Evidence for Calpain-Mediated Androgen Receptor Cleavage as a Mechanism for Androgen Independence

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Abstract
Prostate carcinoma is the most commonly diagnosed cancer in men and the second leading cause of death due to cancer in Western civilization. Androgen ablation therapy is effective in treating androgen-dependent tumors, but eventually, androgen-independent tumors recur and are refractory to conventional chemotherapeutics. Hence, the emergence of androgen independence is the most challenging problem in managing prostate tumors. We report a novel mechanism of androgen independence: calpain cleaves the androgen receptor (AR) into an androgen-independent isoform. In vitro and in vivo analyses show that calpain removes the COOH-terminal ligand binding domain generating a constitutively active molecule. Analysis of human prostate tumors indicates that several tumors express higher levels of this truncated AR than noncancerous prostate tissue. In transient transfection studies, the truncated AR is three to five times more potent than the full-length receptor in transactivating transcription. The androgen-independent Rv1 cells express high levels of the truncated AR, and treatment of these cells with a calpain inhibitor reduces truncated AR expression. In the absence of androgen, inhibition of calpain activity induces apoptosis. The HIV protease inhibitor amprenavir inhibits calpain activity and is also effective in inducing apoptosis in the Rv1 cell line. The cell culture studies were reproduced in a mouse xenograft model, where, in the absence of androgens, amprenavir significantly reduces tumor growth. Together, these studies indicate that calpain-dependent proteolysis of the AR may be a mechanism of androgen independence. The calpain inhibition studies suggest that inhibiting this activity may be a potential treatment for some androgen-independent prostate tumors. [Cancer Res 2007;67(19):9001–5]

Introduction
Most prostate cancers initially present as androgen-dependent neoplasms and therapy relies on androgen ablation aimed at blocking androgen receptor (AR) cell signaling. Although initially successful, androgen-independent tumors that are refractory to such treatments eventually emerge (1). Many androgen-independent prostate cancers continue to express the AR and exhibit reinstatement of its function. Several mechanisms may account for AR activation in low levels of androgen: (a) AR mutations that require low levels of androgen; (b) activation of AR by nonsteroid ligands, such as growth factors and cytokines (2); (c) overexpression/amplification of AR or its coactivators (3); (d) locus-wide histone transcriptional activation at some, but not all, AR targets (4); and (e) proteolytic processing of the AR to an androgen-independent isoform.

The AR consists of four functional domains: an NH2-terminal regulatory region, a DNA-binding domain, a hinge domain, and a COOH-terminal ligand binding domain (LBD; ref. 5). The binding of hormone to the LBD allows for translocation of the receptor into the nucleus and recruitment of proteins to the transcription complex (6). Previous reports show that deletion of the LBD generates an androgen-independent transcriptional activator (7).

Calpains, calcium-dependent proteinases, are ubiquitously expressed. In general, calpains cleave proteins at a limited number of sites to generate large polypeptides (8). Substrate specificity is based on sequence and substrate conformation (9). Calpain activity is regulated by multiple mechanisms, including calcium modulation, autoproteolysis, phosphorylation, intracellular distribution, and inhibition by calpastatin (8). Interestingly, calpain 2 levels are elevated in invasive prostate tumors and are highest in metastatic neoplasms (10).

Materials and Methods
Cell culture. LNCaP, Rv1, PC3, and MCF-7 cells were obtained from American Type Culture Collection and propagated in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37°C and 5% CO2.

Western immunoblot analysis. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer containing E64, leupeptin, and calpeptin (Calbiochem), and protease inhibitor cocktail P8340 (Sigma). Proteins (30–50 μg) were separated on 10% SDS-PAGE gels, transferred to BA-83 membrane (Schleicher & Schuell), and blocked with 5% nonfat dry milk in PBS/0.1% Tween. The following antibodies were used: AR (central) 441 (Ab-1; Lab Vision Corp.), AR (COOH-terminus) C-19 (Santa Cruz Biotechnology, Inc.), AR (NH2-terminus) PG21 (Upstate), PSA-ER-PR8 (Neo Markers), and focal adhesion kinase (FAK; clone 4.47; Upstate). Proteins were detected using chemiluminescence (Amersham Pharmacia).

