Tip60 Is a Co-activator Specific for Class I Nuclear Hormone Receptors

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The nuclear hormone receptor superfamily is composed of a group of hormone-dependent transcription factors that play prominent roles in homeostatic events in vertebrates. A prerequisite for steroid hormone receptor activity is the binding of co-activator molecules to the activation function-2 domain of the receptor. The LXXLL motif/nuclear receptor box, contained within a number of co-activator molecules, mediates the interaction with nuclear hormone receptors. Tip60 (Tat-interactive protein 60 kDa), previously shown to bind to and enhance androgen receptor (AR)-mediated transactivation, contains a single nuclear receptor box at its extreme C terminus. We demonstrate that unlike members of the p160 co-activator family that interact predominantly with the N terminus of the AR in an LXXLL motif-independent manner, the LXXLL motif of Tip60 is required and is sufficient for AR interaction. Furthermore, by using the mammalian two-hybrid system and transient transfection experiments, we show that Tip60 preferentially interacts with and up-regulates class I nuclear receptors, suggesting that Tip60 is a steroid hormone receptor-specific co-activator. We conclude that Tip60 may specifically regulate a subset of nuclear hormone receptors, giving an indication to how regulated nuclear receptor activation can be achieved.

The androgen receptor (AR), a member of the steroid hormone receptor family, is a ligand-inducible transcription factor that plays a key role in the growth, development, and transformation of the prostate gland (1, 2). The AR, in common with other members of the nuclear hormone receptor (NR) family, is a modular protein containing distinct functional domains (for review, see Ref. 3). DNA interaction involves the centrally positioned double zinc finger-containing DNA-binding domain (DBD) (4, 5), which is highly conserved throughout the family of nuclear receptors (NR). Hormone binding is performed by the C-terminal ligand-binding domain (LBD), a structurally conserved region within the family of NRs, containing 12 α-helices (H1–H12) (6–8), which in addition mediates homodimerization and plays a role in transcriptional activation. This latter function is performed by the extreme C-terminal helix H12 of the LBD, termed activation function-2 (AF-2) (9), a ligand-dependent transcriptional activator which, in combination with the N-terminal transactivation domain (TD), is able to activate receptor-mediated gene expression. Although not fully defined, the AF-2 domain of NRs has been proposed to mediate direct interactions with a number of co-activator proteins necessary for the full transcriptional activity of the receptor (8, 10). To date, numerous co-activator molecules have been characterized, such as members of the p160 family, including SRC-1 (steroid receptor co-activator-1) (11) and GRIP-1 (glucocorticoid receptor-interacting protein-1) (12); the CREB-binding protein (CBP)/p300 proteins (13, 14) and p300/CBP-associating factor (15). The finding that many of these co-activator molecules are histone acetyltransferases (HAT), an activity strongly implicated in the remodeling of chromatinized DNA (16), suggests that these intermediary molecules function via the AF-2 to rearrange the promoter template, increasing access to the DNA for the RNA polymerase machinery (17–19).

A short α-helical, leucine-rich peptide sequence LXXLL, termed the NR box, found within a number of co-activators mediates interaction with the AF-2 domain of NRs (20–23). Studies of p160 family members have shown that the individual leucine residues of the motif make up a hydrophobic interface capable of intercalating within a shallow, hydrophobic groove, made up of residues from helices H3, H4, H5, and H12 of the LBD (21, 24). Evidence from both SRC-1 and GRIP-1 suggests that both the hydrophobic nature and structural integrity of the LXXLL motif/NR box is important for a direct AF-2 interaction (22, 23). The fact that these and many other co-activators, such as CBP/p300, SRC-1 (21), and TIF-2 (25, 26), contain multiple NR boxes, each of which is flanked by differing peptide sequences, may explain the broad specificity of nuclear receptor contacts made by these proteins.

The finding that p160 co-activators interact with and co-activate the AR in an LXXLL motif-independent manner suggests that the mechanism of co-activator interaction with the AR is distinct from those of other NRs (27, 28). Indeed, SRC-1 has been shown to interact predominantly with a region of the N-terminal TD via a glutamine-rich domain, indicating that the AR AF-2 domain is redundant for p160 co-activator function (27).

