Androgen ablation therapy is effective in treating androgen-dependent prostate tumors; however, tumors that can proliferate at castrate levels of androgen eventually arise. We previously reported that in CWR22Rv1 (Rv1) cells, the protease calpain 2 can cleave the androgen receptor (AR) into a constitutively active ~80,000 low molecular weight (LMW) form. In this study, we further dissect the mechanisms that produce the AR LMW forms using Rv1 cells and the related CWR22-R1 (R1) cells. The 39-amino acid insertional mutation in the Rv1-AR (E3DM-AR) sensitizes this AR to calpain 2 proteolysis. R1 cells encode the same AR molecule as the parental CWR22 xenograft. Using calpain 2 small interfering RNA and calpeptin, we find that calpain 2 plays a role in the generation of the LMW-AR in R1 cells. Furthermore, LMW-AR expression is regulated by the activation of calpain 2 by ERK 1 and 2. Inhibition of ERK phosphorylation or small interfering RNA-mediated decrease of ERK expression reduces LMW-AR levels in R1 cells. Conversely, activation of the MAPK pathway results in increased ERK phosphorylation and increased levels of LMW-AR. Finally, analyses of human tumor samples found that LMW-AR levels are higher in tumors that have an increased calpain/calpastatin ratio and/or increased levels of phospho-ERK (pERK). This suggests that a higher calpain/calpastatin ratio collaborates with activated ERK to promote the generation of the LMW-AR.

Prostate cancer is a commonly diagnosed malignancy that is treated with hormonal therapy aimed at blocking signaling through the androgen receptor (AR). Initially, androgen ablation therapy is effective, but eventually, this treatment leads to the development of aggressive relapsed tumors that thrive in the absence of androgens. Analysis of clinical samples revealed that >90% of the relapsed tumors express AR (1–4). The AR, a member of the steroid hormone superfamily of ligand-activated transcription factors (5, 6) is central to the initiation and growth of prostate tumors and their responses to therapy. In the absence of ligand, the AR is retained in the cytoplasm. The binding of hormone alters the conformation of AR to promote translocation of the AR into the nucleus, where it regulates gene transcription (6–8).

Aberrant AR activity has been postulated to promote proliferation of tumor cells in reduced levels of androgen. Studies have shown that 25–30% of androgen-independent tumors that arose following androgen ablation have AR gene amplification (9, 10). AR mutations are more commonly observed in androgen-independent tumors (11, 12) and usually broaden ligand specificity (13). The AR present in CWR22 xenograft cells has a mutation in the ligand binding domain (LBD; H847Y) that enhances responsiveness to estradiol and progesterone (14). Structure function analysis of the AR showed that deletion of the LBD generates a constitutively active AR molecule (15). A subsequent study identified a nonsense mutation at Q640 that results in a truncated constitutively active AR in a tumor refractory to androgen ablation therapy (16). We and others previously reported that calpain cleaves the AR molecule to produce various LMW isoforms (17–19), including an ~80,000 C-terminal truncated AR. We found that the ~80,000 LMW-AR is present in some human prostate tumors (18). Using the androgen-independent Rv1 cell line that expresses high levels of the LMW-AR, we demonstrated that inhibition of calpain activity induces apoptosis in cells cultured in the absence of androgen. These studies implied that calpain-dependent proteolysis of the AR may play an important role in conferring androgen independence in a subset of prostate cancer cases (18). In this study, we show that calpain 2 and ERK collaborate in the generation of the LMW-AR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Pharmacological Agents**—LNCaP, Rv1, PC3, and DU145 cells were obtained from American Type Culture Collection. R1 cells were provided by Dr. Elizabeth Wilson (University of North Carolina). Rv1, PC3, DU145, and R1 cells were propagated in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mmol/liter l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37 °C and 5% CO₂. LNCaP cells were propagated in 10% fetal bovine serum. RWPE, pRNS-1-1, and PZ-HPV-7, obtained from Dr. Ralph...
For MEK inhibition studies, cells were treated with 20 µM U0126 (Cell Signaling) and 200 nM of each primer for a total volume of 10 µl. GAPDH was used as the standard. PCR conditions were as follows: a 20-s initial denaturation step at 95 °C; 40 cycles at 95 °C for 3 s, 60 °C for 30 s, followed by a melt curve at 95 °C for 15 s, 60 °C for 15 s, an increase to 95 °C over 20 min; an additional 95 cycles starting at 60 °C with a 0.5 °C increase per cycle for melt curve analysis. The Eppendorf Mastercycler ep Realplex was used for this study. Primer sequences: GAPDH: 5'-TCAGGCCGCTGAAACTGCTTA-3' and 5'-AGAGCGAGGATGATGGTCT-3'; CLDN4: 5'-AACCTGACTTTGGGATCTG-3' and 5'-AGATGCCGACAGACAGGTG-3'; HPRT1: 5'-TGACACCTGGCATAACATGCA-3' and 5'-GGTCCTTTTCACCAAGCT-3'.

