Transcriptional Synergy between Melanoma Antigen Gene Protein-A11 (MAGE-11) and p300 in Androgen Receptor Signaling*

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Androgen receptor (AR)–mediated gene regulation involves interactions with coregulatory proteins that include the melanoma antigen gene protein-A11 (MAGE-11). To understand the functional significance of sequence similarity between MAGE-11 and the adenovirus early protein E1A, we determined whether MAGE-11 contributes to AR transcriptional activity through an interaction with p300, a potent and ubiquitous transcriptional regulator. Here, we report that MAGE-11 interacts with the NH2-terminal region of p300 through the MAGE-11 MXXIF motif, with transcriptional activity depending on the MAGE-11 F-box and MAPK phosphorylation. The MAGE-11- and p300-dependent increase in AR transactivation required the NH2-terminal regions of AR and p300, p300 acetyltransferase activity, and the AR FXXLF motif interaction with MAGE-11. MAGE-11 linked AR to p300 and the p160 coactivator, transcriptional intermediary protein 2 (TIF2). The p300 NH2-terminal FXXLF motif was required for transcriptional activation by TIF2. Increased expression of p300 decreased the ubiquitinylation of MAGE-11 and transiently increased endogenous MAGE-11 levels. Autocatalysis p300 and decreased acetylation of TIF2 were evident in the MAGE-11, p300, and TIF2 complex. The studies suggest that MAGE-11 links NH2-terminal domains of AR and p300 to promote transcriptional synergy through a cadre of FXXLF-related interacting motifs.

The androgen receptor (AR) regulates gene transcription required for male sex development by binding androgens with high affinity and interacting with coregulatory proteins. AR transcriptional activity derives from activation function 1 (AF1) in the NH2-terminal region and the activation function 2 (AF2) hydrophobic surface in the ligand binding domain (Fig. 1). AR AF2 interacts with p160 coactivator LXXLL motifs and the AR NH2-terminal FXXLF motif that mediates the androgen-dependent NH2- and carboxyl-terminal (N/C) interaction (1). The competitive relationship between AR FXXLF and p160 coactivator LXXLL motif binding to AF2 exerts a regulatory effect on p160 coactivator-induced AF2 activity that favors transactivation from the AR NH2-terminal AF1 region (2, 3). These findings, together with the greater AR AF2 binding affinity for the AR FXXLF than coactivator LXXLL motifs (4), suggest that AF1 in the AR NH2-terminal region is the principal AR activation domain.

Although regulation of steroid receptors through the AF2 site is relatively well described, the molecular mechanisms by which coregulatory proteins mediate AR AF1 activity are not understood. One recently described AR coregulator that interacts with the AR NH2-terminal region, modulates the AR N/C interaction, and increases AR transcriptional activity is the melanoma antigen gene protein-A11 (MAGE-11). MAGE-11 binds the AR NH2-terminal FXXLF motif and interacts directly with p160 coactivators to increase AR transcriptional activity (Fig. 1) (5, 6). The interaction between AR and MAGE-11 is mediated by a MAGE-11 F-box and modulated by epidermal growth factor (EGF)-induced phosphorylation of Thr-360 within the F-box and monoubiquitinylatation at lysine residues outside the F-box (6, 7).

The functional importance of MAGE-11 as an AR coregulator is supported by its regulated expression in normal human reproductive physiology and deregulated expression in cancer. MAGE-11 levels increase by ~50-fold in the epithelium of the normal cycling human endometrium during the window of implantation (8) and to a similar extent in the CWR22 human prostate cancer xenograft during castration-recurrent growth. MAGE-11 levels were increased up to ~1000-fold in a subset of patients with castration-recurrent prostate cancer (9). Increased expression of MAGE-11 provides a mechanism to enhance AR signaling and is consistent with the functional importance of AR during prostate cancer progression.

The ability of MAGE-11 to increase the constitutive activity of AR (1–660), an NH2-terminal and DNA binding fragment, when MAGE-11 itself lacks inherent transcriptional activity, provided support for the idea that MAGE-11 functions as an...
AR coregulator through direct interactions with coactivators. Based on sequence similarity between MAGE-11 and the adenovirus early protein E1A, an oncogenic protein that interacts strongly with p300, we investigated the possible interaction between MAGE-11 and p300, a ubiquitous transcriptional regulator and potent acetyltransferase (Fig. 1). p300 activates gene transcription by acetylating histones and transcription factors and as a coactivator of promoter-specific nuclear receptors (10). Studies described here address the mechanisms underlying the MAGE-11-dependent increase in AR transcriptional activity arising from AR NH2-terminal AF1.

EXPERIMENTAL PROCEDURES

Expression Plasmids and Reporter Vectors—pCMV-hAR (11), pCMV-AR-(1–660) (12), VP-AR and VP-AR-(1–660) with wild-type sequence, L26A,F27A (LFAA) and Δ9–28 mutants (1, 13), pSG5-MAGE-11 (pSG5-MAGE), VP-MAGE-11 (2–429) (VP-MAGE), and pCMV-FLAG-MAGE-11 (2–429) (FLAG-MAGE) (5), pSG5-HA-MAGE-(112–429) and pCMV-FLAG-ubiquitin (FLAG-Ub) (7), wild-type and F-box mutants of pSG5-HA-MAGE fragments and pSG5-HA-MAGE-(112–429) (6), pSG5-Transcriptional intermediary factor 2 (TIF2) (14), and pCMV-FLAG-TIF2 (6) expression plasmids were described. Reporter vectors included prostate-specific enhancer–luciferase (PSA-Enh-Luc) that contains the −3935 to −4326 upstream enhancer region of the PSA gene cloned upstream of an E4 TATA box in pGL3 basic (15) and 5×GAL4Luc3 that contains five copies of the GAL4 upstream enhancer element (16, 17). pCMV, pSG5, VP16 activation domain, and GAL-DNA binding domain expression vectors contain the SV40 origin of replication for plasmid amplification in SV40-transformed simian COS cells.

