Biochemical and Biophysical Research Communications **276,** 144–150 (2000)
doi:10.1006/bbrc.2000.3424, available online at http://www.idealibrary.com on IDEAL®

Inhibiting Proteasomes in Human HepG2 and LNCaP Cells Increases Endogenous Androgen Receptor Levels

Lowell Sheflin, Brendan Keegan, Wan Zhang, and Stephen W. Spaulding
VA Western New York Healthcare System and Department of Medicine and Department of Physiology and Biophysical Sciences, State University of New York at Buffalo, Buffalo, New York 14215

Received July 27, 2000

Treating HepG2 cells with MG132 for 4 h to inhibit proteasomal activity increased androgen receptor immunoreactivity in two major bands with molecular weights of 102 and 110 kDa by 77% each ($P < 0.05$). MG132 treatment also increased the overall level of polyubiquitinated proteins between 66 and 220 kDa by 140% ($P < 0.05$). Antiubiquitin immunoreactivity comigrating with the androgen receptor bands was also increased by MG132 treatment. Two other proteasome inhibitors, lactacystin and epoxomycin, caused similar increases in the androgen receptor in HepG2 cells. Proteosome-inhibition studies conducted in LNCaP cells also showed that the two major androgen receptor bands with molecular weights of 102 and 110 kDa were increased by 85 and 115%, respectively ($P < 0.05$ for both) by MG132 treatment. Overall levels of polyubiquitinated proteins with molecular weights between 66 and 220 kDa increased 365%. Ubiquitin immunoreactivity comigrating with the androgen receptor bands was also significantly increased. Thus inhibiting proteasomes in two human androgen-responsive cell lines increases endogenous androgen receptor levels as well as androgen receptor-associated ubiquitin-modified immunoreactivity. The regulation of steady-state levels of endogenous androgen receptor by proteasomal degradation could be involved in its rapid turnover in the absence of ligand and would provide a mechanism for limiting androgen responses. A PEST sequence similar to one in the vitamin D receptor is present in the hinge region of all known mammalian androgen receptors, suggesting that it may function in proteasome-mediated androgen receptor turnover.

Key Words: **ubiquitination; MG132; GSK3β; PEST; β-catenin; androgen receptor; proteasome; HepG2; LNCaP.**

It is clear that endogenous unoccupied androgen receptors are labile (1–3), and that their level can be regulated post-transcriptionally in several tissues and cell culture models, independent of changes in receptor mRNA level (4, 5). The synthesis and maturation of steroid hormone receptors involves the binding of a variety of chaperones that promote proper folding and trafficking. The mature unliganded apo-androgen receptor remains bound to several chaperone proteins that apparently hold it in a conformation that permits high-affinity ligand binding (6, 7). When androgens bind to the receptor they stabilize it, decreasing the turnover about 6-fold (1, 3). The binding of ligand alters the androgen receptor's interaction with chaperones and accessory proteins, and enhances its ability to activate androgen-responsive gene expression (8–11).

The LNCaP human prostate cell line has been extensively studied as a model for androgen-responsive prostate cancer. The liver is another organ which is an important target of androgen action, as well as being a site of androgen metabolism. The HepG2 human heptablastoma cell line maintains many features of hepatocytes: it expresses immunoreactive androgen receptor and can respond to androgen (12, 13). Since the androgen receptor displays rapid turnover in many human cells (1, 4), we looked for evidence of proteasomal involvement in its metabolism in these two androgen-responsive human cell lines. We found that the proteasome inhibitor MG132 increases androgen receptor immunoreactivity detected with a C-terminal antibody on Western blots of whole cell lysates from both cell lines. Recent evidence for ubiquitin-mediated proteasomal degradation has been presented for the estrogen receptor, the vitamin D receptor and the chicken progesterone receptor (14–18), so our findings on the androgen receptor in two human cells suggest that proteasome-dependent degradation may play a common role in steroid hormone receptor metabolism.

Further, we note that all known mammalian androgen receptors contain a strong PEST (proline-, glutamate-, serine-, and threonine-rich) sequence (19) located in the hinge region (Table 1). The presence of a similar PEST element in the hinge-ligand-binding domain of vitamin D receptor suggests that this degra-

0006-291X/00 $35.00

TABLE 1

Species	PEST sequence	PEST score[a]
Human	KLQEEGEA SSTT**S**P* TEETTQK[b]	18.31
Rabbit	KLQEEGES SSAS**S**P* TEDTTQK	17.92
Rat	KLQEEGEN SSAG**S**P* TEDPSQK	13.84
Mouse	KLQEEGEN SNAG**S**P* TEDPSQK	11.73

[a] As detailed in Ref. (19).

[b] ProSwiss sequence P10275, P49699, P15207, and P19091, respectively.