**Statistics**—Analyses using a two-tailed Student's t test were used to compare two groups. p < 0.05 was considered statistically significant.

**RESULTS**

**Characteristics of the Rv1 and R1 Cell Lines**—Two castrate-resistant cell lines, R1 and Rv1, were derived from two independent CWR22 relapsed tumors. The cellular phenotypes of the Rv1 and R1 cells are similar. In the presence of androgen the cells tend to grow in clusters, whereas in the absence of androgen, they tend to be more scattered and less adhesive (Fig. 1A). The AR in both lines has the same LBD mutation as the CWR22 xenograft (20, 21). As previously reported, R1 and Rv1 cells express the LMW AR forms (Fig. 1B) (20, 21). Western immunoblot analysis indicated that R1 cells expressed higher levels of AR than Rv1 cells, but the ratio of the LMW to full-length (FL)-AR was higher in Rv1 cells. The size of the FL-AR in the R1 cells is smaller than the FL-AR in the Rv1 cells, because R1 cells do not have the 39 amino acid duplication of exon 3. Closer inspection revealed that the ~80,000 LMW forms could be resolved into several discrete bands (Fig. 1B). The MTS proliferation assay confirmed that the R1 and Rv1 cell proliferation rates were only slightly slower in androgen-depleted media compared with cells grown in the presence of androgen (Fig. 1C). The proliferation assay conducted in the presence of 10 µM Casodex indicated that R1 and Rv1 cells were refractory to the effects of this AR inhibitor (Fig. 1D). Although all three lines are responsive to androgen, only LNCaP cells are dependent on androgen to sustain growth.

**Generation of the LMW-AR Involves Calpain**—We have reported previously that the inhibition of calpain activity by calpeptin reduces the expression of the LMW-AR in Rv1 cells (18). Likewise, treatment of R1 cells, proliferating in the presence or absence of androgen, with calpeptin reduced the levels of LMW-AR in R1 cells (Fig. 2A). We previously showed that proteolysis of the calpain substrate focal adhesion kinase (FAK) is a good indicator of calpain activity (22). Calpeptin treatment of R1 cells reduced the levels of LMW-FAK (Fig. 2A). To further analyze the role of calpain in the generation of LMW-AR, calpain 2 expression was analyzed in several tumor derived, as well as immortalized, prostate cell lines. R1 cells expressed much higher levels of calpain 2 than Rv1 and LNCaP cells (Fig. 2B). Interestingly, the two AR negative and highly metastatic cell lines, PC3 and DU145, expressed the highest levels of calpain 2. Given that calpain activity is regulated by its endogenous inhibitor calpastatin,
we analyzed calpastatin levels as well, and found that expression was comparable in all the cell lines (Fig. 2B). R1 cells had higher amounts of proteolyzed FAK, indicating greater calpain activity (Fig. 2C). The extent of FAK cleavage was greater in the absence of androgen, suggesting that calpain activity may be higher under androgen-depleted conditions.