pSG5-HA-p300 was prepared by blunt-end cloning a NotI/HindIII fragment of CMVb-NHAp300 (provided by Dr. David Livingston, Dana-Farber Cancer Institute) into the BamHI site of pSG5. pSG5-HA-p300-D1399Y, I97A,F98A, and I188A,F189A were created by QuikChange site-directed mutagenesis (Stratagene) using pSG5-HA-p300 as template. GAL-MAGE fragments were created by PCR mutagenesis. pCMV-FLAG-p300 fragments were created by PCR amplifying pSG5-HA-p300, digesting with EcoRI/Clal (FLAG-p300 (2–300) and (2–357)) or HindIII/Clal (FLAG-p300 (270–357), (300–670), (357–670), and (450–670)) and ligating into the same sites of pCMV-FLAG. Additional FLAG-p300 fragments were created by ligating EcoRI/XbaI fragments of GAL-p300 (2–270) and (2–300) and a Sall/XbaI fragment of GAL-p300 (230–670) into the same sites of pCMV-FLAG. GST-p300 (2–357) was created by inserting the EcoRI/Sall fragment from FLAG-p300 (2–357) into the same sites of pGEX-4T-1.

GAL-p300 (2–300), (230–670), (501–999), (1000–1600), (1601–1900), and (1901–2414) were created by PCR amplifying the indicated regions of pSG5-HA-p300 and cloning Sall/Clal-digested fragments into the same sites of GAL0. GAL-p300 (2–357) was created by digesting FLAG-p300 (2–357) with EcoRI/XbaI and ligating the fragment into the same sites of GAL0. GAL-p300 (2–300)–L36A,F37A was created using QuikChange site-directed mutagenesis and GAL-p300 (2–300) as template. GAL-p300 (230–670) was created by PCR amplifying pSG5-HA-p300, digesting with Sall/Clal, and inserting the fragment into GAL0. Other GAL-p300 fragments were created by PCR amplifying GAL-p300 (2–300), digesting the fragments with Sall/Clal (GAL-p300 (2–270), (2–230), (2–180), (90–300), and (90–270)) or EcoRI/Clal (GAL-p300 (48–300)), and ligating into the same sites of GAL0. All PCR-amplified regions were verified by DNA sequencing.

Expression Studies—Monkey kidney CV1 cells (4×105/6-cm dish) were transfected using calcium phosphate DNA precipitation (3) with 0.1 μg of pCMV-AR and the indicated amounts of wild-type and mutant pSG5-MAGE, pSG5-TIF2, and pSG5-HA-p300 and 3 or 5 μg PSA-Enh-Luc. After transfection and 24 h later, cells were placed in serum-free, phenol red-free media in the absence and presence of 1 nM dihydrotestosterone (DHT). The next day, the medium was replaced, and 24 h later cells were extracted in 0.25 ml of luciferase lysis buffer containing 1% Triton X-100, 2 mM EDTA, and 25 mM Tris phosphate, pH 7.8. Luciferase activity was measured using an automated Lumistar Galaxy multiwell plate luminometer (BMG Labtech). Luciferase activity shown is representative of at least three independent experiments, and the graphs show the mean ± S.E.

Two-hybrid transfection assays were performed in HeLa human epithelial cervical carcinoma cells using FuGENE 6 (Roche Applied Science) by plating 5×104 cells/well in 12-well plates (4). Cells were transfected with 0.1 μg/well 5×GAL4 Luc3 and 0.05 μg GAL-p300 fragments with 0.05 μg of pVP16 empty vector or VP-MAGE. The day after transfection, cells were transferred to serum-free medium and incubated overnight at 37 °C before luciferase activity was measured.

SiRNA was transfected into COS cells (4×104/well in 6-well plates) using Lipofectamine 2000 (Invitrogen) in the absence of antibiotics with 1 μg of pSG5-MAGE, 1 μg of pSG5-TIF2, or 2 μg of pSG5-HA-p300 in the absence and presence of 5 nM MAGE-11 siRNA-2 GCACUGUAUCCUGCAUGCUAUU,
MAGE-11 siRNA-3 CAACUGUCUUUUGGCAUUGGUU, 5 nm TIF2 siRNA-3 GAUCAGAUGACUAUUAA, 10 nm p300 siRNA-1 GAAUUAGGUUACACAACA, p300 siRNA-2 GGACUCCCAAUGAAGUA, p300 siRNA-3 GAACAGUCUCCAAACCA, p300 siRNA-4 GCACAGUUGCAGUGAUAAU, and nonspecific siRNA-5 UGGUUAACUGACUAA (Dharmacon RNA Technologies). The day after transfection, cells were harvested or transferred to serum-free medium and harvested 24 h later for immunoblot analysis. For siRNA experiments in HeLa cells, cells were plated in antibiotic-free medium and transfected using Lipofectamine 2000.

