

Androgen-Independent Expression of Adrenomedullin and Peptidylglycine α -Amidating Monooxygenase in Human Prostatic Carcinoma

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Most of the locally advanced and metastatic prostate carcinomas (PCs) treated with antiandrogenic therapy eventually become refractory to this treatment. Locally produced factors may control prostate tumor biology after androgen withdrawal. Adrenomedullin (AM) is expressed in the prostate and could control cell growth in androgen-independent conditions. AM needs to be amidated by the enzyme peptidylglycine α -amidating monooxygenase (PAM) to become fully active. The objective of the present study was to analyze whether the expression of proadrenomedullin (preproAM) and PAM in PC is regulated by androgens. For this purpose, human *in vitro* and *in vivo* PC models were grown in the presence or absence of androgens, and the expression of AM and PAM was examined by immunohistochemistry, Western blotting, RT-PCR, and Northern blotting. Furthermore, immunohistochemical analysis of AM in clinical specimens was performed to test if its expression is related to Gleason score and antiandrogenic therapy. In PC cell lines and xenografts, mRNA and protein AM levels were similar in the presence or absence of androgens. PAM expression seemed to be induced by androgen-withdrawal. Our results in clinical samples showed no relationship between AM expression and Gleason score or antiandrogenic treatment. In conclusion, our results demonstrate that preproAM and PAM expression in the human prostate is androgen-independent. In addition, we also report for the first time the expression of a novel PAM transcript in PC, which has not been previously described in other tissues. © 2003 Wiley-Liss, Inc.

Key words: androgens; cancer; adrenomedullin; peptidylglycine α -hydroxylating monooxygenase; prostate

INTRODUCTION

Prostate growth, differentiation, and maintenance strongly depend on the presence of androgens [1]. Since prostatic neoplastic cells are initially responsive to androgens, conventional treatment for advanced and disseminated prostate carcinoma (PC) includes androgenic suppression. However, many tumors become refractory to this treatment, which implies a very poor prognosis for the patient [2]. In the prostate, factors that are locally produced contribute to its homeostatic control. Prostate epithelial secretory cells and fibroblasts produce several growth factors (GFs) [3], and neuroendocrine cells synthesize diverse neuropeptides [4]. Some studies suggest that some of these GFs and neuropeptides can regulate prostatic epithelial cell growth in the absence of androgens [5,6]. Some local GFs and epithelial cell receptors suffer dramatic changes in expression after androgen removal [3]. The study of these factors becomes critical, because they could favor prostate cancer tumor regrowth under androgen-free conditions.

Proadrenomedullin N-terminal 20 peptide (PAMP) and adrenomedullin (AM) are regulatory peptides originating from the post-translational processing of proadrenomedullin (preproAM) precursor [7]. AM is a pluripotent peptide that has been related to cell growth control [8,9]. AM and other GFs and neuropeptides expressed by prostatic cells require post-translational amidation to be completely bioactive [10,11]. COOH-terminal α -amidation is

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Abbreviations: AM, adrenomedullin; bp, base pair; FCS, fetal calf serum; FCS-DCC, dextran-charcoal-stripped FCS; GF, growth factor; GSS, Gleason sum score; PAL, peptidyl- α -hydroxyglycine α -amidating lyase; PAM, peptidylglycine α -amidating monooxygenase; PAMP, proadrenomedullin N-terminal 20 peptide; PBS, phosphate buffer saline; PC, prostate carcinoma; PHM, peptidylglycine α -hydroxylating monooxygenase; preproAM, proadrenomedullin; RP, radical prostatectomy; SCLC, small cell lung carcinoma; TURP, transurethral resection of the prostate.

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sequentially performed by two enzymatic activities included in peptidylglycine α -amidating monooxygenase (PAM): peptidylglycine α -hydroxylating monooxygenase (PHM) and peptidyl- α -hydroxyglycine α -amidating lyase (PAL) [12].

PAMP has been found in neuroendocrine cells of normal and malignant prostate [13,14]. On the other hand, normal and neoplastic nonneuroendocrine epithelial cells show AM-immunoreactivity [13–15]. The amidating enzyme PAM is mainly produced by normal and malignant neuroendocrine cells and, to a lesser extent, by tumor cells [13–15]. Rocchi et al. [15] showed that AM slightly promotes cell growth in DU-145 cells. However, we have recently shown that overexpression of the *preproAM* gene in PC-3 cells results in a significant decrease in cell growth rates [16].

In the present work, we have used human PC experimental models (xenografts and cell lines) to study whether the expression of AM and PAM is altered by androgen deprivation. We have also analyzed whether AM expression changes in relationship to tumor grade or antiandrogenic therapy in clinical PCs.