* Consensus proline-directed GSK3β phosphorylation motif; **S** indicates the location of Ser 650 in the human receptor as numbered in Ref. (2).

dation motif may be involved in regulating the steady-state ubiquitin-mediated turnover of steroid hormone receptors *in vivo.*

METHODS

Cell culture/reagents. HepG2 cells were obtained from ATCC (Rockville, MD, passage 77) and initially expanded in Minimum Essential Media (MEM, Eagle) with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine, Earle's buffered salt solution, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 100 mg/penicillin/streptomycin at 37°C and 5% CO_2. Cells were then divided and grown in 6-well plates in MEM/5% FBS to ~80% confluency (2–3 days). LNCaP cells, also obtained from ATCC (passage 22) were initially cultured in T-25 flasks in RPMI-1640 medium with 300 mg L-glutamine and 25 mM HEPES (pH 7.2), 1 mM pyruvate, 10% FBS and 100 mg/penicillin/streptomycin at 37°C and 5% CO_2. These cells were then divided and grown in 6-well dishes in RPMI-1640 medium until they reached 60–70% confluency. Both cell types were then treated with 20 μM *N*-carboxybenzyl-L-leucinyl-leucinyl-L-leucinal (MG132, Biomol, Plymouth Meeting, PA) in ethanol (0.05% final concentration), or mock-treated with vehicle alone in fresh MEM/5% FBS (or RPMI/10% FBS for LNCaP cells) at 37°C for 4 h. Cells were rinsed with phosphate-buffered saline (PBS) and then scraped with a rubber policeman into high salt buffer (300 mM NaCl, 20 mM HEPES, 5 mM magnesium acetate, 5 mM potassium acetate, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 0.4 mM PMSF, 0.4 mM leupeptin, 1 mM *N*-ethylmaleimide, 1% Triton X-100, 1% NP-40). After homogenizing with 10–15 strokes of a Dounce homogenizer, the homogenate was briefly vortexed, incubated on ice for 20 min, then clarified by centrifugation at 10,000*g* for 10 min at 4°C.

Antibodies. Rabbit polyclonal antibody to the unique 21 amino acid peptide sequence at the C-terminal of the androgen receptor was obtained from Santa Cruz Biotechnology (#sc815, Santa Cruz, CA), anti-ubiquitin antibody was from Sigma (St. Louis, MO), and monoclonal antibody to β-catenin was obtained from Transduction Laboratories (#C19220, Lexington, KY).

SDS–PAGE and Western blotting. Protein concentrations in the samples were determined with bicinchoninic acid (Pierce, Rockford, IL) using BSA as a standard. Samples were boiled in SDS–PAGE sample buffer (final concentrations 62.5 mM Tris–Cl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), briefly centrifuged, and 50 μg supernatant protein/lane was separated on SDS–polyacrylamide gels (10%), using a mini Protean II tank system (Bio-Rad, Hercules, CA). Samples and molecular weight markers (e.g., Sigma 6H) were transferred to 0.45-μm pore size PVDF membrane (Immobilon P, Millipore, Bedford, MA) in transfer buffer (0.025 M Tris, pH 8.3, 0.192 M glycine, 20% methanol) using a mini TransBlot tank system (Bio-Rad). Uniformity of protein transfer was established by Ponceau-staining the membranes in 10% methanol. Tris-buffered saline with 0.1% Tween 20 (TBS-T) was used for all incubations and washes. Blots were blocked for 1 h at 20°C with 5% BSA (for anti-androgen receptor), or with 5% membrane blocking agent from Bio-Rad (for anti-ubiquitin and anti-β-catenin), and then incubated with the appropriate primary antibody for 1 h or overnight at 4°C (for anti-ubiquitin). The blots were washed three times for 15 min in TBS-T after each incubation step. Immunoreactive bands were visualized using a secondary antibody conjugated to alkaline phosphatase, and detected using enhanced chemifluorescence (ECF) according to the manufacturer's instructions (Amersham Life Science, Buckinghamshire, England). Immunoreactivity, measured in arbitrary fluorescence units, was quantified with a STORM imaging system (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Version 5.0). For photographic presentation of the immunoreactivity of the androgen receptor and ubiquitin in the LNCaP cells, blots were also probed with HRP-conjugated secondary antibody, using enhanced chemiluminescent (ECL; Amersham Life Sciences) and exposed to X-ray films which were then scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). Results are expressed as means ± 1 SEM, and the statistical significance of differences was determined by analysis of variance (ANOVA, StatView + Graphics, Abacus Version 1.03). Before reprobing blots with a second antibody, they were washed in methanol for 30 min to remove fluorophore, then the previous antibody was stripped by incubation in 0.2 N NaOH for 30 min.