To further confirm the involvement of calpain 2 in the generation of the LMW-AR forms in R1 cells, we used calpain 2 siRNA to reduce calpain 2 expression. A previous study reported that calpain 2 has a very long half-life of 5 days (23). A 6-day treatment resulted in an ~60% reduction of calpain 2 protein levels in R1 cells (Fig. 2D) and reduced levels of the LMW-AR forms (Fig. 2D). This treatment also reduced FAK proteolysis indicating that calpain 2 activity was reduced. This analysis indicates that calpain 2 plays a role in the generation of the LMW-AR in R1 cells.
In R1 cells, the expression of claudin 4 (CLDN4) is highly repressed by the addition of androgen (Fig. 2E). If calpeptin treatment reduces the levels of LMW-AR, then in the absence of androgen the expression of androgen repressed genes may be further activated. In the absence of androgen calpeptin treatment of R1 cells further increased the expression of CLDN4, thus arguing the LMW-AR has a role in transcription of certain genes. The Exon 3 Duplication Sensitizes E3DM-AR to Calpain Proteolysis—Rv1 cells express higher levels of the LMW-AR but have low expression of calpain 2 protein and calpain activity (Fig. 2). We hypothesized that the exon 3 duplication sensitizes the E3DM-AR to calpain cleavage. The AR-null PC3 cells expressing high levels of calpain 2 were transfected with cDNA plasmids encoding either the wild-type or E3DM-AR. As expected, the E3DM-AR was slightly larger than the wild-type receptor (Fig. 3A). Additionally, the LMW forms generated in cells transfected with the E3DM-AR were larger than the LMW forms generated from the wild-type AR cDNAs. To test the hypothesis that the E3DM-AR is more sensitive to calpain-dependent proteolysis, extracts prepared from the transfected cells were treated with CaCl₂ to activate endogenous calpain activity. As shown in Fig. 3B, the AR was progressively cleaved into the smaller forms by the addition of CaCl₂. The amount of FL-AR remaining was quantitated and indicated that the E3DM-AR was degraded more rapidly than the wt AR. The inclusion of calpeptin retarded proteolysis, indicating that proteolysis was calpain-dependent (Fig. 3B). While the ~80,000 forms were present initially and throughout the time course, as proteolysis progressed, the LMW-AR was further proteolyzed to smaller peptides. In vivo, the ~80,000 LMW-AR forms that are generated by proteolysis can translocate into the nucleus, where they would be less susceptible to further proteolysis. In vitro, as was previously observed (17) activated calpain proteolyzes the AR to still smaller forms. The mutant E3DM-AR was cleaved more rapidly than the wild-type FL-AR, resulting in the disappearance of the FL-AR (compare lanes 4 and 9). The Expression of the LMW-AR Is Regulated by ERK—Calpain activity is tightly regulated by various mechanisms, including phosphorylation. Previous studies have shown that ERK can phosphorylate calpain 2 to stimulate protease activity (24). ERK expression was analyzed in immortalized (RWPE-1, PZ-HPV-7, and pRNS-1-1) and tumor derived (PC3, LNCaP, Rv1, R1, and DU145) cell lines. All of the tumor-derived cell lines had higher levels of ERK in comparison to the immortalized cell lines (Fig. 4A). A comparison of R1 and Rv1
cells proliferating in the absence and presence of androgen showed that R1 cells had higher levels of the active form of the protein (pERK) under both conditions (Fig. 4B).

ERK is phosphorylated and activated by MEK, a dual threonine and tyrosine kinase (24). Treatment of R1 cells with the MEK inhibitor U0126 for 24 or 48 h reduced ERK phosphorylation (Fig. 4C). An analysis of AR in the same extracts (Fig. 4C) indicated that inhibition of ERK activity reduced the levels of LMW-AR. Similar results were found in Rv1 cells (data not shown). To confirm that LMW-AR expression is dependent on ERK, cells were treated with control siRNA and ERK siRNA. Inhibition of ERK expression resulted in decreased levels of LMW-AR (Fig. 4D). This analysis established that ERK activation has a role in the etiology of the LMW-AR forms.

Because the protein kinase C activator TPA can result in ERK phosphorylation (25), Rv1 and R1 cells were treated with TPA in the absence of androgen for 1 or 2 h to stimulate ERK activity. This treatment promoted an increase in levels of the LMW-AR indicating that activation of this pathway resulted in enhanced AR proteolysis (Fig. 5A). TPA treatment of Rv1 cells also resulted in decreased levels of the FL-AR; after a 2-h TPA treatment, the FL-AR was barely discernable, arguing that in vivo, the Rv1 AR is more sensitive to proteolysis.