In vivo calpain induction. LNCaP cells were plated, cultured overnight, pretreated with 40 μmol/L of calpeptin or DMSO for 15 min, and then treated with 10 μmol/L of ionomycin (Calbiochem) for 20 min. Cells were harvested, lysed, and assayed as described above.

In vivo calpain inhibition. Rv1 cells (2 × 105) were plated in 35-mm plates and cultured overnight. Cells were treated with DMSO, 40 μmol/L calpeptin, or 30 μg/mL amprenavir, for 24 or 48 h, washed with cold PBS, and harvested. Amprenavir (GlaxoSmithKline) was provided by D.M.A. For analysis of calpain inhibition in the presence or absence of androgens, cells were plated at 105 in 35-mm plates and propagated in androgen-containing or androgen-depleted media (phenol red-free media,charcoal-stripped...
serum) for 48 h before addition of calpeptin, ampranavir (at 15 or 30 μg/mL), or DMSO. Cells were refed daily for 3 days. Floating and adherent cells were harvested, washed in PBS, and fixed in 4% paraformaldehyde/PBS (pH 7.4) overnight at 4°C. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI; 0.2 μg/mL in 1% Triton X-100/2% paraformaldehyde) for 30 min at 4°C in the dark, washed with PBS, spotted onto slides, dried, coverslipped, and examined by fluorescence microscopy.

In vitro calpain assay. LNCaP cells were resuspended in calpain assay buffer [50 mmol/L HEPES buffer (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100]. Calpain was activated with CaCl2 and reactions were incubated at 25°C for 60 min. Alternatively, LNCaP or Rv1 extracts were treated with increasing concentrations of calpain 2 (Calbiochem) for 60 min at 25°C. Reactions were terminated by boiling.

Plasmid construction. The truncated form of AR (tr-AR) was generated by PCR amplification of the sequences encoding amino acids 1 to 648 using the primers, 5′-GGATGGAAAGTGGTTAAGGSC-3′ and 5′-GGTGGCTGGAAGCCTCTCCTTT-3′, followed by cloning into pcDNA3 (Invitrogen). Cloned sequence was excised by XbaI and BamHI and cloned into the XbaI and BamHI sites of pcDNA3 (Invitrogen).

Transfection and luciferase assays. PC3 cells were propagated in control or androgen-depleted media. Cells were transfected using Effectene (Qiagen) and analyzed as described previously (11).

Cell proliferation assay. Cellular proliferation was assessed using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-tetrazolium (MTS) assay (Promega) following manufacturer’s recommendations.

Human prostate specimens. Tumor tissue samples, flash frozen in liquid nitrogen, were obtained from the University of California Davis Cancer Center Tissue Bank. Cellular extracts were prepared as described previously (11).

Animals and xenograft studies. Rv1 xenograft studies were done as described previously (12). Three days after castration, 4-week-old athymic mice (Harlan Sprague-Dawley) were injected with 1.5 × 106 Rv1 cells in peanut oil, or vehicle daily. Animal studies were approved by each Institution’s Animal Care and Use Committee approved.

Statistics. Analyses using a two-tailed Student’s t test were used to compare two groups. P < 0.05 was considered statistically significant. Error bars represent SE.

### Results

**Calpain cleavage of the AR.** To investigate the link between calpain and prostate tumorigenesis, we examined the AR for potential cleavage sites. Theoretically, the consensus calpain cleavage site (9) between residues 648 and 649 of the human AR would generate two polypeptides: an ~80-kDa polypeptide consisting of the transactivation, DNA binding, and hinge domains and a ~30-kDa LBD. To test this possibility, LNCaP cell extracts were treated with increasing concentrations of CaCl2 to activate calpain. Addition of calcium promoted AR proteolysis to an ~80-kDa truncated form (tr-AR; Fig. 1A). Addition of calpain inhibitors calpeptin or EGTA prevented proteolysis. Calpain activation was confirmed by analysis of FAK (8). Addition of calcium promoted FAK cleavage from a 120- to a 90-kDa form (Fig. 1A). The high calcium levels required for proteolysis implicated calpain 2 (8). Indeed, addition of calpain 2 resulted in the appearance of the tr-AR and a disappearance of the full-length AR (FL-AR; Fig. 1B). In addition, analysis using antibodies directed against the LBD showed that calpain proteolysis caused the disappearance of FL-AR and appearance of a 30-kDa fragment (Fig. 1B), confirming deletion of this region.