Tip60 (Tat-interactive protein-60 kDa), first identified in complex with the Tat protein of HIV-1 (29) and further shown to possess intrinsic HAT activity (30), has been shown to interact with the AR and enhance androgen, estrogen, and progesterone receptor-mediated transactivation (31). Tip60 contains a single NR box at its extreme C terminus, which was proposed to mediate interaction with the AR (31). We show that Tip60...
interacts with the AR in an LXXLL-motif-dependent manner, which is also a requisite for Tip60-mediated AR up-regulation, indicating that Tip60 binds the AR in a manner distinct from that of the p160 family of co-activators. Furthermore, we demonstrate that Tip60 specifically interacts with class I NRs, including estrogen receptor-α (ERα), estrogen receptor-β (ERβ), and glucocorticoid receptor (GR), and fails to up-regulate class II NRs, suggesting a specific role for Tip60 in class I NR-mediated gene expression.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis of Tip60—The plasmid pACT2-Tip60 (70–513) (31), designated as wt-Tip60, was used as the template for the generation of the Tip60 NR box mutants. In the first round of a two-step polymerase chain reaction method, using Bio-X-Act proofreading DNA polymerase enzyme (Bioline), the following primer combinations were used to generate 600-bp-base pair 3′-fragments of Tip60 containing the individual NR box leucine to alanine mutations: L493A, ATTCGAAAGGCGCTCTCCGG-pACT2 and reverse (pACT2R) GGTTTTTCATGATCTCA; L496A, ATTCGCAAAGGCGCCATCCGG-pACT2R; and L497A, ATTCGCAAGGGCGCCATCAG-pACT2R. These fragments were then used in combination with the pACT2 forward primer (pACT2F, GGATGAGACATGAACCCCAA) in a second round of PCR, with pACT2-wt-Tip60 as template, to generate the Tip60 (70–513) mutant DNAs: Tip60L493A, Tip60L496A, and Tip60L497A, respectively. The PCR products were cloned into Zero-Blunt vector (Invitrogen) and then subcloned into the yeast vector pACT2 via the BamHI site, generating pACT2-Tip60L493A, pACT2-Tip60L496A, and pACT2-Tip60L497A.

To generate Tip60 (70–363), PCR was performed with the reverse primer, CTCGAGATGCAGGCCACATTGTAG (which anneals to a site upstream from the NR box of Tip60), and pACT2F, using pACT2-wt-Tip60 as template. The product was cloned into the TA vector (Invitrogen) and subsequently subcloned into the yeast vector pACT2 via BamHI/XhoI sites. pAS21-1AR-DS vector containing the DNA- and hormone-binding domains of the human AR has been described previously (31).

For the mammalian two-hybrid system, pVIP16AD-wt-Tip60, pVIP16AD-Tip60L493A, pVIP16AD-Tip60L496A, and pVIP16AD-Tip60L497A fusion proteins were generated by inserting the BamHI fragment from the respective pACT2 clones, utilizing a BamHI site located in the 3′-untranslated region of Tip60, downstream from the VP16 activation domain of pVP16 (CLONTECH). For pVIP16AD-Tip60 (70–363) subcloning, the PCR product was cut with BamHI and HpaI sites and was used. The NR box-containing fragment of Tip60 (70-481–513) was generated by PCR using NR-F, GGATCCATGGCCACAGATCACCATC, and pACT2R primers, using the pACT2-wt-Tip60 as template, to generate the Tip60-(70–513) construct. The amplified product was cloned into Zero-Blunt vector (Invitrogen) and subsequently subcloned into the yeast vector pACT2 via BamHI/HindIII sites. For the mammalian expression vector (CLONTECH) PCR was performed with GR-F, GGATCCCTCTTAAACTCTGCCTC (anneals upstream from LBD of GR), and GR-R, GGATCCTCTACTATTCTGGCT (anneals downstream from LBD of GR), and pACT2R, using the pACT2-wt-Tip60 construct as template. The amplified product was cloned into the TA vector (Invitrogen) and subsequently subcloned into the p mammalian expression vector (CLONTECH) via BamHI site to generate Tip60L493A.

pVIP16AD-DR was created by inserting the EcoRI fragment of pSG5-TR-LBD-424 (gift from K. Chatterjee, University of Cambridge, UK) into the EcoRI site of pVIP16AD. PCR was performed with VDR-F, GGATCGAGGAGGCGGCCATTGTAG (which anneals to a position flanking the VDR LBD), and VDR-R, GGATCCCGATGGAGAGGCGGCCATTGTAG (corresponds stop codon of VDR), using pSG5-VDR (gift from P. McDonald, Case Western Reserve University) as template to generate VDR-LBD.

For experiments incorporating ERα and ERβ, cells were incubated in phenol red-free RPMI 1640 with 10% FCS stripped with dextran-coated charcoal. After 48 h, the cells were harvested and lysates assayed as above.

Analysis of Tip60-AR Interaction Using the Yeast Two-hybrid System—To determine the effect of mutating the individual leucine residues of the NR box of Tip60, the pAS21-1AR-DS vector was co-transformed into the yeast strain PJ69-4A together with pACT2-wt-Tip60 and individual leucine mutant constructs pACT2-Tip60L493A, -L496A, and -L497A. For control, empty pACT2 vector was transformed together with pAS21-1AR-DS. Subsequent yeast two-hybrid analysis was performed as described previously (31).