**Immunoblotting**—Relative expression levels of wild-type and mutant vectors were determined by transfecting COS cells (2 × 10^6/10-cm dish) with 4–8 μg of DNA using DEAE-dextran (4, 18). The next day, cells were transferred to serum-free media with or without 1 μM MG132 proteosome inhibitor (Sigma) and 24 h later harvested in phosphate-buffered saline. Cell pellets were extracted in 0.1 to 0.2 ml of immunoblot lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.5% deoxycholate, 0.15 M NaCl, 0.5% NaF, 2 mM sodium vandate, 50 mM Tris-HCl, pH 7.5, with 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and complete protease inhibitor mixture (Roche Applied Science). Cell extracts were analyzed on 8, 10, or 12% acrylamide gels containing SDS. Proteins were transferred overnight to nitrocellulose membranes at 4 °C, and transblots were probed with the following antibodies: rabbit anti-human AR-32 immunoglobulin G (0.4 μg/ml); rabbit anti-GAL4-DNA binding domain (Santa Cruz Biotechnology sc-577, 1:500 dilution); rabbit polyclonal FLAG-MAGE antibody-1 (0.5 μg/ml) raised against purified baculovirus-expressed FLAG-tagged human MAGE-11; affinity-purified rabbit polyclonal human p300 (C-20) antibody (Santa Cruz Biotechnology sc-585, 1:75–200 dilution); mouse anti-β-actin (Abcam, 1:5000 dilution); mouse anti-TIF2 (BD Transduction Laboratories, 1:100–250 dilution); rabbit anti-MAGE-11 peptide antibodies MAGE-Ab-13–26, 59–79, and 94–108 immunoglobulin G (4–10 μg/ml); mouse anti-human AR-32 immunoglobulin G (0.4 μg/ml); rabbit anti-GAL4-DNA binding domain (Santa Cruz Biotechnology sc-577, 1:500 dilution); rabbit polyclonal FLAG-MAGE antibody-1 (0.5 μg/ml) raised against purified baculovirus-expressed FLAG-tagged human MAGE-11; affinity-purified rabbit polyclonal human p300 (C-20) antibody (Santa Cruz Biotechnology sc-585, 1:75–200 dilution); mouse anti-β-actin (Abcam, 1:5000 dilution); mouse anti-TIF2 (BD Transduction Laboratories, 1:100–250 dilution); rabbit anti-MAGE-11 peptide antibodies MAGE-Ab-13–26, 59–79, and 94–108 immunoglobulin G (4–10 μg/ml); mouse anti-FLAG M2 monoclonal antibody (Sigma F3165, 1:500–2000 dilution); rabbit polyclonal acetylated lysine antibody (Cell Signaling 9441, 1:1000 dilution); rabbit anti-hemagglutinin tag (Abcam ab9110, 1:5000–20000 dilution); rabbit anti-GFP (Santa Cruz Biotechnology sc-577, 1:500 dilution); rabbit anti-HA antibody (Abcam ab1100, 1:5000–20000 dilution); rabbit anti-Myc antibody (Santa Cruz Biotechnology sc-577, 1:500 dilution); rabbit polyclonal FLAG-MAGE antibody-1 (0.5 μg/ml) raised against purified baculovirus-expressed FLAG-tagged human MAGE-11; affinity-purified rabbit polyclonal human p300 (C-20) antibody (Santa Cruz Biotechnology sc-585, 1:75–200 dilution); mouse anti-β-actin (Abcam, 1:5000 dilution); mouse anti-TIF2 (BD Transduction Laboratories, 1:100–250 dilution); rabbit anti-MAGE-11 peptide antibodies MAGE-Ab-13–26, 59–79, and 94–108 immunoglobulin G (4–10 μg/ml); mouse anti-FLAG M2 monoclonal antibody (Sigma F3165, 1:500–2000 dilution); rabbit polyclonal acetylated lysine antibody (Cell Signaling 9441, 1:1000 dilution); rabbit anti-hemagglutinin tag (Abcam ab9110, 1:5000–20000 dilution); rabbit anti-VP16 activation domain (Abcam ab4809, 1:2000 dilution). Gels were calibrated using EZ-Run prestained Rec protein ladder (Fisher Bioreagents), and immunoreactivity was detected by chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Pierce).

Immunoprecipitation was performed by transfecting COS cells as above using two 10-cm plates/group. For protein acetylation assays, COS cells were treated for 1 h prior to harvest with histone deacetylase inhibitor 5 mM nicotinamide with and without 5 mM sodium butyrate. Cell lysates were prepared in 0.25 volume of immunoprecipitation (IP) lysis buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.15 M NaCl, 0.05 M NaF, 1 mM EDTA, and 50 mM Tris, pH 7.6, with 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, complete protease inhibitor mixture (Roche Applied Science) with or without histone deacetylase inhibitors 5 mM nicotinamide and 5 mM sodium butyrate. Lysates were diluted 4-fold with IP buffer lacking deoxycholate and precleared for 1 h at 4 °C with 0.1 ml of Sepharose CL-4B (Sigma). To communoprecipitate endogenous p300 with FLAG-MAGE, human embryonic kidney 293 (HEK293) cells maintained in minimal essential medium containing 2 mM L-glutamine, 10% fetal bovine serum, and penicillin and streptomycin (15 × 10^6 cells/10-cm dish, seven dishes/group) were transfected using DEAE-dextran. HEK293 cell extracts were prepared in 0.5 volume of IP lysis buffer and diluted with an equal volume of IP buffer without deoxycholate prior to preclearing for 10 min at 4 °C with 50 μl of Sepharose CL-4B. COS and HEK293 cell extracts were transferred to 15 μl of anti-FLAG M2-agarose (Sigma) for 2 h or overnight incubation at 4 °C. The next day, samples were pelleted and washed with IP lysis buffer without deoxycholate, resuspended in 0.05 ml of 2× SDS sample buffer containing 3.3% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.12 M Tris-HCl, pH 6.8, incubated for 5 min at 90 °C, and analyzed on immunoblots as described above.

In **Vitro Binding Assays**—Glutathione S-transferase (GST) interaction assays were performed using pGEX-4T-1 empty vector and pGEX-4T-1-p300-(2–357) (GST-p300-(2–357)). 35S-MAGE-11 was prepared using pSG5-MAGE and the TnT T7 Quick-coupled transcription-translation system (Promega) (1, 6, 19). GST fusion proteins were expressed in BL-21 DE3 Escherichia coli with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h at 37 °C. Cell pellets were resuspended in GST binding buffer containing 0.5% Nonidet P-40, 1 mM EDTA, 0.1 M NaCl, 10% glycerol, 20 μg/ml bovine serum albumin, and 20 mM Tris-HCl, pH 8.0, with 1 mM dithiothreitol and complete protease inhibitor mixture (Roche Applied Science). Glutathione-Sepharose 4B (GE Healthcare) was incubated for 1.5 h at 4 °C with GST-0 and GST-p300-(2–357) cell lysates after sonication. Beads were washed with GST buffer, combined with 25 μCl of 35S-MAGE-11, and incubated overnight at 4 °C. The resin was washed, eluted with SDS, and analyzed on an 8–16% gradient minigel containing SDS (Invitrogen). The dried gel was exposed to x-ray film for 48 h.