MATERIALS AND METHODS

Prostatic Clinical Specimens

Normal prostates ($n = 2$) were obtained from 17- and 23-yr-old healthy individuals who died in traffic accidents. Samples were kindly donated by Dr. Luis Santamaria (Autonomous University of Madrid). Neoplastic tissues were obtained through radical prostatectomy ($n = 30$) or transurethral resection of the prostate (TURP, $n = 10$) in the University Hospital Rotterdam (The Netherlands). From the TURPs, four prostates were obtained from patients who were subjected to antiandrogenic therapy and six were obtained from untreated patients. Most of the specimens from radical prostatectomy (RPs) ($n = 25$) and all the samples from TURPs were fixed in 10% buffered formalin and paraffin embedded. Tumor grade was determined according to Gleason's system [17]. Gleason sum scores (GSS) assigned to the patients were 4 ($n = 2$), 5 ($n = 1$), 6 ($n = 6$), 7 ($n = 5$), 8 ($n = 5$), 9 ($n = 4$), and 10 ($n = 2$) for the RPs. Gleason sum score for the TURPs ranged from 7 to 10. Five samples from RPs were frozen at -80°C for protein extraction. All tissue procurement protocols were approved by the relevant institutional committees.

Prostatic Tumor Cell Lines and Culture Conditions

Androgen-independent DU 145 and PC-3 cell lines and androgen-dependent LNCaP and PC-346C cell lines were studied. DU 145, PC-3, and LNCaP were established from PC metastasis [18–20]. The PC-346C line was derived from the human prostate tumor xenograft model PC-346 [21] and corresponds to a primary tumor. This cell line was donated by the

Department of Experimental Urology of the Erasmus University (Rotterdam, The Netherlands). DU 145, PC-3, and LNCaP were obtained from the American Type Culture Collection (Manassas, VA).

Cell lines were cultured in 5% CO_2 at 37°C . All cell lines but PC-346C were grown in RPMI 1640 medium with Glutamax-I (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 7.5% fetal calf serum (FCS) (Bodinco B.V., Alkmaar, The Netherlands), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco). PC-346C was grown in DMEM/F12 with L-glutamine (Biowhittaker Europe, Verviers, Belgium) supplemented with 2% FCS, penicillin, streptomycin, 10^{-10} M synthetic androgen R1881 (NEN Life Science, Zaventem, Belgium) and various GFs as previously described [22].

Androgen-deprivation experiments were carried out with the androgen-dependent LNCaP cell line. Cells were cultured in RPMI 1640 medium supplemented with antibiotics and dextran-charcoal-stripped FCS (FCS-DCC), for 3, 7, or 14 d. A time-point experiment was also included to examine the re-expression of the target molecules after androgen-replacement. Thus, some LNCaP cells were cultured for 7 d with FCS-DCC-supplemented medium, and subsequently with FCS-supplemented medium for 3 d. These cells cultured in hormone-replaced conditions were called LNCaP 7 + 3. LNCaP cells cultured in FCS-DCC medium stopped proliferating, thus indicating the effectiveness of androgen ablation in the medium. To verify further the success of androgen ablation, protein levels of androgen receptor (AR) were analyzed by Western blotting. As expected, the intensity of the androgen receptor-specific 110 kDa band decreased in FCS-DCC cultured cells compared to controls (result not shown). This observation coincides with others previously reported for LNCaP [6] and other androgen-dependent prostatic experimental models [23,24].

Cells were harvested by trypsinization. Pellets were either stored at -80°C for RNA isolation and protein extraction or processed for immunohistochemistry. In the latter case, cells were fixed in Bouin's fluid for 2 h at 4°C , and then mixed with 1–2% agarose at 55°C . Once solidified, agarose blocks were paraffin-embedded.

PC-310 Human Prostate Tumor Xenograft Model

The PC-310 model was established from a human primary PC at the Department of Experimental Urology in Erasmus University [21]. Small pieces of tumor were subcutaneously implanted in NMRI athymic nude mice (Harlan Company, Horst, The Netherlands). Mice were androgen-supplemented through Silastic implants containing testosterone (Sigma, St. Louis, MO). Castration was performed and the mice were sacrificed at day 0, 2, 5, 7, 14, and 21 postcastration [25]. Androgen-withdrawal success was tested by measuring the level of the

androgen-dependent prostatic specific antigen (PSA) in the sera of the tumor-bearing mice. The prostatic specific antigen concentration in the control (non-castrated) host mice was approximately 200 ng/mL, but it dropped rapidly postcastration and was maintained around zero after 14 d [25].

Prostatic tumors were either fixed in 4% buffered formalin and paraffin-embedded for immunohistochemical detection or snap-frozen in liquid nitrogen and stored at -80°C for Western blot analysis.