Transactivation Assays—To assess the role of the LXXLL motif in the enhancement of AR-mediated transactivation, BglII fragments of the pACT2-wt-Tip60 and pACT2-Tip60L493A and -L496A, and -L497A were subcloned into the pCMV mammalian expression vector, downstream from the CMV promoter, producing the pCMV-wt-Tip60 and pCMV-Tip60L493A constructs. COS-7 cells were transiently transfected, using Superfect, with the following DNA: pCMV-β-Gal control plasmid (200 ng), pPSAluc (200 ng), and pVIP16AD-AR, -DS, -TR-LBD, and -VDR-LBD were used.

To determine the specificity of Tip60-mediated co-activation, COS-7 cells were transiently transfected as above with pCMV-β-Gal, pCMV-β-Gal control plasmid (200 ng), pPSAluc (200 ng), pCDNA3-AR-(1–918) (50 ng), and either pCMV-wt-Tip60 or pCMV-Tip60L493A (50 ng). To determine the effect of Tip60 on the AR-mediated transactivation, COS-7 cells were transiently transfected as above with pCMV-β-Gal, pCMV-β-Gal control plasmid (200 ng), pPSAluc (200 ng), pCDNA3-AR-(1–918) (50 ng), and either pCMV-wt-Tip60 or pCMV-Tip60L493A (50 ng).

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Light and electron microscopic studies were performed as described above for all hybrid constructs. For analysis of Tip60 expression, COS-7 cells were incubated in phenol red-free RPMI 1640 with 10% FCS stripped with dextran-coated charcoal. After 48 h, the cells were harvested and lysates assayed as above.

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Tip60 in Class I NR Activation

FIG. 1. Schematic representation of functional domains of Tip60. Tip60 clone used in the study lacks 70 amino acids from N terminus of the published sequence. Regions identified in Tip60 are as follows: HAT, NR box, and zinc finger, the putative zinc finger. Sequence of the flanking residues of the LXXLL are shown. The names of the various Tip60 constructs are denoted to the left of the figure, with the nature of the LXXLL motif indicated above each corresponding line.

In Vitro Transcription and Translation of Proteins and Immunoprecipitation—pRSET-C-Tip60L497A was constructed via excision of a BamHI fragment from pACT2-Tip60L497A and subcloned into pRSET-C-Tip60L497A, pASG4-Sap-1a, and pT7-hAR-(1–363) constructs were transfected into COS-7 cells with 200 ng of UASTKLuc, a Gal4-responsive reporter, and 200 ng of pCMV-β-gal reporter per 350-μmol well in the presence and absence of synthetic 10 μM ligand, mibolerone (black and white bars, respectively). Cells were harvested 48 h after transfection, assayed for luciferase activity, and corrected for β-galactosidase activity to give the relative activity. Results shown are the mean of three independent experiments performed in triplicate ± S.D.

RESULTS

Removal of the NR Box Renders Tip60 Incapable of AR LBD Interaction—It was demonstrated previously (31) that Tip60 is capable of a direct, hormone-dependent interaction with a C-terminal portion of the androgen receptor (AR) containing both the DNA- and hormone-binding domains (DS domain) and can enhance AR-mediated transactivation, implying that Tip60 is a bona fide co-activator molecule for the AR.

In an attempt to define further the function of Tip60 as a co-activator for the AR, we sought to examine the mechanism of Tip60-AR interaction. Tip60 contains a single LXXLL motif between residues 493 and 497. LXXLL motifs have been shown to bind directly a region within the AF-2 of NRs (22–24), and together with the NR box, Tip60 mediates the AR LBD interaction.

To this end, we generated a C-terminal deletion of Tip60, Tip60-(70–363) (Fig. 1), which lacks the extreme 150 C-terminal residues including the LXXLL motif, and together with wt-Tip60, tested for the ability to bind the AR DS domain, using the mammalian two-hybrid system in COS-7 cells. In the presence of 10 nM synthetic androgen, mibolerone, wt-Tip60 interacted specifically with the DS domain of the AR, whereas Tip60-(70–363) failed to interact (Fig. 2). The values obtained in the presence and absence of mibolerone for Tip60-(70–363) were comparable, demonstrating a complete lack of binding upon removal of the C-terminal 150 amino acids, suggesting that the LXXLL motif is a potential requisite for AR LBD interaction.

To delineate further the region involved in receptor binding, a shorter LXXLL-containing fragment was generated, encompassing 12 amino acids N-terminal and 16 residues C-terminal to the NR box, Tip60-(481–513) (Fig. 1), and we tested for AR LBD binding using the mammalian two-hybrid system. We demonstrated that in the presence of mibolerone, the Tip60-(481–513) fragment interacts with the DS domain of the AR to levels observed with wild-type Tip60 (Fig. 2), supporting the notion that the single NR box of Tip60 is sufficient for AR LBD interaction.