Chromatin **Immunoprecipitation** (ChIP)—ChIP assays were performed using LAPC-4 human prostate cancer cells (10 × 10^6 cells/10-cm dish, three dishes/group) plated in RPMI 1640 medium (Invitrogen) supplemented with 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, penicillin, and streptomycin. Cells were treated 72 h after plating for 24 h at 37 °C with and without 10 nM DHT and cross-linked using 1% formaldehyde for 10 min at room temperature followed by 0.125 M glycine. After 10 min at room temperature, cells were harvested and washed with phosphate-buffered saline and lysed in 1.2 ml of 1% SDS, 5 mM EDTA, 1 mM phenylmethlysulfonyl fluoride, and 50 mM Tris-HCl, pH 8.1, with and without 10 mM DHT. After a 10-min incubation at 4 °C, cells were sonicated 12 times for 5 s at 50% power to obtain 100–1000-bp DNA fragments. Lysates were clarified by centrifugation and 0.45 ml diluted 10-fold with 1% Triton X-100, 2 mM EDTA, 0.15 M NaCl, 1 mM phenylmethlysulfonyl fluoride, and 20 mM Tris-HCl, pH 8.1, with and without DHT. Samples were precleared for 4 h at 4 °C with 20 μl of protein-A-agarose (Sigma) and pelleted. Immunoprecipitation was performed overnight at 4 °C by adding 10 μg of the following antibodies to 0.75-ml sample: rabbit anti-AR H-280 (Santa Cruz Biotechnol-
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MAGE-11 Interacts with the NH₂-terminal Region of p300—Mammalian two-hybrid assays were performed to investigate an interaction between MAGE-11 and p300. VP-MAGE was expressed with a GAL4-luciferase reporter and GAL4-DNA binding domain fusion proteins containing different regions of p300 (Fig. 3A). MAGE-11 interacted with the p300-(2–300) NH₂-terminal region to a greater extent than with p300-(230–670) (Fig. 3B). MAGE-11 did not interact with the p300-(501–999) Kix domain, 1000–1600-residue Bromo domain, PHD and histone acetyltransferase domains (20–22), or the 1601–1900-residue ZZ and Taz2 domains (23). Constitutive activity of the carboxyl-terminal fragment p300-(1901–2414) that included an IBiD domain (24) was only slightly increased by MAGE-11.

To investigate the influence of MAGE-11 on transcriptional activity from the p300 NH₂-terminal region, additional GAL-p300 fragments were expressed with a MAGE-(112–429) carboxyl-terminal fragment in transient transfection assays using the GAL-luciferase reporter (Fig. 3C). Similar to the two-hybrid interaction results (Fig. 3B), MAGE-(112–429) increased transcriptional activity from GAL-p300-(2–357), -(2–300), -(2–270), and -(230–670) compared with other p300 NH₂-terminal fragments (Fig. 3C). Overall activity decreased with deletion of the p300 NH₂-terminal region in GAL-p300-(48–300) or with mutations in a p300 NH₂-terminal FXFL motif at Ser329–369 shown previously to interact with the AR FXLF motif (4, A and B) (6).

p300 Interacts with the Carboxyl-terminal Region of MAGE-11—Based on the ability of MAGE-(112–429) to increase GAL-p300-(2–300) activity (Fig. 3C), we determined which MAGE-11 regions were required for this effect. Coexpression of full-length MAGE-11 caused less of an increase in GAL-p300-(2–300) activity than MAGE-(112–429), and there was no increase with MAGE-11 fragments that did not include F-box amino acid residues 329–369 shown previously to interact with the AR FXLF motif (Fig. 4, A and B) (6).

A requirement for the MAGE-11 F-box to increase GAL-p300-(2–300) transcriptional activity was supported by the loss in activity associated with a series of single amino acid MAGE-11 F-box mutants (Fig. 4C) that also decreased MAGE-11 binding to the AR FXLF motif (6). Mutation of MAPK site Ser-174 blocked the MAGE-(112–429)-dependent increase in GAL-p300-(2–300) activity and was recovered with...
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FIGURE 3. MAGE-11 interacts with the p300 NH2-terminal region. A, schematic diagram of p300 interaction domains and fragments analyzed for interaction with MAGE-11. B, indicated GAL-p300 vectors (0.05 μg) were transfected into HeLa cells with 0.1 μg of 5×GAL4Luc3 and 0.05 μg of pVP16 empty vector (−) or VP-MAGE. Lower panel, COS cells were transfected with 8 μg of GAL-p300 vectors and incubated with 1 μM MG132 for 24 h prior to harvest. Cell extracts (50 μg of protein/lane) were analyzed on immunoblots using anti-GAL4-DNA binding domain antibody. C, indicated GAL-p300 vectors (0.05 μg) were transfected into HeLa cells with 0.1 μg of 5×GAL4Luc3 and 0.1 μg of pSG5 empty vector (−) or pSG5-HA-MAGE-(112–429). Lower panel, COS cells were transfected with 5 μg of GAL-p300 vectors and incubated with 1 μM MG132 for 24 h and 1 h prior to harvest. Cell extracts (60 μg of protein/lane) were analyzed on the transblot using GAL4-DNA binding domain antibody. HAT, histone acetyltransferase.

The results suggest that the carboxyl-terminal region of MAGE-11 interacts with the NH2-terminal region of p300 and increases p300 transcriptional activity through mechanisms that depend on the MAGE-11 F-box and MAPK phosphorylation. The apparent inhibitory effect of the MAGE-11 NH2-terminal region on GAL-p300 transcriptional activity (Fig. 4B) suggested the presence of an auto-regulatory mechanism.

MAGE-11 MXXIF Motif Interacts with p300—To further characterize the MAGE-11 interaction with p300, transcriptional activation of GAL-luciferase was measured after expressing full-length p300 with a series of GAL-MAGE fragments (Fig. 5A). GAL-MAGE and GAL-MAGE-(112–429) were activated to only a limited extent by p300 (Fig. 5B). In contrast, p300 strongly activated GAL-MAGE-(112–205), -(140–205), -(161–205), -(85–205), and -(85–152) but not -(100–152) (Fig. 5B).

Within the MAGE-(85–205) region that was activated by p300 are two (M/I)XXIF motifs, 94ITQIF98 and 185MDAIF189 (Fig. 5A). The functional importance of the 185MXXIF189 motif was suggested by the decrease in GAL-MAGE-(85–205)-I188A,F189A activity in the presence of p300 and by the lower activity of GAL-MAGE-(140–205)-I188A,F189A that lacked the 94LXXIF98 motif (Fig. 5C). Disruption of the 94LXXIF98 motif in GAL-MAGE-(85–205)-I97A,F98A, in which the 185MXXIF189 motif was intact, had no effect on p300-dependent activity. However, activity decreased with the GAL-MAGE-(85–152)-I97A,F98A,4LXXIF98 motif mutant that lacked the 185MXXIF189 motif (Fig. 5C).