Antibodies and Peptides

Immunoreactivity for AM was demonstrated using a polyclonal antiserum raised in rabbit to the amidated AM₂₂₋₅₂ synthetic human sequence. The specificity of this antiserum has been previously assessed [26]. Optimal dilution determined for immunohistochemistry was 1:1000 (for clinical and xenograft tissues) and 1:4000 (for cell lines). In Western blot analysis, the anti-AM antibody was applied at a 1:2000 dilution for tissues and at a 1:4000 dilution for cell lines. To detect PHM/PAM immunoreactivity, we used a monoclonal antibody (clone G8) raised against the bovine PHM region (bPAM₂₈₈₋₃₁₀). This antiserum recognizes human PAM [14] and was applied at a 1:1000 dilution for immunohistochemistry and at a 1:1500 (tissue extracts) or a 1:3000 (cell extracts) dilution for Western blotting. Antigenic peptides, amidated-AM₂₂₋₅₂ and bPAM₂₈₈₋₃₁₀, were used for the absorption controls, at a concentration of 10 μM .

Immunohistochemistry

Sections 4 μm thick were obtained from the paraffin-embedded tissues and cells and placed on Vectabond (Vector Laboratories, Burlingame, CA) pretreated slides. To detect AM in the clinical and xenograft tissues, we used a previously described protocol [25]. For evaluating AM and PHM/PAM expression in the cell lines, we applied a detection method based on the EnVisionTM + System (Dako, Carpinteria, CA). Cell slides were deparaffinized and rehydrated, and endogenous peroxidase was inhibited in a 3% H₂O₂ solution in deionized H₂O for 10 min. Microwave pretreatment was applied for antigen retrieval [25]. Slides were blocked with normal goat serum (1:20) at room temperature for 30 min and incubated overnight with the specific antiserum at 4°C. Sections were incubated at room temperature for 30 min with horseradish peroxidase labeled polymer conjugated to goat antirabbit or antimouse (according to the primary antibody applied) immunoglobulins (EnVision). Peroxidase activity was detected with 3-3'-diaminobenzidine. Slides were counterstained with Harris' hematoxylin, dehydrated and embedded in DPX. Pictures were taken with a Nikon Eclipse E800M microscope.

For absorption controls, optimal dilutions of primary antisera preincubated at 4°C with the corre-

sponding antigen at 10 μM were applied onto the slides instead of the primary antiserum.

Protein Extraction and Western Blot

Prostatic tissues were crushed in a metal cylinder cooled down at -80°C , and cell pellets were kept on ice for 5 min. Then, lysis buffer [25] containing a cocktail of protease inhibitors (CompleteTM; Roche Diagnostics, Penzberg, Germany) was added to both types of samples. After centrifugation (12 000 $\times g$, at 4°C, 10 min), supernatants were recovered and the protein concentration was spectrophotometrically measured by the binciconic acid method (BCATM protein assay kit; Pierce, Rockford, IL).

For AM detection, each sample was transferred to 10–20% tricine SDS-PAGE gels (Invitrogen, Carlsbad, CA). Bovine adrenal medulla extracts were loaded as positive control [10]. For PHM/PAM analysis the samples, and H69 small cell lung carcinoma (SCLC) cell line as control [27], were loaded in NuPAGE 3–8% tris-acetate SDS-PAGE gels (Invitrogen). Cells or tissue samples consisted of 30 or 50 μg of protein extracts, respectively. Unstained Mark12TM Wide Range Protein Standard (Invitrogen) was used as molecular weight standard. Proteins were electrophoretically separated, and blotted as previously described [13] for the AM detection.

Blots were blocked overnight at 4°C with 5% milk in phosphate buffer saline (PBS; pH 7.5). Anti-AM or anti-PHM antisera diluted in PBS/milk was added subsequently, and membranes were incubated for 1 h on an orbital shaker at room temperature. Membranes were rinsed with 0.1% Tween-20 (Fluka Chemika, Switzerland) in PBS. An incubation with horseradish peroxidase-conjugated antirabbit (for AM) or antimouse (for PHM/PAM) secondary antibodies (Amersham, Life Science, UK) diluted 1:5000 or 1:4000 respectively in PBS/milk was performed for 1 h at room temperature. Membranes were rinsed with PBS/Tween and Western blotting luminol reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added for 1 min. Blots were then exposed to high performance chemiluminescence film (HyperfilmTM ECLTM; Amersham). Finally, membranes were stained with Serva Blue R (Serva Electrophoresis, Heidelberg, Germany) in order to assess the uniform loading of protein samples.

Cell RNA Isolation and RT-PCR

Total RNA from prostatic cell samples was obtained with the UltraspecTM RNA Kit (Biotecx, S. Loop E. Houston, TX), following the manufacturer's instructions. The RNA concentration was spectrophotometrically determined.