RESULTS

Response of Androgen Receptor and Polyubiquitinated Protein Levels to MG132: In HepG2 Cells

Treatment with the proteasome inhibitor MG132 for 4 h resulted in a significant increase in the levels of the two major androgen receptor bands detected in HepG2 cell extracts (Fig. 1). Both the ~102 and ~110 kDa molecular weight immunoreactive androgen receptor bands present in HepG2 cell lysates increased by 77% ($n = 6$, $P < 0.05$) following MG132 (Fig. 1A). MG132 also increased slower-migrating higher molecular weight androgen receptor immunoreactive bands that run above the androgen receptor bands, a finding consistent with the endogenous androgen receptor being polyubiquitinated. Thus inhibition of the proteasome in HepG2 cells increases the level of the androgen receptor protein as well as the level of higher molecular weight immunoreactive androgen receptor bands, findings consistent with polyubiquitination of the androgen receptor and proteasome mediated degradation.

After stripping, the blot was reprobed with anti-ubiquitin antibody. MG132 increased the overall level of ubiquitinated protein running between 66 and 220 kDa by 140% (from 4.9 to 11.8 fluorescence units, $n = 6$, $P < 0.05$) (Fig. 1B). To confirm these findings, additional experiments were conducted in HepG2 cells using lactacystin and epoxomycin, two other proteasomal inhibitors which also caused similar increases in the level of the androgen receptor and higher molecular weight immunoreactive androgen receptor bands (not shown).

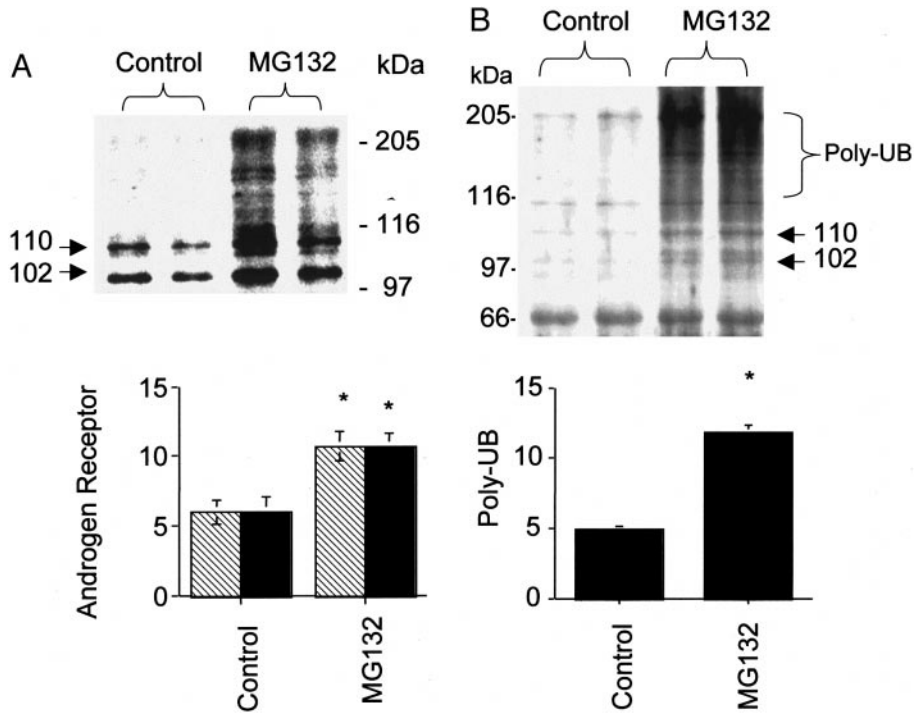

FIG. 1. Responses of androgen receptor and polyubiquitinated protein in HepG2 cells to MG132. (A) Western blot of androgen receptor. Total homogenates (50 μg total protein) from HepG2 cells treated for 4 h with 0.05% ethanol (controls) or MG132 (20 μM in 0.05% ethanol) were separated by 10% SDS–PAGE, and probed with anti-androgen receptor. Immunoreactive bands were detected with a secondary antibody labeled with alkaline phosphatase and ECF substrate (Amersham Life Sciences). Two representative samples from each group (n = 6) are shown. The relative mobility of molecular weight (MW) standards corresponding to 205, 116, and 97 kDa (Sigma 6H) are shown. (Bottom) Graph of androgen receptor levels. Immunoreactivity in the ~102 kDa band (hatched bar) and ~110 kDa band (solid bar) from the six samples in each group were quantified using the ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA). Mean androgen receptor levels (expressed in arbitrary fluorescence units) are shown ±1 standard error. *$P < 0.05$. (B) Western blot of polyubiquitinated protein. The blot shown in A was reprobed using anti-ubiquitin. (Bottom) Graph of polyubiquitinated protein levels. The overall mean immunoreactivity in proteins from 66 and to 220 kDa are plotted ±1 standard error. *$P < 0.05$.