The MAGE-11 185MXXIF189 motif was also required for p300 to increase AR transcriptional activity. Mutations in either the MXXIF or LXXIF motif in full-length MAGE-11 did not significantly decrease AR transcriptional activity when assayed using the PSA-Enh-Luc reporter without coexpression of p300 or TIF2 (Fig. 5D). However, mutations in the 185MXXIF189 motif minimized or eliminated the MAGE-11-dependent increase in AR transcriptional activity with the expression of p300 and/or TIF2. AR activity was similar to wild-type with mutations in the MAGE-11 94LXXIF98 motif.

The results suggest that MAGE-11 MXXIF motif sequence 185MDAIF189 is a primary interaction site for p300 (Fig. 1), and the LXXIF motif sequence 94ITQIF98 is a secondary interaction site. The inability of p300 to increase GAL-MAGE or GAL-MAGE-(112–429) activity while smaller fragments were strongly activated by p300 (Fig. 5B) suggested that the MAGE-11 carboxyl-terminal region has an inhibitory effect.

the MAGE-(112–429)-S174D phosphomimetic (Fig. 4D). A predicted MAGE-11 phosphorylation site at Ser-298 when mutated to alanine decreased the MAGE-(112–429)-dependent increase in GAL-p300 (2–300) activity but was only partially restored with the S298D phosphomimetic.

A direct interaction between MAGE-11 and the p300 NH2-terminal region was supported by in vitro binding studies in which GST-p300-(2–357) interacted with35S-labeled MAGE-11 (Fig. 4E). In addition, endogenous p300 communoprecipitated with FLAG-MAGE in HEK293 cells (Fig. 4F). However, the low levels and cell cycle-dependent expression of endogenous MAGE-11 (see Fig. 11A) hindered further analysis of these protein-protein interactions.

The results suggest that the carboxyl-terminal region of MAGE-11 interacts with the NH2-terminal region of p300 and increases p300 transcriptional activity through mechanisms that depend on the MAGE-11 F-box and MAPK phosphorylation. The apparent inhibitory effect of the MAGE-11
Functional Requirement for AR and p300 NH$_2$-terminal FXXLF Motifs—p300 has an NH$_2$-terminal FXXLF sequence 33FGSLF37 in a similar relative position to the AR FXXLF motif (Fig. 6A). Evidence that the p300 FXXLF motif was required to enhance GAL-p300-(2–300) activity (Fig. 3C), in addition to the close proximity of flanking charged residues (Fig. 6A), suggested that the p300 NH$_2$-terminal FXXLF motif was functionally important. Because previous studies demonstrated AR activation of androgen-responsive enhancer/promoters depends on the AR FXXLF motif (25), we investigated the requirement for NH$_2$-terminal FXXLF motifs in the AR interaction with p300.

Mammalian two-hybrid assays using the GAL-luciferase reporter showed that the AR FXXLF motif was required for an interaction between VP-AR-(1–660) and GAL-p300-(2–300). AR interaction with p300 was eliminated with VP-AR-(1–660)-L26A,F27A in which the AR FXXLF motif was mutated (Fig. 6B). Deletion of the p300 FXXLF motif in GAL-p300-(2–300) and 0.1 μg of pSG5 empty vector (−) or pSG5-HA-MAGE-(2–429), -(112–429), -(112–362), -(112–307), -(112–298), or -(112–276); C, 25 ng of GAL-p300-(2–300) and 0.1 μg of pSG5 (−) or wild-type (WT) pSG5-HA-(112–429) or indicated F-box mutant; and D, 0.05 μg of GAL-p300-(2–300) and 0.1 μg of pSG5 (−) or WT pSG5-HA-MAGE-(112–429) or S174A, S174D, S298A, or S298D mutant. B–D, lower panels, COS cells were transfected with 4 μg of pSG5 (−) or the indicated pSG5-HA-MAGE vectors. Cells expressing GAL fusion proteins were treated with 1 μM MG132 for 24 h and 1 h prior to harvest. Transblots of cell extracts for GAL4 fusion proteins (60 μg of protein/lane) and HA-MAGE (30 μg of protein/lane) were probed using GAL or hemagglutinin (HA) antibodies. E, in vitro interaction between MAGE-11 and p300 was performed using GST-p300-(2–357) and 35S-labeled MAGE-11. The input represents 10% of the total reaction. F, endogenous p300 was immunoprecipitated from HEK293 cells transfected with 3 μg of FLAG empty vector (−) (lane 1) or FLAG-MAGE (lane 2). Cells were treated with 0.1 μg/ml EGF and 1 μM MG132 24 h prior to harvest and incubated for 2 h with FLAG affinity resin. Cell extracts (0.15 mg of protein/lane) and immunoprecipitates were analyzed on transblots probed using FLAG and p300 antibodies.
expressed with TIF2 in a transient transcription assay using the GAL-luciferase reporter. GAL-p300-(2–300) was strongly activated by TIF2 (Fig. 6D) in a p300 FXLFL motif-dependent manner. TIF2-dependent GAL-p300-(2–300) activity decreased with GAL-p300-(48–300) in which the p300 FXLFL motif was deleted and with GAL-p300-(2–300)-L36A,F37A in which the p300 FXLFL motif was mutated.