RNA retrotranscription was performed with M-MLV reverse transcriptase (Invitrogen). PCR was performed by using *Taq* DNA polymerase (Invitrogen) in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA). Primers for human *preproAM* cDNA

amplification were designed to allow the elongation of intron 3 in the processed mRNA, if present. These primers for preproAM (Genbank: D14874) were: 5'-AAGAAGTGGAATAAGTGGGCT-3' (sense, nucleotides 250–270) and 5'-TGTGAACTGGTAGATCTGGT-3' (antisense, nucleotides 521–540). In the case of human PAM, selected primers amplify the most variable region among all described PAM transcript variants, which includes the linker region A. Primers for PAM (Genbank: M37721) amplification were: 5'-AGAACCATACCACAGAGG-3' (sense, nucleotides 1203–1221) and 5'-GTCTTCTTCAATTGGTCCG-3' (antisense, nucleotides 1841–1859). PCR products were run in 1% agarose gels and scanned with Gelprinter SuperII (*Tecnología para Diagnóstico e Investigación*, Madrid, Spain) equipped with the Windows Software ScionImage.

Analysis of Amplified cDNA by Cloning and Sequencing

All the PCR products were purified using GeneClean II Kit (Bio101, Carlsbad, CA) and cloned into PCTM II vector of the TA Cloning kit (Invitrogen). The correct size of the cloned inserts was confirmed by digesting the plasmids with *Sac* I and *Eco* R V for PAM, and with *Eco* R I for AM (all enzymes from New England BioLabs, Norwalk, CN). Inserts were sequenced using an Abi Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). Three different clones were sequenced for each PCR band to ensure the accuracy of the results.

Northern Blot Analysis

Northern blot analysis was performed for *preproAM* mRNA detection in PC cell lines. Ten micrograms of total RNA were loaded in 1% agarose 2.2 M formaldehyde gels. Total RNA from H157 SCLC cell line was used as positive control [27]. The procedure used and the characterization of the human *preproAM* cDNA probe applied have been described previously [28]. Relative *preproAM* mRNA levels were normalized with the S9 ribosomal protein mRNA. Autoradiograms were scanned with a Sharp JX-325 apparatus and densitometric analysis was made with the Software ImageMaster 1D (Amersham).

RESULTS

PreproAM Gene Expression in Clinical and Experimental Human Prostatic Carcinoma

Immunohistochemistry for AM

Clinical and experimental PC exhibited cytoplasmic staining for AM (Figure 1A–H). Normal prostate showed immunostaining for AM in the epithelium of the glands and ducts (Figure 1A), as previously described [13]. Glands in the peripheral, central, transition, and periurethral areas were similarly stained. A heterogeneous pattern of intensity of staining was observed in normal glands and

ducts, which was independent of the zone of the prostate. Thus, glands with different intensity of staining could be observed in the same zone of the prostate (result not shown).

In PC clinical samples, 34 of 35 specimens showed immunoreactivity for AM. In RPs, AM immunostaining was observed in either well, moderately, or poorly differentiated neoplastic glands (Figure 1B–F). However, the intensity of the staining was heterogeneous and both strong and faint immunoreactivity was found, independently of the tumor differentiation grade and GSS (result not shown). Absorption controls demonstrated the specificity of the immunolabeling (Figure 1E,F). Semiquantification of AM was not performed, given the variability of the staining intensity and the apparent lack of association between AM immunoreactivity and GSS.

We also analyzed TURPs samples from patients treated with antiandrogenic therapy and untreated patients. Results in TURPs were similar to those observed for RPs (Figure 1G). All the samples were immunoreactive for AM, with no clear association between GSS or tumor differentiation grade and intensity of AM staining. Antiandrogenic therapy did not change the pattern of immunoreactivity compared to untreated patients (Figure 1G).

In the prostate cancer, model PC-310 xenografted into nude mice with normal levels of testosterone (Figure 1H), AM immunoreactivity was found in the neoplastic epithelium of all the animals studied. To determine if AM expression was androgen dependent, a group of mice were castrated and AM expression was analyzed by immunohistochemistry. After castration, tumor cells were positive for AM in all the mice analyzed, and the pattern and intensity of AM staining remained as for those of control mice (noncastrated). PC-310 tumors were positive for AM staining even 21 d postcastration (data not shown). These results strongly suggest that AM expression in prostate cancer is independent of circulating levels of androgens.

PC cell lines (DU 145, PC-3, LNCaP, and PC-346C) grown in the presence of androgens showed immunoreactivity for AM (representative examples are shown in Figure 2). To determine whether expression of AM was mediated by androgens in *in vitro* settings, we cultured the androgen-dependent LNCaP cell line with an androgen-depleted medium. Expression of AM did not change in LNCaP cells cultured in androgen-free conditions (even after 14 d) compared to controls (Figure 2). This result supports the *in vivo* data on the androgen independence of AM expression.