In LNCaP Cells

The LNCaP cells displayed two major immunoreactive androgen receptor bands with mobilities similar to the androgen receptor bands detected in HepG2 cells above and in LNCaP cells (11). However, when compared side-by-side with HepG2 cell homogenates on the same blot, the relative levels of the two androgen receptor bands were much greater in LNCaP cells (not shown). Despite the higher steady-state level in LNCaP cells, the proteasome inhibitor MG132 significantly increased the levels of both immunoreactive androgen receptor bands (Fig. 2A). The ~102 kDa band increased by 85% (from 5.5 to 10.3 fluorescence units; $n = 3$, $P < 0.05$) while the ~110 kDa band increased by 115% (from 5.6 to 12.0 fluorescence units, $n = 3$, $P < 0.05$) (Fig. 2A). Slower-migrating immunoreactive bands also were detected after MG132 treatment (Fig. 2A), again resembling the findings in HepG2 cells.

After stripping the LNCaP blot, it was reprobed with anti-ubiquitin. MG132 treatment resulted in a 365% ($n = 3$, $P < 0.05$) increase in the level of polyubiquitinated proteins running between 66 and 220 kDa (Fig. 2B). A prominent ubiquitinated band with the same mobility as the ~110 kDa androgen receptor increased after MG132 treatment of LNCaP cells.

Thus the results obtained in LNCaP cells closely resemble those obtained in HepG2 cells, and collectively they indicate that endogenous androgen receptor in two human androgen responsive cell lines are ubiquitinated and degraded via the proteasome.

MG132 Stabilizes Endogenous Levels of β-Catenin in HepG2 and LNCaP Cells

As an independent means of assessing proteasomal inhibition, we also measured the response of β-catenin to MG132 in both cells lines (20–23). As shown in Fig. 3, wild-type β-catenin increased by 81% following MG132 treatment ($P < 0.01$, $n = 6$) in HepG2 cells (Fig. 3A) and by 84% ($P < 0.05$, $n = 3$) in LNCaP cells (Fig. 3B). HepG2 cells also coexpress an endogenous mutant isoform, which lacks the GSK3β phosphorylation site required for proteasome-mediated degradation of β-catenin. The level of the mutant β-catenin isoform is ~3-fold higher than the wild type in the