A contribution of MAGE-11 to the TIF2-dependent increase in GAL-p300-(2–300) activity was further suggested through the use of siRNAs to lower protein levels. MAGE-11 siRNA-2 reduced MAGE-11 levels (Fig. 7A, top panel) and inhibited inherent GAL-p300-(2–300) transcriptional activity but did not eliminate the increase with TIF2 (Fig. 7B). MAGE-11 siRNA-3 was less effective in decreasing MAGE-11 levels (Fig. 7A), and the TIF2-induced increase in GAL-p300-(2–300) activity was similar to that seen in the absence and presence of nonspecific siRNA. TIF2 siRNA-3 decreased both TIF2 expression (Fig. 7A, bottom panel) and the TIF2-
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Dependence of GAL-p300-(2–300) transcriptional activity on MAGE-11 and TIF2. A, inhibition of MAGE-11 (upper panel) and TIF2 (lower panel) expression by siRNA was performed by transfecting COS cells using Lipofectamine 2000 with 0.1 μg of pSG5 empty vector (lane 1), 1 μg of pSG5-MAGE (top panel), or pSG5-TIF2 (bottom panel) in the absence and presence of 5 nm MAGE-11 siRNA-2 or -3, TIF2 siRNA-3, and nonspecific (NS) siRNA-5. The next day, cells were harvested (TIF2) or transfected to fresh serum-free medium and harvested 24 h later (MAGE-11). Cell extracts for MAGE-11 (10 μg of protein/lane) and TIF2 (40 μg of protein/lane) were analyzed on transblots probed with MAGE-Ab (94–108), TIF2, and β-actin antibodies. B, HeLa cells (10^7 well in 12-well plates) were transfected using Lipofectamine 2000 with 0.1 μg of 5×GAL4Luc3 and 0.05 μg of p300-(2–300) in the absence and presence of 0.1 μg of pSG5 empty vector (−) or pSG5-TIF2 in the absence and presence of 5 nm nonspecific siRNA-5 (NS-5), MAGE-11 siRNA-2 or -3, and TIF2 siRNA-3.

The requirement for endogenous p300 in AR N/C interaction-dependent AR transactivation was demonstrated further using p300 siRNA. p300 siRNA-1, -3, and -4 each decreased p300 levels (Fig. 8B) and decreased the androgen-dependent activity of AR-(507–919) and AR-(1–503) coexpression with MAGE-11 in the presence of DHT (Fig. 8C). Specificity of siRNA inhibition was indicated by wild-type activity associated with the AR-(507–919) alone (26). AR-(507–919) alone did not activate a PSA-Enh-Luc reporter in the presence of DHT with or without coexpression of MAGE-11 or p300 alone or together (Fig. 8A). However, expression of AR-(507–919) with AR-(1–503), an AR NH2-terminal fragment that lacks the AR DNA and ligand binding domains, resulted in androgen-dependent activity that was increased by expressing MAGE-11 or p300 and was highest when all were expressed together. An interaction between AR-(507–919) and AR-(1–503) was required for transcriptional activation and depended on AR FXXLF motif binding to AF2 in the ligand binding domain (1). Dependence on the AR N/C interaction for the increase in activity by MAGE-11 and p300 was shown by loss of AR-(507–919) activity expressed with AR FXXLF motif mutant AR-(1–503)-L26A,F27A with or without MAGE-11 or p300 alone or together.

To demonstrate further that MAGE-11 functionally links AR and p300 through the AR FXXLF motif, GAL-AR-(16–36), which contains only the AR NH2-terminal FXXLF motif region, was expressed with wild-type or mutant MAGE-11 and p300 alone or together in the presence of a GAL-luciferase reporter.

proteins and that the p300 FXXLF motif facilitates transcriptional activation by TIF2 (Fig. 1).

Multiple Functions of the AR FXXLF Motif—Previous studies have demonstrated the requirement for the androgen-dependent AR N/C interaction between the AR FXXLF motif and AF2 in AR activation of androgen-responsive enhancer/promoters (1, 25). These findings, together with the requirement for the AR FXXLF motif in functional interactions involving MAGE-11 and p300, provided evidence that the AR N/C interaction influences AR transactivation involving p300.

To investigate this further, we determined the effect of MAGE-11 and p300 using an experimental paradigm that depended on the androgen-dependent AR N/C interaction. AR-(507–919) is an AR DNA and ligand binding domain fragment that lacks the NH2-terminal region and retains high affinity androgen binding (26). AR-(507–919) alone did not activate a PSA-Enh-Luc reporter in the presence of DHT with or without coexpression of MAGE-11 or p300 alone or together (Fig. 8A). However, expression of AR-(507–919) with AR-(1–503), an AR NH2-terminal fragment that lacks the AR DNA and ligand binding domains, resulted in androgen-dependent activity that was increased by expressing MAGE-11 or p300 and was highest when all were expressed together. An interaction between AR-(507–919) and AR-(1–503) was required for transcriptional activation and depended on AR FXXLF motif binding to AF2 in the ligand binding domain (1). Dependence on the AR N/C interaction for the increase in activity by MAGE-11 and p300 was shown by loss of AR-(507–919) activity expressed with AR FXXLF motif mutant AR-(1–503)-L26A,F27A with or without MAGE-11 or p300 alone or together.

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Neither MAGE-11 nor p300 alone increased GAL-AR-(16–36) activity but together were strongly synergistic (Fig. 8D). Mutations in the MAGE-11 189–MXXIP189 motif that was required for MAGE-11 to interact with p300 (Fig. 5) reduced p300 activation of GAL-AR-(16–36), although there was only a slight decrease with mutations in the MAGE-11 194–LXIP194 motif, in agreement with the results in Fig. 5. Dependence of MAGE-11 and p300-induced GAL-AR-(16–36) activity on the AR FXXLF motif interaction with MAGE-11 was demonstrated by loss of transcriptional activity with the L358A and T360A MAGE-11 F-box mutants and with the MAGE-11 ubiquitylation site mutant K240A,K245A, where each mutation disrupted MAGE-11 binding to the AR FXXLF motif (6, 7). Dependence on the AR FXXLF motif was shown by the absence of GAL-AR-(16–36)-L26A,F27A FXXLF motif mutant activity when expressed with MAGE-11 and p300. GAL-AR-(16–36) activity in the presence of MAGE-11 was also reduced when coexpressed with p300-D1399Y (Fig. 8D), a p300 acetyltransferase mutant (27).

The results suggest that AR transcriptional activity is enhanced by the ability of MAGE-11 to link AR and p300 by binding the same AR LF motif required for MAGE-11-dependent GAL-AR-(16–36) activity with the p300 acetyltransferase mutant led us to investigate the requirement for p300 acetyltransferase activity in the MAGE-11-dependent AR activity. AR transcriptional activity in the presence of MAGE-11 or TIF2 alone or together increased with p300 but not with p300-D1399Y when assayed using a PSA-Enh-Luc reporter (Fig. 9A). Similarly, the p300-dependent increase in GAL-MAGE-(85–205) and -(140–205) activity was eliminated with the p300-D1399Y acetylation mutant (Fig. 9B).