Western blotting for AM

Protein extracts from PC samples (five clinical specimens, the four cell lines, and the PC-310 model) and from adrenal medulla (positive control) showed two AM-immunoreactive bands with approximate

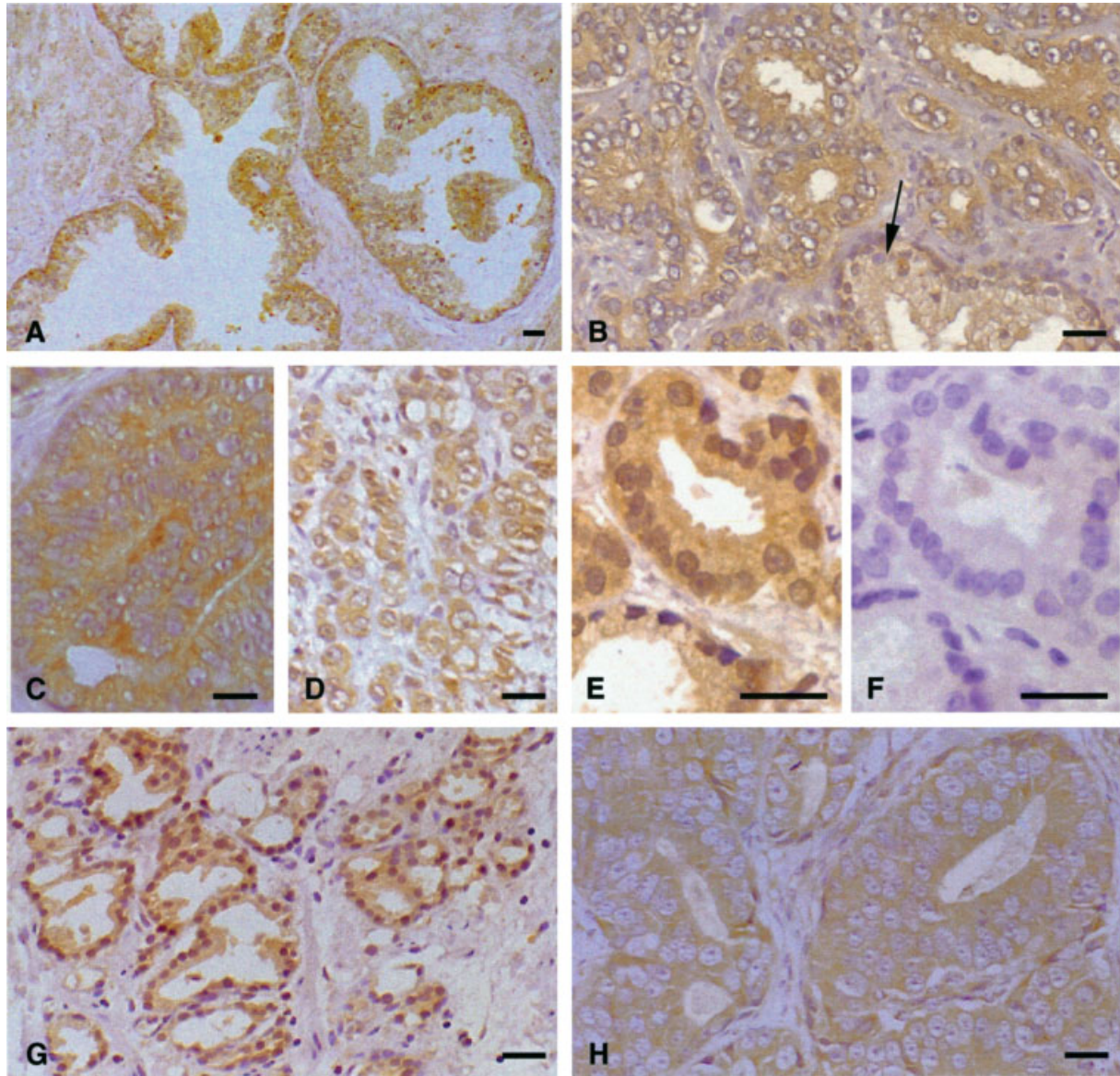


Figure 1. Sections of clinical PC and experimental PC models immunostained for AM (A–H). In the normal prostate, epithelial cells of the glands (A) and ducts are positive for AM. The staining is stronger in the basal area of the cells in many glands. In clinical specimens, AM immunostaining is found in the cytoplasm of tumor cells in well (B), moderately (C), or poorly (D) differentiated neoplastic glands and also in normal glands (arrow in B). AM adsorption control is shown in F (consecutive section to E). TURPs from patients treated

with antiandrogenic therapy also show AM-immunostaining in tumoral glands (G). No differences in the pattern or intensity of staining is observed between samples from patients treated and untreated. PC-310 tumor xenografted into nude mice also shows AM-immunolabeling (H), which was independent of levels of circulating testosterone. Gleason sum scores: B = 8; C = 8; D = 9; E/F = 4; G = 10. Bar size corresponds to 25 μ m.

molecular weights of 13 and 22 kDa (Figure 3). In most cases we also detected another immunoreactive band of 15 kDa (Figure 3). None of the prostatic samples exhibited the 6 kDa band corresponding to the fully processed AM, whereas the adrenal medulla did (Figure 3A). All bands were absent in the adsorption controls (figure not shown).