To test our hypothesis that an increase in calpain 2 and ERK activity collaborate in promoting LMW-AR expression, we examined calpain 2, calpastatin, and pERK levels in 6 of 13 tumor samples previously analyzed for the expression of the LMW-AR. Three of the thirteen samples that had the highest levels LMW-AR (01, 31, and 94) and three that had low levels of LMW-AR (21, 25, and 28) were used in the analysis (Fig. 5B). The expression of LMW-AR was defined as percent of total. Interestingly, the levels of the endogenous calpain inhibitor calpastatin was variable. It was higher in samples 21 and 25, which have lower levels of LMW-AR and lowest in Sample 01. Samples 01 and 31 had high levels of pERK (Fig. 5C). The remaining samples had low pERK levels. Therefore, the three samples that had the highest LMW-AR had high levels of pERK or a high amount of calpain 2. Conversely, samples that had low LMW-AR levels had little pERK and elevated calpastatin levels. This limited analysis suggests that in human tumors an increased ratio of calpain to

FIGURE 4. Inhibition of ERK phosphorylation reduces the expression of the LMW-AR. A. Western blot analysis of ERK expression in nontransformed and tumor-derived cell lines. B, the pERK levels are higher in R1 than Rv1 cells in the presence or absence of androgen. C, R1 cells were treated with 20 μM of the MEK inhibitor U0126 (I) or vehicle (C) for 24 or 48 h. The top portion of the blot shown in the top panel was used to detect AR. Inhibition of ERK phosphorylation reduced the expression of the LMW-AR relative to FL-AR by 32% in 24 h and 51% in 48 h. The arrows denote the FL and ~80,000 LMW-AR. D, ERK-specific siRNA reduced the expression of pERK and the levels of LMW-AR relative to FL-AR to 51.8% in the presence of androgen (AD+) and 21% in the absence of androgen (AD−). Ab, antibody; si, small interfering; siC, control siRNA.

FIGURE 5. ERK activation and calpain/calpastatin ratios collaborate to promote expression of the LMW-AR. A, treatment of R1 and Rv1 cells with TPA (10 nM) for 1 and 2 h increases the expression of the LMW-AR forms (top panel). Control cells were treated with dimethyl sulfoxide. The bottom panel shows that TPA treatment increases pERK levels. Arrows denote the full length and LMW AR or pERK. B, higher calpain/calpastatin and pERK levels together correlate with higher expression of LMW-AR in tumor samples. Arrows denote tumors with highest percent of LMW-AR. C, quantitation of the protein levels in B. The calpain/calpastatin ratios multiplied by levels of pERK were calculated for tumors that express high levels of LMW-AR (01, 30, and 94) and samples that had low levels of LMW-AR (21, 25, 28). The average calpain/calpastatin × pERK levels are significantly higher in samples with elevated levels of LMW-AR. Error bars represent S.D. p < 0.05. canp2, calpain 2; cast, calpastatin.
calpastatin and increased ERK activity, work in concert contribute to increased LMW-AR expression.