Because protein stability can be influenced by acetylation or ubiquitylation of lysine residues (28), we determined whether p300 acetyltransferase activity influenced MAGE-11 ubiquitylation. Coexpression of HA-MAGE-(112–429) with FLAG-ubiquitin followed by immunoprecipitation using a FLAG-affinity resin demonstrated the ubiquitylation of MAGE-11 (Fig. 9C) as reported previously (7). Coexpression of p300 or the p300-D1399Y acetyltransferase mutant inhibited the ubiquitylation of MAGE-11 (16–429). However, there was no evidence that MAGE-11 was acetylated by p300 (data not shown), although acetylated forms of TIF2 and p300 were present in the complex with MAGE-11 that were not detected with p300-D1399Y and MAGE-11 (Fig. 10A). Acetylation of TIF2 was reduced with the coexpression of MAGE-11 and was not detected with p300-D1399Y (Fig. 10B).

The results indicate that p300 acetyltransferase activity is required for MAGE-11 and p300 to increase AR transcriptional activity. Although MAGE-11 was not acetylated by p300 even though there was evidence for the acetylation of both p300 and
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The time- and p300-dependent transient increase in endogenous MAGE-11 suggested that p300 influences the cell cycle expression of MAGE-11.

To obtain evidence that endogenous p300 is associated with AR and MAGE-11 at an androgen-responsive gene enhancer, ChIP assays were performed using LAC-P prostate cancer cells that express AR, MAGE-11, p300, and increased levels of PSA in response to androgen. The extent of amplified DNA using PSA upstream enhancer-specific primers indicated an increased association of AR, p300, and MAGE-11 in the presence of 10 nM DHT.

DISCUSSION

AR Transcriptional Regulation by MAGE-11 and p300—We have demonstrated transcriptional synergy between AR, the AR coregulator MAGE-11, and p300, a ubiquitous and potent transcriptional regulator and acetyltransferase (22, 29, 30). MAGE-11 links the NH2-terminal regions of AR and p300 and increases AR transcriptional activity dependent on p300 acetyltransferase activity. A transcription complex involving AR, MAGE-11, p300, and increased p300, a ubiquitous and potent transcriptional regulator and acetyltransferase (22, 29, 30). MAGE-11 links the NH2-terminal regions of AR and p300 and increases AR transcriptional activity dependent on p300 acetyltransferase (22, 29, 30). MAGE-11 links the NH2-terminal regions of AR and p300 and increases AR transcriptional activity dependent on p300 acetyltransferase (22, 29, 30). MAGE-11 links the NH2-terminal regions of AR and p300 and increases AR transcriptional activity dependent on p300 acetyltransferase (22, 29, 30). MAGE-11 links the NH2-terminal regions of AR and p300 and increases AR transcriptional activity dependent on p300 acetyltransferase (22, 29, 30).
Transactivation Synergy of AR, MAGE-11, and p300

**A**

![Bar graph](image)

**B**

![Diagram](image)

- **Transactivation Synergy of AR, MAGE-11, and p300**

- **Requirement for p300 Acetyltransferase Activity**—p300 has a modular structure of protein interaction sites. These include a cysteine- and histidine-rich Taz1 domain at residues 332–417, a Kix domain (residues 566–646) that interacts with CBP-binding protein CREB, a Bromo domain that interacts with p53 (residues 1053–1156), a PHD domain (residues 1198–1278), a glutamine-rich carboxyl-terminal region that interacts with the p160 coactivator activation domain-1 (14, 35, 36), and a lysine acetyltransferase domain between amino acid residues 1284 and 1517 (22).

The NH$_2$-terminal region of p300 was implicated previously in ligand-dependent nuclear receptor signaling, although the interaction sites were not defined (10, 37). The closely related cyclic AMP-response element-binding protein-binding protein (CBP) (10, 29) mediated ligand-independent effects of the AR NH$_2$-terminal region through the CBP 271–452-amino-acid region (31), and the CBP steroid receptor coactivator 1 (SRC1) interaction domain carboxyl-terminal residues 2058–2130 interact with SRC1 activation domain 1 (AD1) residues 926–960 conserved among p160 coactivators (36, 38). We found that the ability of MAGE-11 and p300 to increase AR transcriptional activity depended on the p300 NH$_2$-terminal FXXLF motif sequence $^{33}$FGSLF$^{37}$. However, the p300 FXXLF motif did not interact directly with MAGE-11 but was required for a functional interaction with the NH$_2$-terminal region of TIF2, and for the MAGE-11-dependent increase in GAL-p300 transcriptional activity.

- **p300 acetyltransferase activity has multiple targets that include acetylation of histone tail lysine residues that open the chromatin structure for active transcription. p300 also acetylates non-histone proteins such as p160 coactivators, p53 and β-catenin (29, 39–42). TIF2 was reported to be acetylated by CBP, and the p160 activator for thyroid hormone and retinoid receptors ACTR was acetylated by p300, which modulated the interaction of activator for thyroid hormone and retinoid receptors with nuclear receptors (43). p300 acetyltransferase activity was also linked to AR signaling.**

- **Increased the interaction with full-length AR and was influenced by the AR FXXLF motif. This suggested that an F-box-like sequence in the NH$_2$-terminal region of p300 may interact with another protein that competes with AR binding to p300. The existence of multiple overlapping motifs and binding surfaces is consistent with MAGE-11 and p300 coordinating temporal interactions during AR transactivation.**

- **Both MAGE-11 and p300 are downstream targets of MAPK (6, 33) and p300 is phosphorylated in a time-dependent manner in response to EGF (34). In response to serum stimulation, MAGE-11 is phosphorylated by MAPK at Ser-174, a conserved residue in the MAGE-A subfamily (6). The MAGE-S174A mutant eliminated the functional interaction between MAGE-11 and p300 that was restored with the S174D phosphomimetic. The MAGE-S174A mutation also diminished the MAGE-11 interaction with the AR FXXLF motif (6). Inhibition of GAL-p300 activity by MAGE-S174A suggests that phosphorylation influences the ability of p300 to function with MAGE-11 to modulate AR transcriptional activity. The results suggest that MAPK phosphorylation at Ser-174 regulates several important aspects of MAGE-11 function.**
Lys-632 and -633 by p300 and p/CAF, a p300-associated protein (44). However, these basic residues are part of the AR bipartite nuclear targeting signal (45) so that inhibitory effects on AR nuclear transport may complicate interpretation of the consequences of acetylation on AR transcriptional activity. The dependence of MAGE-11 on p300 acetyltransferase activity to increase AR transcriptional activity was associated with the acetylation of p300 and TIF2.

