (ER) α was used as the NR. Similar tests were conducted to determine whether the coactivator function of p300 for ER relied on the GRIP1 AD1 site, to which p300 binds. This question is of particular interest, because current data has not resolved whether CBP and p300 act as coactivators through direct contact with NRs, through their interaction with the AD1 region of p160 coactivators, or both. CBP and p300 have been shown to bind directly to ER and other NRs and in some studies enhanced NR function in the absence of co-expressed p160 coactivators (22). On the other hand, deletion of AD1 has been shown to eliminate CBP and p300 binding and to cause a substantial reduction in the coactivator activity of p160 coactivators (12, 18, 21). Use of the AD1 and AD2 deletion mutants of GRIP1 also allowed us to determine whether the coactivator functions of CARM1 and p300 depended on, or were independent of, each other. In addition, because they all serve as NR coactivators and can physically interact, we tested whether the effects of GRIP1, p300, and CARM1 on ER function were synergistic, additive, or redundant.

EXPERIMENTAL PROCEDURES

Plasmids—Proteins with N-terminal hemagglutinin A (HA) epitope tags were expressed in transient mammalian cell transfections and in vitro from pSG5.HA, which has SV40 and T7 promoters (14). The following proteins were expressed: previously described pSG5.HA derivatives: GRIP1, SRC-1a, and CARM1 (14); GRIP1AD1 (full-length GRIP1 with amino acids 1057–1109 deleted) and GRIP1AD1D2 (GRIP1$_{1041-1121}$) (21). Vector pSG5.HA-GRIP1AD1AD2 was constructed as follows: The C-terminal region was removed from the wild type pSG5.HA-GRIP1 vector by using a natural XhoI site at codon 765 and the XhoI site in the vector’s cloning site following the GRIP1 AD1 stop codon. A new C-terminal GRIP1 cDNA fragment containing the AD1 and AD2 deletion mutations was generated by PCR using pSG5.HA-GRIP1AD1 AD1 as a template. The downstream primer for the PCR reaction included a stop codon and a SalI site after codon 1121. This PCR product was cut at the natural XhoI site at codon 765 and at the new SalI site and ligated with the XhoI sites of the pSG5.HA-GRIP1 vector fragment described above, thus generating pSG5.HA-GRIP1AD1+AD2. Plasmids encoding Ga4 DBD fused to various GRIP1 fragments were constructed by subcloning the following cDNA fragments into the EcoRI-SalI sites of pM (CLONTECH): an EcoRI-XhoI fragment encoding GRIP1$_{1057-765}$ and a PCR-generated EcoRI-SalI fragment encoding GRIP1$_{1043-1121}$, pM.GRIP1$_{1122-1462}$ (pMLGRIpC) was described previously (14). pHE0, encoding human ERα, was described previously (28), and cell-free luciferase reporter plasmids MMTVpLacZ, pEGFP-GRIP1, and G1K (controlled by Gal4 response elements) (19) and the β-galactosidase (β-gal) reporter plasmid pCMV, pβgal (30). The pcDNA3-pβgal expression vector for p300 was kindly provided by Dr. Vittorio Sartorelli (National Institutes of Health, Bethesda, MD).

Cell Culture and Transfection Assays—CV-1 cells (31) were transfected, and cell extracts were assayed for luciferase and β-gal activity essentially as described previously (14). Approximately 18 h prior to transfection, 5 × 10⁴ CV-1 cells were seeded into each well of 12-well dishes. Cells were transiently transfected by the SuperFect transfection reagent (Qiagen) according to manufacturer’s protocol with a total amount of 1.0 μg of plasmid DNA per well, including 50 ng of pCMV, pβgal, included as an internal control to monitor transfection efficiency. 2 h after transfection, 20 nm estradiol or an equivalent volume of the ethanol vehicle was added to the medium containing charcoal-treated serum (Gemini Bio Products). Approximately 48 h after transfection, cell extracts were made and assayed for luciferase and β-gal activity as described previously (15, 21). The luciferase data, expressed as relative light units (RLU), shown are the mean and standard deviation for triplicate points and are representative of at least three independent transfection experiments. Because some of the coactivators enhance the activity of the cytomegalovirus promoter and many other so-called constitutive promoters, β-gal activity was determined to monitor the uniformity of transfection efficiency, but the luciferase activities were not normalized with the β-gal activity.