Mutation to the Individual Leucine Residues of the NR Box of Tip60 Abolishes AR LBD Interaction—Secondary structure analysis of several co-activator molecules has revealed that the individual leucine residues of the LXXLL motif align upon one face of an α-helix, generating a hydrophobic interface for LBD interaction (21, 24). Disruption of the hydrophobic peptide sequence, through replacement of the leucine residues with alamines, has proved to abolish NR binding of several co-activator molecules, including SRC-1, RIP-140 (21), and GRIP-1 (12, 32).

By having established that a 33-amino acid C-terminal peptide of Tip60 is sufficient to bind to the DS domain of the AR, we hypothesized that binding was mediated predominantly through the NR box of Tip60. We introduced individual leucine residue mutations into the single NR box of Tip60 to assess directly the role of the LXXLL motif in ligand-dependent AR interaction. Each of the leucine residues was replaced with alamines forming three separate Tip60 NR box mutants as follows: Tip60L493A, Tip60L496A, and Tip60L497A which lack the first, second, and third leucine residues of the NR box, respectively, but retain the rest of the wild-type sequence (Fig. 1).

By using the yeast two-hybrid system, the NR box mutants were tested for hormone-dependent AR LBD binding. As shown in Fig. 3, in the presence of dihydrotestosterone, the relative binding activity of the three mutants remained at basal level, whereas an expected increase in AR-binding was observed with wt-Tip60, indicating that the NR box mutants were incapable of a direct interaction with the AR LBD.

To confirm the above results, the NR box mutants were tested for AR interaction in the mammalian two-hybrid system. In the presence of mibolerone, Tip60L493A, Tip60L496A, and Tip60L497A failed to interact with the AR LBD, whereas wt-Tip60 specifically interacted with the AR LBD (results not shown). Furthermore, in an attempt to define a region of the AR targeted for Tip60 binding, we found wt-Tip60 incapable of interaction with the AR LBD mutant, AR-LBDΔTV4 (results not shown), which contains a glycine to valine substitution at position 743 and has been shown to disrupt the integrity of the LBD, suggesting that Tip60 may target a region encompassing Gly-743 of the LBD for binding. Together, results from the yeast and mammalian two-hybrid system provide strong evidence that the Tip60 LXXLL motif mediates AR LBD interaction.
systems confirm that each of the leucine residues of the LXXLL motif are required for successful AR LBD interaction, and a region at the extreme C terminus, including residues of the AF-2, may be involved in Tip60 interaction.

Mutation to the LXXLL Motif of Tip60 Abolishes Full-length AR Interaction in Vitro—Previous studies (27, 32) of p160 co-activators have demonstrated that both N- and C-terminal domains of the AR are important for p160 protein interaction. Indeed, although the central nuclear interacting domain of SRC-1, containing three LXXLL motifs, interacts directly with the AR LBD fused to a heterologous DNA-binding domain, SRC-1 has been shown to interact primarily with the AR TD in the context of the full-length AR (27). Therefore, although we have demonstrated that Tip60 interacts with the LBD via a single LXXLL motif, Tip60 may also interact directly with regions outside of the LBD to stabilize or mediate the protein interaction with the full-length receptor.

Thus, we next sought to define the interaction of Tip60 with the full-length AR in vitro. We figured that if the LXXLL motif of Tip60 was the lone requirement for full-length AR interaction, abrogating the NR box of Tip60 would abolish full-length AR binding. By using radiolabeled proteins transcribed and translated in vitro, we attempted to co-immunoprecipitate both wt-Tip60 and the NR box mutant Tip60L497A with the AR, in the presence of miobolone, using a polyclonal anti-AR antibody. The transcription factor Sap-1α, an Ets family protein, was incorporated as a negative control for this experiment (as described previously in Ref. 31).

Before immunoprecipitation, labeled wt-Tip60, Tip60L497A, and Sap-1α were combined with the rabbit reticulocyte lysate extract containing AR (Fig. 4; lanes 3–5, respectively). Lane 2 shows the control sample, where immunoprecipitation was performed on extracts containing Tip60 alone in order to determine the level of Tip60 immunoprecipitated that was not specifically associated with AR.

Following immunoprecipitation, we observed that wt-Tip60 interacted specifically with the AR (lane 8), confirming previous data (31), whereas in comparison, Tip60L497A failed to co-immunoprecipitate any AR (lane 9), suggesting that the LXXLL motif of Tip60 was the main determinant for interaction with the full-length AR. Importantly, Sap-1α did not interact with AR in this experiment (lane 10), confirming the specificity of the immunoprecipitation procedure.