Protein stability and function are influenced by the competing and complementary effects of acetylation and ubiquitinylation at lysine residues (28). For example, p300 regulates the levels of p53 by promoting p53 degradation through an association with the MDM2 E3-like ubiquitin ligase (46). Although MAGE-11 was not acetylated by p300, p300 inhibited MAGE-11 ubiquitinylation through an acetylation-independent mechanism. The transient increase in endogenous MAGE-11 when p300 was expressed appeared to be cell cycle-regulated, in agreement with evidence that MAGE-11 is cell cycle-regulated in CWR-R1 prostate cancer cells (6).

MAGE-11 is monoubiquitinylated at Lys-240 and -245 and undergoes polyubiquitinylation in association with its degradation (7). Although the E3 ligase that ubiquitinates MAGE-11 has not been identified, some evidence suggests that MAGE-11 itself functions as part of a ubiquitin ligase complex. MAGE-11 contains an F-box similar to the F-box in S-phase kinase-associated protein 2 (Skp2) and MAGE-11 interacted with Skp1 (6). p300 was reported to have E4 ubiquitin ligase activity (47) that could influence the ubiquitinylation of MAGE-11.

A Primate-specific AR Transcriptional Scaffold—The evolution of MAGE-11 has resulted in its exclusive expression within the primate lineage. This suggests a novel mechanism has arisen in primates to increase AR signaling that is facilitated by interactions between AR, MAGE-11, and p160 coactivators. MAGE-11 may function as a transcription factor scaffold assembly protein for AR. A similar function as a transcription factor nucleation site has been attributed to p300 that may increase gene expression at select enhancers by bridging between DNA-binding nuclear receptors and transcription factors to the basal transcriptional machinery (48, 49). Many transcription factors, nuclear receptors, and signaling proteins interact with p300, and the levels of p300 are thought to be rate-limiting for transcription (50). Competition may exist in recruiting p300 to nuclear receptor-regulated enhancers. Interaction between MAGE-11 and p300 could enhance AR-mediated gene regulation through the more efficient recruitment of a limiting set of proteins that include p300, p160 coactivators, and components of the general transcriptional machinery.

p300 and CBP were shown to increase androgen-dependent and ligand-independent AR transactivation in prostate cancer (44, 51, 52). However, p300 protein levels decrease in prostate cancer cells in response to androgen (53), and p300 mRNA levels were unchanged in the CWR22 human prostate cancer xenograft during tumor progression to castration-recurrent growth when AR mRNA levels increase ~5-fold (9). In contrast, MAGE-11 mRNA levels increased ~50-fold in the castration-recurrent CWR22 tumor, and up to 1000-fold in a subset of castration-recurrent prostate cancer tissue specimens. The increase in MAGE-11 in castration-recurrent prostate cancer provides a mechanism for AR to more effectively recruit p300 and TIF2 and increase transactivation of AR target genes when circulating androgen levels are low.

MAGE-11 and the Adenovirus Early Protein, E1A—p300 was first identified based on its interaction with the adenovirus early oncoprotein E1A that targets p300 during viral infection (31, 35, 54, 55). Sequence similarity between the NH2-terminal regions of MAGE-11 and E1A suggested that MAGE-11 may share properties of E1A and interact with p300. Binding to E1A decreased p300 transcriptional activity through the modification of histone acetyltransferase activity (35, 56–58). MAGE-11 does not interact with the histone acetyltransferase domain of p300 but inhibits p300 acetylation of TIF2. Inhibition of p300 histone acetyltransferase activity was also associated with p300 phosphorylation at Ser-89 (59), suggesting that allosteric effects extend from the p300 NH2-terminal region that could be influenced by MAGE-11.

E1A has a modular structure of interaction motifs for transcriptional regulators associated with the induction of the S-phase of the cell cycle, cell immortalization, cell transformation, and transcriptional regulation (60–62). Based on evidence that E1A binds p300 and promotes cell growth, it was suggested that p300 functions as a tumor suppressor (29). The E1A FX(D/E)XXXL motif sequence 66FPDSVML72 was reported to interact with one of several domains in p300/CBP (63). MAGE-11 contains a similar FX(D/E)XXXL motif sequence 66FREQANL72 at the same relative position to the NH2 terminus as E1A. However, single amino acid mutations in the MAGE-11 FX(D/E)XXXL motif did not interfere with p300 binding (data not shown), suggesting that other MAGE-11 regions possibly homologous to E1A bind p300 (60). Instead, interaction between MAGE-11 and p300 depended on the MAGE-11 MXXIF motif 185MDAIE189 (Fig. 1) and secondarily on LXXIF motif 94ITQIF98, as well as the MAGE-11 F-box and MAPK phosphorylation. The MAGE-11 185MXXIF189 motif and Ser-174 MAPK site are in close proximity and homologous to a region in E1A. The reported E1A 66FX(D/E)XXXL72 interaction site for p300 is preceded by 52QVSQIF66 which, based on sequence homology and position, could be a previously unrecognized E1A interaction site for p300.

Although there are no known human genomic counterparts for E1A, sequence similarity between MAGE-11 and E1A suggests that MAGE-11 shares some functional properties with E1A. The late evolutionary species-specific divergence of the MAGE gene family among primates suggests that like E1A, MAGE-11 may have hijacked transcription factor binding sites required for its function as an AR-selective coregulator.

Acknowledgments—We gratefully acknowledge the technical assistance of John T. Minges, Andrew T. Hnat, and K. Michelle Cobb, and we thank Frank S. French for reviewing the manuscript.

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