RESULTS

Coactivator Function of p300 and CARM1 Is Synergistic and Depends on p160 Coactivators—To explore the functional relationships among GRIP1, p300, and CARM1, we examined their abilities to enhance transcriptional activation by ER in transient transfection assays. Different combinations of the three coactivators were co-expressed with ER in CV-1 cells, and their effect on the expression of an ER-regulated reporter plasmid was measured. In addition, GRIP1 mutants lacking AD1 (the binding site for p300), AD2 (the binding site for CARM1), or both were substituted for wild type GRIP1. This strategy allowed us to address several questions. First, does the coactivator function of p300 with ER depend on its ability to bind to the AD1 region of p160 coactivators through direct contact with ER? Second, does the coactivator function of CARM1 depend on its ability to bind the AD2 region of p160 coactivators? Third, are the coactivator functions of p300 and CARM1 independent of each other? Fourth, are the coactivator effects on ER function by GRIP1, p300, and CARM1 synergistic?
exogenous expression of CARM1 alone (i.e., in the absence of a co-expressed p160 coactivator) had little effect on reporter gene activation by ER (Fig. 1A, histogram 4). Similarly, no enhancement of activity was observed when p300 alone was co-expressed with ER in the absence of a co-expressed p160 coactivator (histogram 3), and varying amounts of the p300 expression vector, from 25 to 500 ng, had no effect (Fig. 2A, unfilled histograms). Co-expression of CARM1 and p300 resulted in a slight enhancement of ER function (Fig. 1A, histogram 5). In contrast, co-expression of GRIP1 with ER elevated reporter gene activity 3.5-fold (compare histograms 2 in Fig. 1, A and B, points on y axis). However, expression of both CARM1 and p300 caused a dramatic enhancement (up to 30-fold) of ER-dependent reporter gene activity (Fig. 3). The synergistic enhancement depended on the amount of p300 and CARM1 expression vectors used, when one was held constant and the other was varied. The dramatic synergistic enhancement caused by p300 and CARM1 was almost entirely dependent on the presence of GRIP1, ER, and estradiol (data not shown).

The synergy was more dramatic when lower levels of ER expression vector were used in the transfections. Under such conditions, neither CARM1 nor p300 effectively enhanced the reporter gene activity observed with ER plus GRIP1; each one caused an enhancement of 2-fold or less (Fig. 3, A and B, points on y axis). However, expression of both CARM1 and p300 caused a dramatic enhancement (up to 30-fold) of ER-dependent reporter gene activity (Fig. 3). The synergistic enhancement depended on the amount of p300 and CARM1 expression vectors used, when one was held constant and the other was varied. The dramatic synergistic enhancement caused by p300 and CARM1 was almost entirely dependent on the presence of GRIP1, ER, and estradiol (data not shown).

CARM1 and p300 Act through Independent Signaling Pathways—Both p300 and CARM1 bound to specific regions of p160 coactivators (12, 14, 18, 21), and both proteins depended on the presence of a p160 coactivator for their ability to enhance ER function (Fig. 1, A and B). These findings suggest that the coactivator functions of p300 and CARM1 depend on their ability to bind specific regions of p160 coactivators. To test this hypothesis further, we used GRIP1 deletion mutants (21) lacking AD1 (the binding site for p300 and CBP) or AD2 (the binding site for CARM1). Reporter gene activation by ER was enhanced by the GRIP1\textsuperscript{DAD1} mutant (compare histograms 2 in Fig. 1, A and C); further enhancement was caused by co-expression of CARM1 with GRIP1\textsuperscript{DAD1} but not by co-expression of p300, either in the presence or absence of CARM1 (Fig. 1C).

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Co-transfection of various amounts of p300 expression vector failed to enhance the activity observed with CARM1 and GRIP1, and high doses of p300 vector caused partial inhibition (Fig. 2).

The GRIP1ΔAD2 mutant enhanced ER function (compare histograms 2, Fig. 1, A and D), and further enhancement was achieved by co-expression of p300 (Fig. 1D, histogram 3). However, co-expression of CARM1 with GRIP1ΔAD2 failed to enhance reporter gene activity either in the presence or absence of p300 (Fig. 1D, histograms 4 and 5). In the presence of GRIP1ΔAD2 and p300, varying amounts of CARM1 expression vector failed to enhance activity, and higher doses of CARM1 vector caused partial inhibition (Fig. 2C). A GRIP1 mutant lacking both AD1 and AD2 was inactive by itself or in the presence of co-expressed CARM1 and p300 (Fig. 1E). Immuno-blot analysis indicated that wild type GRIP1 and all three GRIP1 mutants were expressed at similar levels from the same expression vectors used here (Ref. 21 and data not shown).