LNCaP cells cultured in hormone-depleted or in hormone-replaced media, and PC-310 tumors kept in castrated mice, showed the same pattern of AM-immunoreactive bands (Figure 3B). The intensity of

the bands obtained for both models remained unchanged throughout the experimental time-points (Figure 3B). Therefore, Western blot analysis confirmed our previous immunohistochemical results.

RT-PCR for *preproAM* mRNA in PC cell lines and sequencing of cDNAs

In prostatic cell lines and in H157 SCLC cell line (positive control), a strong band with the expected size for the PCR amplified *preproAM* cDNA (291 bp)

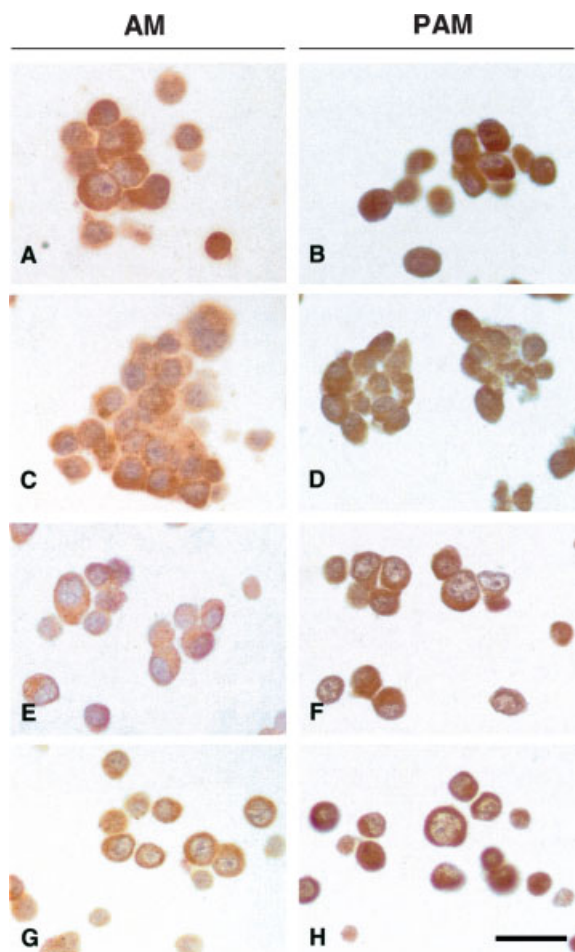


Figure 2. PC cell lines immunostained for AM (left panel) or PAM (right panel). LNCaP cells cultured for 7 d in absence of androgens show similar staining for AM (C) and PAM (D) as do control cells (A and B, respectively). PC-346C cells show AM (E) and PAM (F) immunostaining. Similarly, DU 145 cells are immunostained by AM (G) and PAM (H). Experiments were performed in triplicate. Bar size corresponds to 25 μ m.

was detected (Figure 4A). Two other fainter bands of 441 and 524 bp were observed in all the samples. These three cDNAs were also found for LNCaP cells grown in hormone-depleted or -replaced media (Figure 4A). Water control did not render any band (data not shown).

All amplified cDNAs were cloned and sequenced. The 291 bp band corresponded to the *preproAM* cDNA that was described previously. Sequencing of the 524-bp cDNA showed that this transcript contained the 291-bp band plus the third intron (233 bp) of the *preproAM* gene. Thus, the 524-bp band was identical to that reported for a recently identified alternatively-spliced *preproAM* gene-derived mRNA [29]. Sequencing of the 441-bp cDNA showed that this form corresponded to the 291-bp band plus primer concatemers and, therefore, it did not represent any alternatively spliced mRNA.

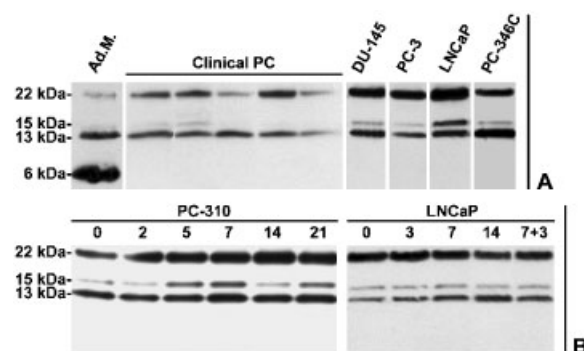


Figure 3. Western blotting for AM. (A) Three AM-immunoreactive bands different of mature AM can be found in clinical PCs and cell lines as well as in a positive control (adrenal medulla). (B) PC-310 and LNCaP models exhibit the same band pattern in every androgen-depletion time-point. Number of androgen-deprivation days is indicated above each lane. 7+3 means that after 7 d of culture in androgen-depleted medium, the cells were cultured for 3 d in androgen-containing medium. Experiments were performed in triplicate.