Mutation to the LXXLL Motif Abolishes Tip60-mediated Co-activation of AR Activity—Numerous studies have shown that mutation to individual NR boxes of p160 co-activators abolishes both binding to and co-activation of NR-mediated transcription (22). However, evidence from SRC-1 and GRIP-1 suggests that p160 co-activators up-regulate AR activity in the absence of an LXXLL motif-AR interaction, suggesting that other regions of the AR are bound by co-activators. Indeed, the finding that SRC-1 interacts directly with a region in the AR TD and up-regulates TD-mediated transactivation indicates that members of the p160 family function through the N terminus of the AR to increase activity (27, 28).

Our finding that the LXXLL motif of Tip60 is necessary and sufficient for full-length AR interaction suggests that Tip60 binds AR in a fashion distinct from p160 proteins and may up-regulate AR activity in an LXXLL motif-dependent manner. In order to confirm these findings, AR co-activation by wt-Tip60 and an NR box mutant Tip60 were compared in transient transfection experiments in COS-7, in the presence of androgen. We hypothesized that if Tip60 interacts solely with the AR LBD, then mutating the LXXLL motif will abolish Tip60-mediated co-activation of AR activity.

As shown in Fig. 5, in the presence of wt-Tip60, AR activity was increased 5-fold over that of basal level, confirming previous data (31). In contrast, co-transfection of the NR box mutant Tip60L497A failed to enhance AR activity over basal levels, confirming the notion that the NR box of Tip60 was the main requisite for binding to the AR and is necessary for the co-activator properties of Tip60 to be elicited.

Tip60 Is Incapable of Homodimerization—It is interesting that the interaction between Tip60 and the AR potentially occurs via one LXXLL motif, whereas many of the co-activator-NR receptor interactions reported to date require several NR boxes to contribute to receptor binding. Recently, however, a novel co-activator called NRC-1 has been characterized, which contains a single NR box that is capable of avid receptor interaction (33). NRC-1 achieves high-affinity receptor binding through homodimerization, in which the two NR boxes of the NRC-1 dimer can interact with NRs.

In an effort to define further the interaction between Tip60 and AR, the potential for Tip60 to homodimerize was thus investigated. We expressed Gal4DBD and VP16AD fusions of Tip60 and tested for Tip60-Tip60 interaction using the mammalian two-hybrid system in COS-7 cells. Comparing interaction between wt-Tip60-Gal4DBD and empty VP16AD with wt-Tip60-Gal4DBD and wt-Tip60-VP16AD, we find no discernible difference, indicating that Tip60 does not homodimerize in vivo (Fig. 6). We thus speculate that a single monomer of Tip60 may be sufficient for efficient AR interaction.
Tip60 Preferentially Interacts with Class I Nuclear Hormone Receptors—We next sought to define the relative nuclear receptor binding activity of Tip60. The fact that many co-activators have been characterized, which share similar nuclear receptor specificities, suggests functional redundancy among co-activator molecules, thus making it difficult to assign functional roles of co-activators to specific activation of hormone receptors.

By using the mammalian two-hybrid system, incorporating members of both class I and class II receptors as Gal4DBD-fusion proteins, the receptor binding activity of Tip60, expressed as a VP16AD-Tip60 fusion protein, in the presence and absence of hormone was tested. As shown in Fig. 7A, in the presence of the respective hormone, VP16AD-Tip60 interacted with Gal4DBD-AR, -ERα, -ERβ, and -GR very strongly with a respective 4-, 4-, 9-, and 5-fold increase in binding strength over that in the absence of ligand (compare 2nd and 4th lanes, 6th and 8th lanes, 10th and 12th lanes, and 14th and 16th lanes), whereas it failed to bind to Gal4DBD-TR and -VDR and only marginally interacted with Gal4DBD-RXR (1.5-fold increase in interaction over basal level) (compare 18th and 20th lanes, 22nd and 24th lanes, and 26th and 28th lanes). This result suggests that Tip60 has a preference for class I nuclear hormone receptor interaction, a specificity unique to any co-activator molecule identified to date.

One concern in this experiment is that the DNA-tethered TR and -VDR have very high levels of inherent transcriptional activity, which could potentially mask any increase in reporter expression due to an interaction between Tip60 and TR or VDR.

To address this, we performed the reciprocal experiment in the mammalian two-hybrid system, incorporating a Gal4DBD-Tip60 fusion and the VP16-TR, -VDR, and -AR constructs. The fact that Tip60 has no inherent transcriptional activity when tethered to DNA (31) suggests that the background level of reporter activity will be low, allowing the detection of potential interactions between Tip60 and both class II receptors.