Our results indicate that binding of p300 to ER is not sufficient to allow p300 to act as a coactivator; rather, the key interaction is through the p160 coactivators. Furthermore, the coactivator functions of both p300 and CARM1 depended on the presence of AD1 and AD2, respectively, in GRIP1. The activity of p300 was independent of CARM1, and vice versa. The synergistic manner in which p300 and CARM1 enhance ER function and the independence of their mechanisms of action indicate that p300 and CARM1 function as coactivators through independent signaling pathways.
fragment containing AD2 had a weak transcriptional activation activity, which was enhanced 7-fold by CARM1 but not by p300 (Fig. 4D). The enhancement of reporter gene activity by the C-terminal GRIP1 fragment increased in parallel with the amounts of the Gal4DBD-GRIP1c and the CARM1 expression vectors used in the transfections (Fig. 5). Thus, CARM1 and p300 each enhanced the activity of only the GRIP1 fragment to which each bound. These results further support the hypotheses that the binding of p300 to GRIP1 AD1 and of CARM1 to GRIP1 AD2 are necessary and sufficient for their coactivator activities and that p300 and CARM1 activate transcription through independent pathways.

**DISCUSSION**

CARM1 and p300 Act through the p160 Coactivators to Enhance ER Function—CBP and p300 can bind directly to ER and other NRs (22) but can also bind to the AD1 domains of p160 coactivators (12, 18, 21). Which of these interactions is required for the coactivator function of CBP and p300 toward NRs? One way to address this question is to determine whether co-expression of p160 coactivators is required for the coactivator effect of p300 or CBP. However, different studies have produced different answers to this question. In some cases CBP or p300 enhanced NR function without co-expression of p160 coactivators (22, 32), whereas in the current study we found little if any enhancement of NR function by p300 alone (see Figs. 1A and 2). It is possible that the results of such experiments may depend on the levels of endogenous coactivators expressed in different cell types, the type of NR under study, and the experimental conditions employed. In the current studies we established conditions under which wild type GRIP1 and p300 acted synergistically as coactivators for ER (Figs. 1B and 3). When a GRIP1 mutant lacking AD1, the p300 binding site, was substituted for wild type GRIP1, p300 no longer enhanced ER function; however, CARM1 could still enhance ER function in conjunction with the GRIP1 AD1 mutant, demonstrating that the deletion of AD1 only caused a specific and selective loss of GRIP1 function.

Similar questions apply to CARM1. Although CARM1 was discovered through its ability to bind the AD2 region of p160 coactivators (14), it can also bind directly to ER and weakly to p300. Which binding interaction potentiates the coactivator function of CARM1 for ER? As previously shown (14), CARM1 enhanced ER function only in the presence of a p160 coactivator (Fig. 1, A and B). Substitution of a GRIP1 AD2 mutant for the wild type GRIP1 eliminated the ability of CARM1 to enhance ER function, but p300 still cooperated with this mutant GRIP1 to enhance ER function (Fig. 1D), demonstrating that the AD2 deletion also caused only specific and selective loss of function (Fig. 1C).

Thus, although p300 and CARM1 can both bind directly to ER, these binding interactions (if they occur under physiological circumstances) are not sufficient in our experimental system to potentiate the coactivator functions of p300 and CARM1. In contrast, our results with the AD1 and AD2 deletion mutants of GRIP1 indicate that the binding interactions of p300 and CARM1, respectively, with these two GRIP1 domains are essential for their abilities to serve as coactivators for ER.
Thus, GRIP1 and the other p160 coactivators act as coactivators through direct binding to ER, whereas p300 and CARM1 apparently rely primarily on their binding to p160 coactivators to recruit them to the promoter or otherwise potentiate their activities as coactivators. The above conclusions are consistent with two previous findings: deletion of the N-terminal NR binding domain of CBP or p300 had no effect on their ability to enhance NR function, whereas deletion of the p160 binding domain of p300 eliminated its ability to serve as a NR coactivator (33, 34).

Additional indications that p300 and CARM1 enhance ER function by directly mediating the signaling function of p160 coactivators was provided by experiments using three different fragments of GRIP1 fused to Gal4 DBD (Fig. 4). In these experiments, where no NR was involved, p300 only enhanced the transcriptional activation activity of the GRIP1 fragment containing AD1 (the p300 binding site), and CARM1 only enhanced the transcriptional activation activity of the GRIP1 fragment containing AD2 (the CARM1 binding site). In other words, p300 and CARM1 only enhanced the activity of the fragments to which they physically bound. These findings further support the conclusion that p300 and CARM1 enhance ER function by mediating the coactivator function of p160 coactivators.