Our previous study identified a novel AR mutation in Rv1 cells, a line derived from the relapsed CWR22Rv1-2152 human xenograft. Rv1 cells contain a duplication of the third exon (13) that was not detected in the parental, androgen-dependent CWR22 tumor (14), and express an 80- to 85-kDa tr-AR that is missing the LBD. Treatment of the Rv1 extracts with calpain 2 completely converted the FL-AR to the tr-AR (Fig. 1C).

**In vivo inhibition and activation of AR proteolysis.** Treatment of LNCaP cells with the calcium ionophore ionomycin activated endogenous calpain resulting in proteolysis of the AR to an ~80-kDa isoform (Fig. 2A). In a complementary experiment, calpeptin treatment of intact Rv1 cells reduced the level of the tr-AR (Fig. 2B). These in vivo results establish that the AR is a calpain substrate.

**Truncated AR is more efficient in transactivating transcription.** An expression plasmid encoding tr-AR was generated to

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**Figure 1.** The AR is a calpain substrate. A. Western immunoblot analysis (Ab: AR441) revealed that addition of CaCl2 to LNCaP extracts resulted in cleavage of the AR to a truncated isoform. Addition of calpeptin or EGTA inhibited AR proteolysis. Addition of CaCl2 activates proteolysis of FAK to a 90-kDa isoform. B, addition of purified calpain 2 (0.17 units/L) to LNCaP extracts promoted AR proteolysis, which was inhibited by calpeptin. An antibody directed against the COOH-terminal region of the AR (C-19) detected the full-length and 30-kDa COOH-terminal fragments. C, treatment of CWR22Rv1 extracts with calpain 2 resulted in the proteolysis of the FL-AR to the truncated isoform.

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determine the efficacy of the tr-AR in transactivating transcription. In transient transfection studies, tr-AR was three to five times more robust than FL-AR in transactivating an AR-dependent prostate specific antigen (PSA) promoter and acted in a ligand-independent manner (Fig. 2C). A Western blot analysis of FL-AR- and tr-AR-transfected cells shows that the levels of AR expression is comparable; therefore, the increase in tr-AR activity can be attributed to enhanced transactivation function (Fig. 2D).

Truncated AR expression in prostate tumors. To establish the potential clinical relevance of these observations, the AR was examined in normal and malignant prostate. Nonmalignant tissue (benign prostatic hyperplasia) expressed very low levels of tr-AR.

Figure 2. In vivo inhibition of activation of calpain activity. A, treatment of LNCaP cells with ionomycin promotes proteolysis of the AR to the truncated isoform. B, treatment of CWR22Rv1 cells with 40 μM calpeptin for 24 or 48 h reduces the expression of the tr-AR. C, the tr-AR efficiently transactivates transcription of the androgen-responsive PSA promoter. PC3 cells were plated (1.0 x 10⁶ cells/35-mm dish), propagated in control or androgen-depleted media, and transfected with PSA-luciferase 0.03 μg, 0.050 μg of FL-AR, tr-AR, or vector pCDNA3, and 0.020 μg of the CMV-β-galactosidase plasmids. In the absence of androgens, the tr-AR is ~8-fold more effective than the FL-AR in transactivating transcription. Columns, fold transactivation; bars, SE. D, a Western blot analysis of cells transfected with the control pCDNA3, tr-AR, or FL-AR plasmid along with the luciferase expression plasmid and CMV-β-galactosidase plasmids shows the expression levels of the tr-AR and FL-AR. Loading was normalized to β-galactosidase activity.