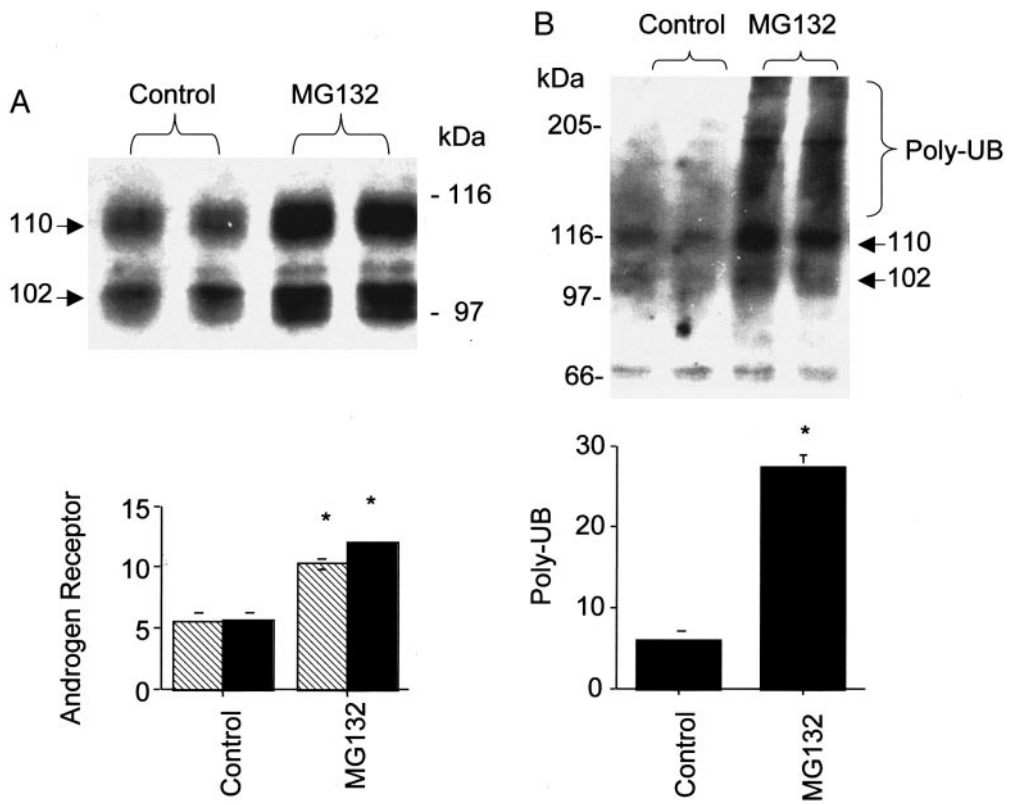

FIG. 2. Responses of androgen receptor and polyubiquitinated protein in LNCaP cells to MG132. (A) Western blot of androgen receptor. Total homogenates (50 μg total protein) from LNCaP cells treated for 4 h with 0.05% ethanol or MG132 (20 μM in 0.05% ethanol) were separated by 10% SDS–PAGE and immunoreactive androgen receptor bands were detected as above. Two representative samples from each group ($n = 3$) are shown. (Bottom) Graph of androgen receptor levels. Mean immunoreactivity in the ~102 kDa (hatched bar) and ~110 kDa (solid bar) forms of the androgen receptor from 3 samples from each group of ± 1 standard error is shown. $^*P < 0.05$. (B) Western blot of polyubiquitinated protein. The blot shown in A was reprobed using anti-ubiquitin antibody. (Bottom) Graph of polyubiquitinated protein levels. The overall mean immunoreactivity in protein migrating between 66 and 220 kDa ± 1 standard error is shown. $^*P < 0.05$.

control HepG2 homogenates, but unlike the wild type, the level of the mutant did not change as a result of MG132 treatment (Fig. 3A, graph). We note that MG132 caused slower-running bands to appear above the wild-type β-catenin present in both cell types (Figs. 3A and 3B), consistent with monoubiquitination of this isoform.

DISCUSSION

Our finding that MG132 increases androgen receptor immunoreactivity in both HepG2 and LNCaP cells is consistent with recent reports that estrogen, progesterone and vitamin D receptors are also degraded via the proteasome (14–18). MG132 treatment also increased the amount of ubiquitinated protein comigrating with androgen receptor immunoreactive bands. Continuous degradation of the unliganded androgen receptor via the proteasome would provide cells with the means to limit the size and duration of their response to androgen, yet newly synthesized unliganded receptors would always be readily available to transactivate gene expression in response to hormone.

The heat shock proteins/chaperones Hsp90, 70 and 56 interact with the androgen receptor (7, 8), and their presence affects the receptor's hormone-binding affinity (6, 7). The binding of inducible Hsp72 to unfolded proteins is thought to be nonspecific, but its binding to the androgen receptor may be facilitated via more specific interactions of the receptor with co-chaperones like DnaJ (Hsp40), which also promotes the proper folding and transactivating function of the androgen receptor (24). Parenthetically, mutant androgen receptors with expansion of the poly-Q region are found in patients with spinal bulbar muscular atrophy, and appear to result in increased accumulation of the receptor along with ubiquitin and Hsp72 in the nuclei of motor neurons (25). Taken with the data presented in this report, it is possible that the normal degradation of the androgen receptor via the proteosome is blocked by the expansion of this poly-Q stability sequence, resulting in misfolding and/or enhanced interaction with Hsp72, to form aggregates (25). Conversely, a shortened poly-Q stability region is found in the androgen receptors of some prostate cancers, and has been implicated in the oncogenic process (26).