Northern blotting for *preproAM* mRNA in PC cell lines

By Northern blot analysis, cell lines showed a band of approximately 1.4 kb, the expected size for the *preproAM* mRNA (Figure 4B). The androgen-independent cell line DU 145 exhibited a stronger signal than the androgen-dependent cell line LNCaP. These latter cells cultured in hormone-deficient medium up to 14 d produced a similar amount of *preproAM* mRNA to that of cells cultured in androgen-replaced medium (Figure 4B). Relative *preproAM* mRNA levels were normalized to those of the ribosomal protein S9 (Figure 4C). Normalization showed no variation in *preproAM* mRNA levels during the androgen-withdrawal experiment. This experiment shows that lack of androgens does not alter AM mRNA levels in the androgen-dependent LNCaP cell line.

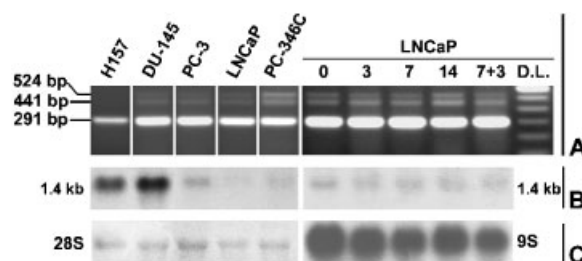


Figure 4. (A) RT-PCR for *preproAM* mRNA detection in PC cell lines and in positive control (H157). D.L., 100 bp DNA ladder. (B) Northern blot for *preproAM* mRNA detection in the same samples studied in (A). DU-145 shows the strongest signal. LNCaP cells cultured in androgen depleted medium at different time points show the same intensity of AM mRNA expression. (C) Loading controls for the Northern blot. The similar intensities of the 28S (for the different cell lines) and 9S bands (for the androgen-removal experiment in LNCaP cells) show that the amount of mRNA loaded was similar in the wells. Three replicates of the experiment were performed.

PAM Gene Expression in PC Cell Lines

Immunohistochemistry for PHM/PAM

Expression of PAM in the normal prostate and PC was previously described by our group [14]. All prostatic cell lines studied showed PHM/PAM immunoreactivity (representative examples are shown in Figure 2). PHM/PAM staining was positive for the androgen-dependent LNCaP and PC-346C cells and the androgen-independent DU 145 or PC-3 cells, although the latter ones tended to have stronger staining. LNCaP cells cultured in hormone-depleted or hormone-replaced media showed similar staining for PAM (Figure 2). Adsorption controls lacked immunostaining (figure not shown).

Western blot for PHM/PAM

Proteins immunoreactive for PHM/PAM with estimated molecular weights ranging from 34 to 105 kDa were detected in all the samples (Figure 5A). The pattern and intensity of the PHM/PAM-immunoreactive bands were not identical in all the cell lines, but a 50 kDa band was the most intense in all cases. Band intensities were fainter for the androgen-dependent cell lines LNCaP and PC-346C than for the androgen-independent DU 145 and PC-3 (Figure 5A). Almost all bands were also detected in the positive control, the H69 SCLC cell line (Figure 5A). The PHM/PAM-immunoreactive bands were considered specific, because all the signals were abrogated in the adsorption controls (data not shown).

LNCaP cells also showed PHM/PAM immunoreactivity after hormone depletion (Figure 5A). In fact, the intensity of the bands increased after androgen deprivation, being highest 14 d after androgen removal. However, after androgen replacement, the intensity of the bands did not reach the initial levels of expression (Figure 5A).

RT-PCR for PAM and sequencing of cDNAs

Four cDNAs (336, 390, 519, and 657 bp) were amplified from all the cell lines tested (Figure 5B). The pattern of bands found for LNCaP was not altered after androgen withdrawal or androgen replacement (Figure 5B).

The PCR bands of 336, 390, and 657 bp correspond to the expected sizes for hPAM-B, hPAM-C, and hPAM-A, respectively. To corroborate further the identities of these bands, the amplified cDNA was cloned, purified, and sequenced. Thus, 657- and 336-bp cDNAs are coincident with the hPAM-A and hPAM-B sequences, respectively, described by Glauder [30] and the 390-bp band with Vos' hPAM-C [31]. The 519-bp band had not been previously described. Sequencing of three different clones revealed the existence of a novel PAM gene transcript that was similar to hPAM-A (Figure 6) but lacked the last 138 bp (nucleotides 1534 to 1671, Genbank: M37721) of the exon A, codifying for the region A (a linker zone between PHM and PAL enzymatic domains). The splicing phenomenon that renders this new PAM mRNA transcript occurs between a GT sequence in exon A (nt 1534) and an AG located at the end of the intron preceding the PAL domain. Following the current nomenclature for the PAM proteins, we have called this novel transcript hPAM-E (Figure 6). hPAM-E maintains the translational reading frame and would produce a protein with 46 aminoacids less than hPAM-A. Besides, three point mutations were found in all sequenced 519 bp-band clones, the three of them belonging to exon A: E402E (GAA → GAG), T410H (TAT → CAT), and R448G (AGG → GGG). The first change of nucleotide described is a silent mutation, whereas the other two would lead to an aminoacid change in the translated protein.