Figure 3. Human prostate tumors express the tr-AR. A, tumors 01, 30, and 94 express higher levels of an AR isoform that is similar in size to the isoform detected in CWR22Rv1. Bottom, a longer exposure that shows that although the tumor sample and control prostate tissue express similar amounts of the AR, the tumor samples have much higher levels of the truncated isoform. A Western blot analysis using an antibody directed against the NH₂-terminal region of the AR and tumor sample 01 confirms that the truncated form has a COOH-terminal deletion. Partially cleaved LNCaP AR (lighter exposure) served as a marker. Tumor samples were flash frozen in liquid nitrogen within 30 min of tumor resection and stored in liquid nitrogen to minimize proteolytic activity. B, expression of cleaved FAK (90-kDa form) is higher in tumor samples that have elevated levels of the tr-AR. C, growth curve of Rv1 cells proliferating in AD⁺ media (closed rectangles), AD⁺ media and 40 μM calpeptin (open rectangles), AD⁻ media (closed circles), and AD⁻ media and 40 μM/L calpeptin (open circles). Cellular proliferation was quantitated using the MTS assay. D, Western blot analysis of PSA expression in Rv1 cells proliferating in AD⁺ and AD⁻ media, in the presence or absence of calpeptin. NS, nonspecific band.
Inhibition of calpain activity in the absence of androgen promotes apoptosis. Does calpain inhibition in the absence of androgens cause reversion of the Rv1 cells to the parental androgen-dependent phenotype? Cells proliferated in the absence of androgen but more slowly then in androgen-containing media. Calpeptin-treated cells survived well in the presence of androgen, although calpeptin retarded proliferation (Fig. 3C). However, calpeptin treatment in the absence of androgens resulted in a decrease of viable cells. A 48-h calpeptin treatment in the presence of androgens did not reduce the expression of the endogenous androgen-responsive PSA gene, whereas calpeptin treatment in the absence of androgens reduced PSA expression significantly, suggesting a block in AR-dependent transcription (Fig. 3D). A 72-h calpeptin treatment in the absence of androgens induced apoptosis in ~40% of the cells, supporting the hypothesis that calpain inhibition causes Rv1 cells to revert to an androgen-dependent phenotype (Fig. 4A).

An HIV protease inhibitor inhibits calpain activity. Calpeptin, while effective in cell culture studies, has limited utility for animal studies. A previous report indicated that HIV protease inhibitors inhibit calpain activity (15); therefore, Rv1 cells were treated with amprenavir. The effective amprenavir dose was two or four times the peak plasma level used in anti-retroviral therapy. Amprenavir treatment reduced expression of tr-AR (Fig. 4B). Importantly, treatment of Rv1 cells with amprenavir in the absence of androgens caused the cells to undergo apoptosis (Fig. 4C).

Amprenavir inhibits tumor growth. To test the efficacy of amprenavir in a mouse model, Rv1 tumors were established in castrated nude mice. Amprenavir or vehicle was given daily at a dose that is equivalent to 3.3 times the pediatric dosage used in anti-HIV therapy. Tumor growth was followed for 6 weeks. Amprenavir-treated mice did not exhibit any apparent toxicity and amprenavir treatment resulted in a statistically significant inhibition of tumor growth (Fig. 4D).

Discussion

The role of calpain in the etiology and progression of cancer is supported by reports of increased calpain levels in prostate, renal, and colorectal tumors (10, 16, 17). Calpain cleavage affects various aspects of cell physiology and the consequences of increased calpain activity are determined by cellular context. We and others (18) find that the AR is a calpain substrate. The current study shows that calpain cleaves the AR, removing the LBD to generate a constitutively active molecule that was more robust in transcriptionally activating transcription from the PSA promoter. One potential explanation for increased tr-AR activity is that the FL-AR has to be activated by ligand, whereas the tr-AR is active immediately after translation, hence exhibits enhanced activity. Alternatively, deletion of a domain that interacts with coactivators and corepressors may prevent recruitment of corepressors into the transcription complex, enhancing transcription. Moreover, the tr-AR and FL-AR may interact with a distinct subset of proteins and potentially transactivate distinct cohorts of genes.

Western blot analysis of AR in tumor tissue detected elevated expression of the truncated AR isofrom in some tumors. This analysis provides compelling evidence that expression of this
isoform is not a rare event restricted to relapsed cell lines derived from a single tumor (CWR22) but a previously uncharacterized feature of certain prostate tumors. Higher levels of FAK cleavage in these samples suggest higher calpain activity. The role of the truncated AR in the etiology of androgen independence is buttressed by the identification of a Q640 termination mutation in an androgen-independent prostate tumor (19). This mutation results in the expression of a tr-AR missing the LBD.

Although HIV protease inhibitors were developed to specifically inhibit a HIV protease, they also possess anti-calpain activity (15). We have shown that ampravir mimics the effects of calpeptin. If increased calpain activity contributes to prostate tumorigenesis, then inhibition of this activity would be an attractive therapeutic target. The results of the current study raise the possibility that HIV protease inhibitors may have efficacy in reducing the growth of certain prostate tumors.

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