As shown in Fig. 7B, in the presence of androgen, VP16AD-AR failed to affect basal promoter activity, implying that the AR fusion is incapable of inherently activating the Gal4-responsive reporter (compare 1st and 2nd lanes). Co-transfection of Gal4DBD-Tip60 and VP16AD-AR in the presence of hormone activated reporter activity 2.3-fold over that in the absence of hormone, implicating an interaction between the proteins (compare 3rd and 4th lanes). It is interesting to note that the fold interaction between Tip60 and the AR in this experiment is lower than that observed in the reciprocal experiment (Fig. 7A, compare 2nd and 4th lanes). It is possible that slight allosteric changes to the AR when tethered to the VP16AD, which could potentially change the Tip60-binding site, may result in the lower binding affinity between Tip60 and AR.
VDR-mediated transcription. In the presence of D₃, VDR dra-
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activator for class II NRs, was also incorporated.

In transient transfection experiments in COS-7 cells, using both VDR and TR. SRC-1, a co-
co-activation of class II NRs was examined in transient transfection presence and absence of 10 nM vitamin D (pGFP-VDR, pCMV-Tip60, -SRC-1, or empty pCMV for control was co-trans-
ungenetically increased basal promoter activity 100-fold (compare 1st and 2nd lanes), confirming that Tip60 specifically interacts with class I nuclear receptors.

**Tip60 Is a Class I NR-specific Co-activator** —The fact that Tip60 preferentially interacts with members of the class I NR family, and previous work has shown that Tip60 up-regulates AR-, ER- and PR-mediated transcription (31), suggests that Tip60 is a potential class I NR-specific co-activator. Thus, the specificity of Tip60-mediated co-activation for NRs was next examined. Considering that Tip60 fails to interact with TR and VDR and that a direct Tip60-AR interaction is a requisite for enhancing AR-medi-
ted transcription (Fig. 5), we hypothesized that Tip60 would fail to enhance the transcriptional activity of both TR and VDR.

To test this, the ability of Tip60 to enhance the activity of full-length VDR and TR was compared with that of SRC-1 that has been demonstrated previously to up-regulate both VDR and TR activity (22). As illustrated in Fig. 8A, in the absence of D₃, the addition of Tip60 or SRC-1 had little or no effect upon VDR-mediated transcription. In the presence of D₃, VDR dras-
tically increased basal promoter activity 100-fold (compare 1st and 2nd lanes), indicating a strong inherent transcriptional activity of the receptor. In the presence of SRC-1, VDR medi-
ated transactivation was doubled in the presence of D₃, confirming SRC-1 as a co-activator for VDR. However, co-transfection of Tip60 failed to up-regulate transcriptional activity of VDR, suggesting that Tip60 is not a co-activator for VDR.

Similarly, whereas SRC-1 increased TR-mediated transcriptional activity 2-fold in a ligand-dependent manner, Tip60 demonstrated a complete inability to increase TR activity (Fig. 8B). These results further support that Tip60 is a co-activator specific for class I NRs.

**DISCUSSION**

In an effort to define the process of nuclear receptor-mediated gene expression, numerous co-activator proteins have been identified that play an important accessory role to nuclear receptors in transcription. Of these co-activators, two main families have emerged, the p160 and CBP/p300 families of transcription factors (for review see Ref. 34). Members of both groups contain inherent HAT activity, a post-translational modification that has been linked with chromatin remodeling implicated in enhancing the activity of numerous transcription factors, including nuclear hormone receptors.

Whereas many co-activator-NR interactions are mediated via LXXLL motifs, numerous lines of evidence have indicated that p160 co-activators interact with and enhance AR-mediated transactivation in an LXXLL motif-independent manner (27, 28, 32), suggesting that co-activators may interact with the AR in a distinct fashion to that of other NRs.

The data presented here demonstrate that the LXXLL motif of Tip60 is necessary and sufficient for AR interaction. Like other co-activators, the hydrophobic integrity of the LXXLL motif was demonstrated to be vital for AR LBD binding; substitution of the individual leucine residues to alanine resulted in abrogation of AR LBD interaction. Interestingly, the same NR box mutations also abolished full-length AR activation in vitro suggesting that Tip60 interacts with full-length AR in an LXXLL motif-dependent manner. This notion was further supported with the demonstration that the NR box mutant, Tip60L497A, failed to up-regulate AR activity, indicating that the NR box of Tip60 is the sole determinant for AR binding and is a requisite for the co-activator properties of Tip60 to be elicited. These findings are in contrast to the mechanics of p160 co-activator-AR interactions. SRC-1 interacts in an AF-2- and LF motifs, which show high affin-
ity binding to the AF-2 domain and is necessary for the N-C interaction, the hydrophobic integrity of the LXXLL motif-independent manner is intriguing. Considering that the AR AF-2 is deemed to be the target site for the AR N-terminal WXXLF and FXXLF motifs, which show high affinity binding to the AF-2 domain and is necessary for the N-C interaction (35), suggests potential competition between the three motifs for AR interaction. Indeed, our finding² that Tip60 fails to interact with the AR LBDG743V mutant, which has been shown to abolish N-C interaction in the full-length AR context (36), may indicate that Tip60 binds to a site within the LBD that is also required for TD interaction. However, the rapid androgen dissociation kinetics associated with several AR LBD mutants (36) may have distorted our results, such that the failure for a Tip60 interaction may be due to the LBD being in the un-ligated, apo form. Further analysis using an array of LBD mutants may define the exact site for Tip60 interaction.