CARM1 and p300 Function Independently of Each Other—In the presence of wild type GRIP1, p300 and CARM1 each functioned as a coactivator for ER, and together p300 and CARM1 caused a synergistic enhancement of ER function (which was also GRIP1-dependent). Deletion of the AD1 region from GRIP1 completely eliminated the ability of p300 to enhance ER function but had no effect on the ability of CARM1 to cooperate with GRIP1 in enhancing ER function. The AD1 deletion also prevented p300 from cooperating with CARM1 and GRIP1 to produce a synergistic enhancement of ER function. As discussed above, these results show the dependence of p300 coactivator function on GRIP1 AD1 but also lead to two other important mechanistic conclusions. First, the contribution of p300 to p300-CARM1 synergy was also mediated by the binding of p300 to GRIP1 and was not mediated through p300-CARM1 binding or through any indirect pathway. Second, because the AD1 deletion completely eliminated the coactivator function of p300 but did not affect CARM1 function, the function of CARM1 as a coactivator was completely independent of p300.

Results with the AD2 deletion lead to a parallel set of conclusions. The coactivator function of CARM1 is completely dependent on its ability to interact with GRIP1 AD2. This includes the ability of CARM1 to function synergistically with p300 to enhance ER function. Because the AD2 deletion eliminated the function of CARM1 as a coactivator but had no effect on p300 function, the coactivator function of p300 was completely independent of CARM1. Thus, although we can detect a weak binding between CARM1 and p300 in vitro (data not shown), our results suggest that this binding interaction alone (if it occurs in vivo) is not sufficient to allow for their synergistic activity as coactivators for ER.

CARM1 and p300 Enhance ER Function by Different Mechanisms—The results of this study are consistent with the model that NRs recruit p160 coactivators to the promoter and that the p160 coactivators in turn recruit p300 and CARM1 (Fig. 6). The enhancement of NR function by p300 and CARM1 is presumably because of their ability to recruit or activate other components of the transcription machinery. The synergy between p300 and CARM1 indicates that they activate or recruit different downstream targets in the transcription machinery. If p300 and CARM1 enhanced ER function by activating the same signaling pathways or downstream target, we would expect their effects to be additive at best but not synergistic.

What are the downstream targets of p300 and CARM1? Abundant evidence indicates that the protein acetyltransferase activities of CBP and p300 play an important role in their coactivator functions at least in some cases. Histones are at least one of the in vivo targets for these acetyltransferase activities (27), but acetylation of transcriptional activators and
Mechanism of Nuclear Receptor Coactivator Synergy

Although the mechanism of the coactivator function of CARM1 remains to be determined, CARM1 contains an arginine-specific protein methyltransferase activity that can methylate histone H3 in vitro, suggesting that histone methylation might be one mechanism of CARM1 coactivator function (14). Further studies will be required to determine the biological significance of histone methylation, but a steady stream of recent studies have suggested regulatory roles for a variety of different types of covalent histone modification including phosphorylation, lysine and arginine methylation, ADP-ribosylation, and ubiquitination (35, 36). However, in light of the multiple signaling mechanisms that are probably used by CBP and p300, the coactivator function of CARM1 could involve methylation of other components of the transcription machinery, and other activation or signaling domains of CARM1 that work independently of the methyltransferase activity of CARM1 may also contribute to coactivator function.

It seems likely that the synergy between GRIP1, p300, and CARM1 observed in our studies is physiologically relevant. Although the current study focused on ER, we have observed similar synergy among these three coactivators with a variety of other NRs, and careful titration of the NR and coactivator expression plasmids in co-transfection experiments produced an extremely high degree of synergy among these coactivators.

Furthermore, CARM1 and p300 both function as coactivators for ER through interactions with different domains of p160 coactivators, and these interactions are conserved among the three members of the p160 coactivator family. Thus, these three coactivators (presumably in conjunction with other coactivators) may function as a coactivator complex or unit that propagates the activating signal from the NR through multiple signaling pathways to multiple targets in the transcription machinery. If multiple coactivator components are needed to mediate transcriptional activation by NRs, then we might expect to see more than additive effects by using multiple coactivator components together versus individually. Thus, the coactivator synergy we observed is consistent with a requirement for multiple coactivators. It will be interesting to determine whether further synergies can be achieved by addition of more components. For example, the NR coactivator steroid receptor RNA activator (SRA), which appears to function as an RNA molecule, has also been shown to associate with p160 coactivators (37).

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