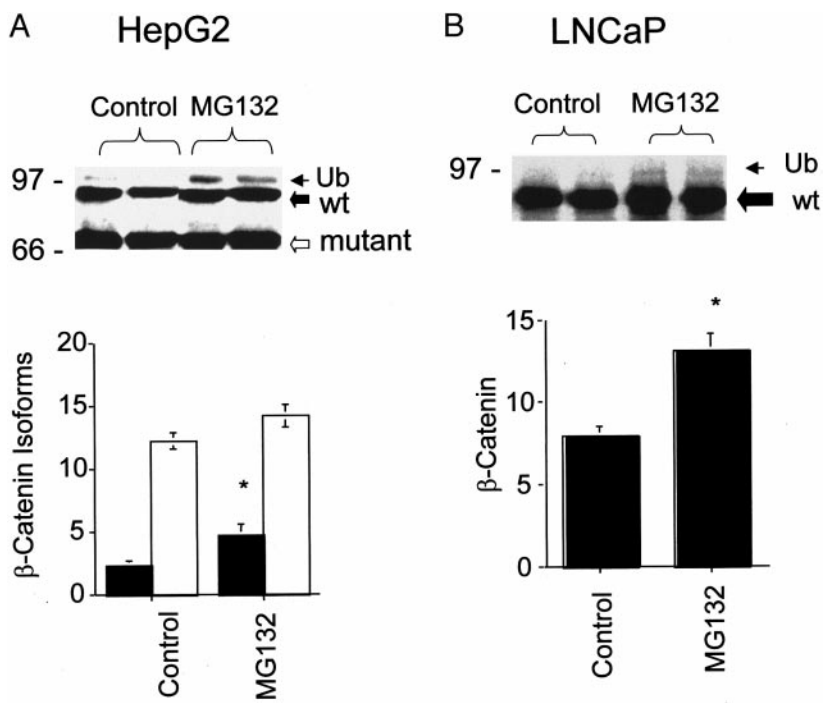

FIG. 3. Responses of β-catenin in HepG2 and LNCaP cells to MG132. (A) Western blots of β-catenin. The blots of HepG2 (A) and LNCaP (B) cells shown in Figs. 1A and 2A were reprobed with anti-β-catenin and overexposed to enhance the visibility of minor slower-migrating bands of β-catenin (thin arrows). The normal mobility of the wild-type isoform of β-catenin is indicated with solid arrows, and the mutant isoform only present in HepG2 cells is indicated with an open arrow. (Bottom) Graph of β-catenin responses to MG132. Mean β-catenin immunoreactivity present in the wild-type isoform (solid bars) and in the mutant present only in HepG2 cells (open bars) was quantified as described in the legend to Fig. 1B ($n = 6$ for each group of HepG2 cells, $^*P < 0.01$ and $n = 3$ for each group of LNCaP cells, $^*P < 0.05$).

Phosphorylation promotes ubiquitination and proteasomal degradation of a number of transcription factors. Phosphorylation via a constitutively active serine kinase, GSK3β, regulates the turnover of a number of transcription factors, including c-jun, c-myb, c-myc, CREB, NFAT, and the heat shock factor HSF1 (23, 27). The androgen receptor and β-catenin are two transcription factors which also contain GSK3β phosphorylation motifs, and our data suggest that a common mechanism might be involved in triggering the ubiquitination and degradation of both these proteins. Phosphorylation of the GSK3β motif has been shown to regulate the levels of IκB as well as β-catenin in other cells (20–22, 28). Our data confirm and extend these studies by demonstrating the importance of the GSK3β phosphorylation site in the ubiquitin-proteasome mediated turnover of β-catenin, since blocking the proteasome with MG132 increases the level of only the wild-type β-catenin isoform but not the mutant isoform present in HepG2 cells.

Many potential phosphorylation sites are present in the androgen receptor (9, 29–31), but only two serine residues have actually been shown to display increased phosphorylation following receptor activation by androgen, and this has been attributed to the stabilizing effect that hormone binding has on the degradation of the receptor (1–4).

The *in vivo* effects of mutating Ser-Pro sequences to Ala-Pro have been studied at four different sites in the androgen receptor (Ser 81, Ser 94, Ser 424 and Ser 650: numbering as in Ref. 2). Only the mutation of the Ser 650 affected the receptor's transcriptional activity in transiently transfected COS cells (2). Ser 650 was originally identified as one of four potential sites for GSK3β-dependent phosphorylation based on the substrate requirement of a pair of Ser residues separated by three amino acids (Ser-X-X-X-Ser(PO_4)) (30). However, GSK3β is also a proline-directed kinase (23, 27) and of these four potential GSK3β sites (Ser 318, 422, 650, and 777), only Ser 650 is directly neighbored by a downstream proline residue, and it is the major site phosphorylated in the C-terminal region of the receptor *in vivo* (2, 31). Phosphorylation of a serine residue situated next to a proline residue causes a major change in the protein backbone (32), and alters its susceptibility to proline-specific proteases (33). Since Ser 650/Pro 651 is located in the androgen receptor's hinge region, changes in the phosphorylation of this site could influence not only the receptor's conformation and its ligand-binding transactivating activity (2, 3, 11, 31), but could also influence its normal turnover by changing its interactions with several proteins known to be involved in protein ubiquitination that bind at or near the hinge region. These include SNURF

(small nuclear RING finger protein) (34), the ubiquitin-protein ligase E6-AP (35), UBC9 (ubiquitin-conjugating enzyme 9) (36) and DnaJ (24). SNURF has been shown to bind between aa 624–644 in the hinge region of the androgen receptor, and it coregulates androgen receptor dependent transcription (34). SNURF contains a RING finger which could facilitate ubiquitin ligation by various E2 ligases (37). Although UBC9 can facilitate the conjugation of ubiquitin to proteins (38), it preferentially conjugates the ubiquitin homologue SUMO-1 to lysine residues to which ubiquitin could otherwise be attached (39). Future studies will help define the functional role of these protein interactions in relation to androgen receptor turnover in the absence and presence of ligand.

The GSK3β phosphorylation motif at Ser 650 (2) is centered within a high-scoring PEST motif (19) that is evolutionarily conserved, displaying a high degree of identity in the four published mammalian androgen receptor sequences (Table 1). This PEST element is only found in mammalian androgen receptors and not in amphibia or fish, suggesting that this sequence evolved later in evolution. PEST motifs are found in a number of labile signaling proteins whose turnover is regulated via phosphorylation and ubiquitin-mediated proteasomal degradation (19), and potential PEST sequences have also been identified in the vitamin D and progesterone receptor (14, 15). The PEST sequence in the vitamin D receptor is also near its hinge/ligand binding domain, and the binding of vitamin D inhibits the degradation of this receptor (14), as reported for the androgen receptor in LNCaP cells (4). Treating HepG2 cells with DHT did increase androgen receptor levels but did not alter the overall level of polyubiquitinated protein (Sheflin and Spaulding, unpublished data). The DHT-mediated increase in androgen receptor was less dramatic than the increase produced by MG132, whereas in the case of the vitamin D receptor, the ligand inhibited the degradation of its receptor to a greater extent than did MG132 (14). It will be important to determine how PEST sequences function in the proteasomal degradation of these signal transduction transcription factors in relation to the stability of the hormone receptor upon binding of its cognate ligand.