Sequence analysis of the NR box of Tip60 and the immediate flanking residues (Fig. 1) show no discernible homology to any

² L. Gaughan, M. E. Brady, S. Cook, D. E. Neal, and C. N. Robson, unpublished information.
LXXLL motifs characterized to date, including the three classes identified using the combinatorial peptide library screen (37), suggesting that Tip60 has a unique NR box that may potentially interact with the AR LBD in a fashion that allows Tip60 binding as well as a stable N-C interaction necessary for AR-mediated transcription. Alternatively, a putative interaction between AF-2-bound Tip60 and a TD-bound p160 co-activator may act to bridge the N-C interaction without the requirement of the FXXLF and WXXLF motifs thus allowing both stable Tip60-LBD and N-C interactions. Although not fully defined, Tip60 and SRC-1 have been demonstrated to interact directly in vitro, suggesting the AR N-C interaction may be facilitated by the interaction of two co-activators bound at distinct regions of the AR.

The finding that FHL2, an AR-specific co-activator, interacts with the AR in an AF-2-dependent manner via several LIM domains (38) confirms an active role for the AR AF-2 in specific co-activator recruitment. Furthermore, the ability for FHL2, Tip60, and members of the p160 co-activator family to each interact with the AR in a specific fashion indicates that diverse mechanisms exist for successful AR interaction that may have evolved to allow fail-safe AR-mediated transcription. For example, mutation to the FHL2-binding site in the AR LBD may still permit p160 and Tip60 interaction, such that AR retains the ability to be up-regulated.

The finding that Tip60 interacts preferentially with and up-regulates class I NRs, including AR, ER, and PR (31), suggests that Tip60 is a co-activator specific for steroid receptors, a specificity that is unique among the numerous co-activators identified to date. Interestingly, a novel co-activator has been identified recently, called NRIF3, that specifically interacts with and enhances the activity of TR and RXX but fails to interact with the receptors ER, GR, PR, retinoic acid receptor, and VDR (39). This limited receptor binding repertoire of NRIF3 has been suggested to contribute to the functional specificities of a subset of nuclear receptors, differing from the activities of the p160 and CBP/p300 family members of co-activators that seem to be relatively liberal in their receptor interactions. Thus, the unique binding and co-activation activity of Tip60 suggests that it may act as a dominant regulatory molecule in class I receptor function.

The ability for Tip60 to interact with several class I NRs, seemingly through the single LXXLL motif, is remarkable when considering that p160 proteins utilize a number of NR boxes for varied NR interactions. It is interesting to speculate that Tip60 contains a unique NR box motif, whose primary peptide sequence facilitates interaction with numerous class I receptors. It may also be pertinent to suggest that the position of the NR box may influence its role in NR interaction. Because it lies at the extreme C terminus of the protein, it may have more freedom of movement and increased exposure than those LXXLL motifs of the p160 family that reside centrally within the protein, putatively implying that subtle allosteric changes to the NR box region of Tip60 may aid in its array of steroid receptor interactions.

In contrast to the binding activity of Tip60 toward class I receptors, Tip60 ultimately fails to interact with class II NRs. We speculate that the single NR box in Tip60, rather than the sequence of the NR box, may aid in class II NR discrimination. Both crystallographic and mutagenesis studies (22, 24) have demonstrated that class II NRs require the intercalation of two NR boxes from a single co-activator for a successful protein-protein interaction. For example, SRC-1 utilizes two NR boxes from the central nuclear interacting domain to interact directly with peroxisome proliferator-activated receptor γ, in which each of the LBDs in the dimer are contacted by a single LXXLL motif (24). The presence of a single NR box in Tip60, and the failure for Tip60 to homodimerize, suggests that only one of the two receptor LBDs has the potential to be bound by Tip60, which may be unable to sustain an avid interaction with class II receptors. It is interesting to consider that such a characteristic of Tip60 has evolved to limit the number of co-activators sharing the capacity to up-regulate class II NRs. Considering that both p300/ebp and members of the p160 co-activator family increase class II NR-mediated transcription, and they undoubtedly share similar patterns of tissue expression, suggests that there is a plentiful supply of co-activators within a given cell to up-regulate class II receptor activity. Furthermore, a more defined and limited subset of co-activators capable of up-regulating a given NR will also lend to greater specificity for NR activation.