**DISCUSSION**

R1 and Rv1 cell lines were derived from relapsed CWR22 tumors and express the FL-AR as well as LMW-AR forms. However, the FL and LMW-AR forms expressed in Rv1 cells is larger than those in R1 cells due to a 39-amino acid insertional mutation at the junction of the DNA binding domain and hinge region (21). Transient expression of the E3DM-AR cDNA in PC3 cells also results in the expression of slightly larger LMW forms than transfection of the wt AR cDNA. Activation of calpain AR-transfected PC3 extracts indicates that the E3DM-AR is more susceptible to proteolysis than the wt AR. In vivo activation of calpain activity through activation of ERK also promotes a more rapid proteolysis of the E3DM-AR. Early studies reported that a serine protease can proteolyze the AR to generate a ~30,000 or ~40,000 fragment containing the LBD (26). More recently, an independent study found that in vitro, calpain proteolyzes the AR to smaller amino-terminal fragments; those fragments include an ~75,000 polypeptide (17). Our data suggest that the junction between the DNA binding domain and LBD might be especially sensitive to proteolysis. Therefore, it is not unexpected that the insertion of 39 additional amino acids near this region would alter AR structure and further sensitizes the molecule to calpain proteolysis (27, 28). Unlike Rv1 cells, R1 cells have an AR that is identical to the AR in the parental CWR22 xenograft. Therefore, we postulated that other molecular alterations must account for the increased expression of the LMW-AR. The current study shows that R1 cells express higher levels of calpain 2 and pERK than Rv1 cells. These two features collaborate to elevate calpain activity and promote proteolysis of the AR and FAK. The role of calpain in the degradation of AR is substantiated by the reduction of LMW-AR caused by inhibition of calpain by calpeptin or a decrease of calpain 2 by siRNA. A comparison of R1 and Rv1 cells indicated that R1 cells had higher levels of ERK and pERK. The participation of ERK in AR proteolysis was demonstrated by an siRNA-mediated decrease of ERK and by the inhibition of ERK phosphorylation by the MEK inhibitor U01286. Therefore, a decrease of ERK levels or ERK activity reduces LMW-AR expression. Activation of ERK by TPA in Rv1 and R1 cells results in a time-dependent increase in the generation of LMW-AR. The short interval required for increased LMW-AR generation is consistent with activation of a signaling cascade that results in the activation of a protease. The MAPK phosphorylation cascade that leads to ERK activation has been well studied and is considered a target for cancer therapeutics (29). Since ERK activation in prostate tumors has been previously reported (30), this is a potential mechanism that could contribute to the expression of LMW-AR in human tumors. Likewise, increased calpain 2 expression has been observed in prostate tumors. Because the activity of calpain 2 is partly regulated by calpastatin, the ratio of calpain/calpastatin affects calpain 2 activity. The expression of calpastatin has not been previously studied in prostate tumors. However, an increase in the calpain/calpastatin ratio has been reported in a study of colorectal cancer (31), which showed that calpastatin levels are high in normal mucosa but decreased in tumor tissue. Moreover, increased expression of calpain 2 was detected in colorectal tumors and polyps, suggesting that the increase of calpain 2 levels may be an early event in the tumorigenesis process. At this point, we cannot rule out that calpain 1 contributes to the generation of the LMW-AR. Interestingly, calpain 1 has been shown to activate ERK (32), and, therefore, all of these molecules may be components of a regulatory pathway. The importance of the calpain/calpastatin equilibrium and the activation of the MAPK signaling pathway in prostate tumorigenesis remain to be defined.

Recent studies reported that the LMW-AR forms expressed in Rv1 cells are derived from an alternatively spliced AR mRNA (33–35). However, the studies do not agree on the identity of the spliced forms that give rise to the LMW-AR forms. Our analysis shows that several LMW-AR forms are expressed in Rv1 and R1 cells. Because we did not completely eliminate the expression of the LMW-AR by inhibiting calpain 2 and pERK, some of the LMW-AR forms could be derived from alternatively spliced AR mRNA. This is analogous to results obtained from studies of cyclin E. In transformed cells, several LMW cyclin E forms can be detected (36). Studies have shown that some of the LMW cyclin E forms are derived from alternatively spliced mRNAs, whereas others are generated by proteolysis of cyclin E protein (22, 37–39). The LMW cyclin E forms have altered cellular localization and are associated with higher kinase activity (40, 41). We agree with the interpretation of Guo et al. (35) that several mechanisms can be employed to generate LMW-AR forms. These LMW-AR forms may not be identical, but they would share critical features including the presence of the activation and DNA binding domains and a deletion of the LBD. Such AR molecules would be able to translocate into the nucleus in an androgen-independent manner, bind to DNA, and activate or repress gene transcription. Furthermore, the interaction of the LMW-AR and FL-AR with various AR-interacting proteins may differ, and, therefore, if the LMW-AR and the FL-AR bind to identical DNA sequences, they may have differential effects on gene transcription.

Multiple calpain substrates have been previously implicated in cellular transformation. This suggests that an alteration of the calpain/calpastatin equilibrium, which is observed in some tumors, would affect multiple pathways that drive tumor progression. The modulation of calpain activity could result in a constellation of changes that would be difficult to attribute to any individual molecule. This feature of calpain-driven deregulation of cell physiology also provides a therapeutic opportunity. The inhibition of calpain activity, even partially, could be sufficient to modify multiple tumor survival and proliferative pathways, which, in synergy with other therapeutics, could be effective in halting tumor progression.

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ERK and Calpain Regulate AR LMW Levels

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