ACKNOWLEDGMENTS

We thank Dr. Amy O'Donnell for providing helpful comments on the manuscript. This work was supported by funds from the Medical Research Service, Department of Veterans Affairs.

REFERENCES

1. Zhou, Z.-X., Lane, M. V., Kemppainen, J. A., French, F. S., and Wilson, E. M. (1995) Specificity of ligand-dependent androgen receptor stabilization: Receptor domain interactions influence ligand dissociation and receptor stability. *Mol. Endocrinol.* **9,** 208–218.
2. Zhou, Z.-X., Kemppainen, J. A., and Wilson, E. M. (1995) Identification of three proline-directed phosphorylation sites in the human androgen receptor. *Mol. Endocrinol.* **9,** 605–615.
3. Kemppainen, J. A., Lane, M. V., Sar, M., and Wilson, E. M. (1992) Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J. Biol. Chem.* **267,** 968–974.
4. Yeap, B. B., Krueger, R. G., and Leedman, P. J. (1999) Differential posttranscriptional regulation of androgen receptor gene expression by androgen in prostate and breast cancer cells. *Endocrinology* **140,** 3282–3291.
5. Wolf, D. A., Herzinger, T., Hermeking, H., Blaschke, D., and Horz, W. (1993) Transcriptional and posttranscriptional regulation of human androgen receptor by androgen. *Mol. Endocrinol.* **7,** 924–936.
6. Pratt, W. B., and Toft, D. O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* **18,** 306–360.
7. Fang, Y., Fliss, A. E., Robins, D. M., and Caplan, A. J. (1996) Hsp90 regulates androgen receptor hormone binding affinity *in vivo. J. Biol. Chem.* **271,** 28697–28702.
8. Veldscholte, J., Berrevoets, C. A., Zegers, N. D., van der Kwast, T. H., Grootegoed, J. A., and Mulder, E. (1992) Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: Use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* **18,** 7422–7430.
9. Brinkmann, A. O., Blok, L. J., de Ruiter, P. E., Doesburg, P., Steketee, K., Berrevoets, C. A., and Trapman, J. (1999) Mechanisms of androgen receptor activation and function. *J. Steroid Biochem. Mol. Biol.* **6,** 807–813.
10. Wilson, C. M., and McPhaul, M. J. (1996) A and B forms of the androgen receptor are expressed in a variety of human tissues. *Mol. Cell. Endocrinol.* **120,** 51–57.
11. Wang, L. G., Liu, X. M., Kreis, W., and Budman, D. R. (1999) Phosphorylation/dephosphorylation of androgen receptor as a determinant of androgen agonistic or antagonistic activity. *Biochem. Biophys. Res. Commun.* **259,** 21–28.
12. Cho, H., and Lim, I. K. (1997) Expression of androgen receptor and its implication in hepatoma cells. *Cancer Lett.* **115,** 135–140.
13. Shan, L.-X., Rodriguez, M. C., and Janne, O. A. (1990) Regulation of androgen receptor protein and mRNA concentrations by androgens in rat ventral prostate and seminal vesicles and in human hepatoma cells. *Mol. Endocrinol.* **4,** 1636–1646.
14. Li, X.-Y., Boudjelal, M., Xiao, J.-H., Peng, Z.-H., Asura, A., Kang, S., Fishe, G. J., and Voorhees, J. J. (1999) 1,25-Dihydroxyvitamin D_3 increases nuclear vitamin D_3 receptors by blocking ubiquitin/proteasome-mediated degradation in human skin. *Mol. Endocrinol.* **13,** 1686–1694.
15. Syvala, H., Vienonen, A., and Zhuang, Y.-H. (1998) Evidence for enhanced ubiquitin-mediated proteolysis of the chicken progesterone receptor by progesterone. *Life Sci.* **63,** 1505–1512.
16. Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L., and O'Malley, B. W. (1999) Proteasome-dependent degradation of the human estrogen receptor. *Proc. Natl. Acad. Sci. USA* **96,** 1858–1862.
17. Nirmala, P. B., and Thampan, R. V. (1995) Ubiquitination of the rat uterine estrogen receptor: Dependence on estradiol. *Biochem. Biophys. Res. Commun.* **213,** 24–31.
18. Alarid, E. T., Bakopoulos, N., and Solodin, N. (1999) Proteasome-mediated proteolysis of estrogen receptor: A novel component in autologous down-regulation. *Mol. Endocrinol.* **13,** 1522–1534.
19. Rechsteiner, M., and Rogers, S. W. (1996) PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21,** 267–271.
20. Koch, A., Denkhaus, D., Albrecht, S., Leuschner, I., von Schwein-

itz, D., and Pietsch, T. (1999) Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the β-catenin gene. *Cancer Res.* **59,** 269–273.

21. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M., and Byers, S. W. (1997) Serine Phosphorylation regulated ubiquitination and degradation of β-catenin. *J. Biol. Chem.* **272,** 24735–24738.
22. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) β-Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16,** 3797–3804.
23. Plyte, S. E., Hughes, K., Nikolakaki, E., Pulverer, B. J., and Woodgett, J. R. (1992) Glycogen synthase kinase-3: Functions in oncogenesis and development. *Biochim. Biophys. Acta* **1114,** 147–162.
24. Fliss, A. E., Rao, J., Melville, M. W., Cheetham, M. E., and Caplan, A. J. (1999) Domain requirements of DnaJ-like (Hsp40) molecular chaperones in the activation of a steroid hormone receptor. *J. Biol. Chem.* **274,** 34045–34052.
25. Stenoien, D. L., Cummings, C. J., Adams, H. P., Mancini, M. G., Patel, K., DeMartino, G. N., Marcelli, M., Weigel, N. L., and Mancini, M. A. (1999) Polyglutamine-expanded androgen receptors form aggregates that sequester heat shock proteins, proteasome components and SRC-1, and are suppressed by the HDJ-2 chaperone. *Hum. Mol. Genet.* **8,** 731–741.
26. Giovannucci, E., Stampfer, M. J., Krithivaas, K., Brown, M., Dahl, D., Brufsky, A., Talcott, J., Hennektens, C. H., and Kantoff, P. W. (1997) The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Clin. Cancer Res.* **3,** 1599–1608.
27. Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., and Calderwood, S. K. (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J. Biol. Chem.* **271,** 30847–30857.
28. DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996) Mapping of the inducible IkB phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell. Biol.* **16,** 1295–1304.
29. Blok, L. J., de Ruiter, P. E., and Brinkmann, A. O. (1996) Androgen receptor phosphorylation. *Endocrine Res.* **22,** 197–219.
30. Chang, C., Saltzman, A., Yeh, S., Young, W., Keller, E., Lee, H. J., Wang, C., and Mizokami, A. (1995) Androgen receptor: An overview. *Crit. Rev. Eukaryotic Gene Expression* **5,** 97–125.
31. Block, L. J., deRuiter, P. E., and Brinkmann, A. O. (1998) Forskolin-induced dephosphorylation of the androgen receptor impairs ligand binding. *Biochemistry* **37,** 3850–3857.
32. Schutkowski, M., Bernhardt, A., Zhou, X. Z., Shen, M., Reimer, U., Rahfeld, J. U., Lu, K. P., and Fischer, G. (1998) Role of phosphorylation in determining the backbone dynamics of the serine/threonine-proline motif and Pin1 substrate recognition. *Biochemistry* **37,** 5566–5575.
33. Kaspari, A., Diefenthal, T., Grosche, G., Schierhorn, A., and Demuth, H. U. (1996) Substrates containing phosphorylated residues adjacent to proline decrease the cleavage by proline-specific peptidases. *Biochim. Biophys. Acta* **1293,** 147–153.
34. Moilanen, A. M., Karvonen, U., Poukka, H., Janne, O. A., and Palvimo, J. J. (1998) Identification of a novel RING finger protein as a coregulator in steroid receptor-mediated gene transcription. *Mol. Cell. Biol.* **18,** 5128–5139.
35. Nawaz, Z., Lonard, D. M., Smith, C. L., Lev-Lehman, E., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999) The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Mol. Cell. Biol.* **19,** 1182–1189.
36. Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J. J., and Janne, O. A. (1999) Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. *J. Biol. Chem.* **274,** 19441–19446.
37. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. USA* **96,** 11364–11369.
38. Huggins, G. S., Chin, M. T., Sibinga, N. E., Lee, S. L., Haber, E., and Lee, M. E. (1999) Characterization of the mUBC9-binding sites required for E2A protein degradation. *J. Biol. Chem.* **274,** 28690–28696.
39. Duprez, E., Saurin, A. J., Desterro, J. M., Lallemand-Breitenbach, V., Howe, K., Boddy, M. N., Solomon, E., deHay, R. T., and Freemont, P. S. (1999) SUMO-1 modification of the acute promyelocytic leukaemia protein PML: Implications for nuclear localisation. *J. Cell. Sci.* **112,** 381–393.