Further regulation of co-activator activity is thought to be achieved through the formation of large multimeric co-activator complexes, such as the vitamin D receptor-interacting proteins, thyroid hormone receptor-associating proteins (40, 41), and p300-CBP-associating factor complexes (42). Interestingly, the yeast orthologue of Tip60, Esa-1p, is the catalytic subunit of the yeast NuA4 (nucleosomal acetyltransferase of histone H4) complex that preferentially acetylates histone H4 to enhance the activity of numerous yeast transcription factors (43, 44). It is therefore important to determine whether Tip60 acts in a larger complex to enhance the activity of nuclear receptors. The fact that we show Tip60 to be unable to dimerize might suggest that only one Tip60 molecule binds per nuclear receptor dimer, in which the single NR box of Tip60 binds to one AF-2 domain of the receptor dimer. It is interesting to speculate that the LXXLL motif-binding pocket of the AR remains suitably exposed when bound to the single NR box of Tip60 such that other co-activators are allowed to bind and further enhance the activity of the receptor through the formation of a larger Tip60-containing complex.

In the past year, Nakatani and co-workers (45) have reported a Tip60-containing complex that is important for DNA repair and apoptosis, two novel activities for an identified co-activator molecule to date. It will be interesting to define the mode of interaction between Tip60 and the putative protein partners in the complex and whether the LXXLL motif of Tip60 is important for such contacts.

In summary, the results suggest that Tip60 interacts in a novel manner with the AR and is a co-activator specific for steroid hormone receptors. The mechanism of Tip60 interaction with ER-α/β, PR, and GR is as yet undefined, but it is likely that the LXXLL motif is involved in the protein-protein interaction. The production of LXXLL-containing peptides specific for NR LBDs has been suggested to constitute a mechanism for blocking co-activator-NR interaction that may have clinical relevance in the treatment of dysfunctional NRs. Tip60 up-regulates endogenous AR activity in LNCaP prostate cancer cells (31), indicating that Tip60 may be important in AR function during normal and transformed cell development. The finding that the lone NR box of Tip60 mediates the Tip60-AR interaction suggests a potential mechanism for inhibiting Tip60-mediated AR enhancement via the production of a peptide encompassing the LXXLL motif of Tip60. Whether such strategies arise in the clinical context will remain to be seen, but it does lend to another level of potential targets in the fight against prostate cancer.

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REFERENCES

1. Gnanapragasam, V. J., Robson, C. N., Leung, H. Y., and Neal, D. R. (2000) Biochim. Biophys. Acta 1498, 106–120
2. Jenster, G. (1999) Semin. Oncol. 26, 457–461
3. Mangelsdorf, D. J., Thummler, C., Beat, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Champon, P., and Evans, R. M. (1999) EMBO J. 18, 6068–6076
4. Hard, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L., P., Carstedtduke, J., Yamamoto, K. R., Gustafsson, J. A., and Kaptein, R. (1990) Science 245, 157–160
5. Bannister, A. J., and Kouzarides, T. (1996) EMBO J. 15, 4608–4618
6. Bevan, C. L., and Gustafsson, J. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12,736–12,741
7. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) EMBO J. 16, 567–574
8. Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) Nature 389, 753–758
9. Torchio, J., Glass, C., and Rosenfeld, M. G. (1998) Curr. Opin. Cell Biol. 10, 373–383
10. Ultey, R. T., Ikeda, K., Grant, P. A., Cote, J., Steger, D. J., Eberharder, A., John, S., and Workman, J. L. (1998) Nature 394, 488–502
11. Onate, S. A., Tsai, S. Y., Tsai, M. J., and Malley, B. W. (1999) Mol. Cell. Biol. 19, 324–332
12. Thompson, J., Saatcioglu, F., Janne, O. A., and Palvimo, J. J. (2001) Mol. Cell. Biol. 21, 1749–1759
13. Ogryzko, V. V., Kotani, T., Zhang, X., Schiltz, R. L., Howard, B. H., Qin, J., and Nakatani, Y. (1998) EMBO J. 17, 2735–2809
14. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1999) Nature Genet. 22, 383–389
15. Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Fletterick, R. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3343–3356
16. Hard, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carstedtduke, J., Yamamoto, K. R., Gustafsson, J. A., and Kaptein, R. (1990) Science 245, 157–160
17. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1999) Nature 395, 137–143
18. Struhl, K. (1998) Genes Dev. 12, 3357–3368
19. Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000) Cell 102, 463–473
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