DISCUSSION

In the present work we demonstrate that most of the studied PC clinical samples and all the PC models show AM-like immunolabelling, independently of the tumor grade, GSS, and androgen levels. The immunoreactive bands detected in PC protein extracts by Western blot (13, 15, and 22 kDa) have also been described in the positive control (adrenal medulla) and are specific, as demonstrated by adsorption controls. These bands probably correspond to the *preproAM* and intermediate processing-derived peptides, as suggested by others [9]. The fact that human PC samples lack fully processed AM but express other AM-immunoreactive peptides could have two possible explanations. Many authors have suggested that mature AM (corresponding to the 6 kDa band) would be rapidly processed from the *preproAM* form and constitutively secreted into the media. This hypothesis is supported by the observation of a high amount of mature 6 kDa AM in the secreted medium of cells where endogenous mature AM is almost absent

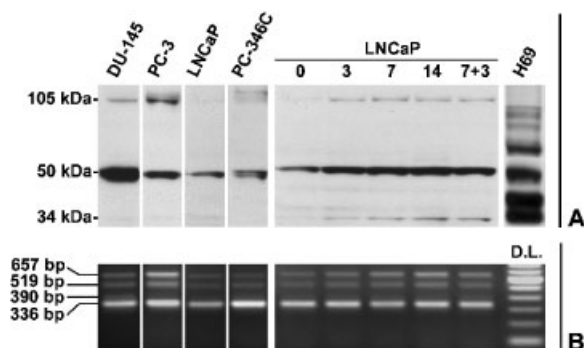
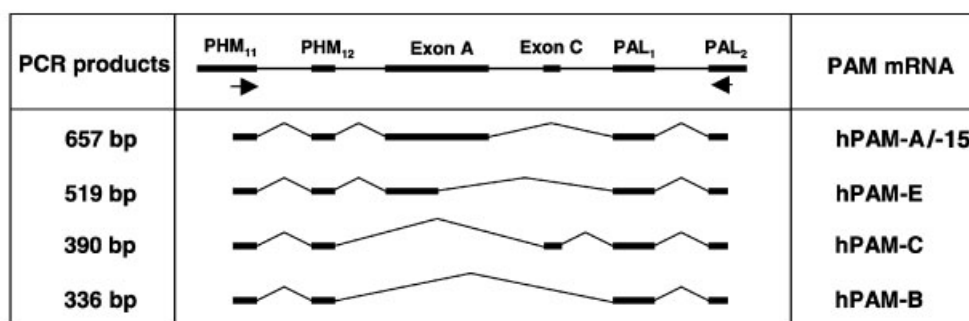


Figure 5. (A) Western blot for PHM/PAM in PC cell lines and in positive control (H69). Several immunoreactive bands are detected. (B) RT-PCR for PAM mRNA detection in the same cases showed in (A). The 519 bp band corresponds to the novel isoform that we have called hPAM-E. D.L., 100 bp DNA ladder. Experiments were performed in triplicate.

A



B

¹²⁰³AGAACCATACCACCAGAGGCCAACATTCCAATCCCGTGAAGTCTGATATGGTTATGATGCATGAACA
 TCATAAAGAAACAGAATATAAAGATAAGATTCTTTACTACAGCAGCCAAAACGAGAAGAAGAAGTGT
 TAGACCAGGGTGATTTCTATTCACTACTTTCCAAGCTGCTAGGAGAAAAGGGAGGATGTTGTTTCATGTGCAC
 AAACATAATCCTACAGAAAAGGCAGAATCAGAGTCAGACCTGGTAGCTGAGATTGCAAAATGTAGTCCAAAA
 AAAGGATCTTGGTCGATCTGATGCCAGAGAGGGTGCAGAACATGAGGGGG . . . ATTTCCACATGGAAGAGG
 CACTGGATTGGCCTGGAGTATACTTGTACCAGGCCAGTTCCTGGGGTGGCTCTAGACCCCTAAGAATAAC
 CTGGTGATTTCCACAGAGGTGACCATGTCTGGGATGGAACTCGTTTGACAGCAAGTTGTTTACCAGCA
 AATAGGACTCGGACCAATGAAGAAGAC¹⁸⁵⁹

C

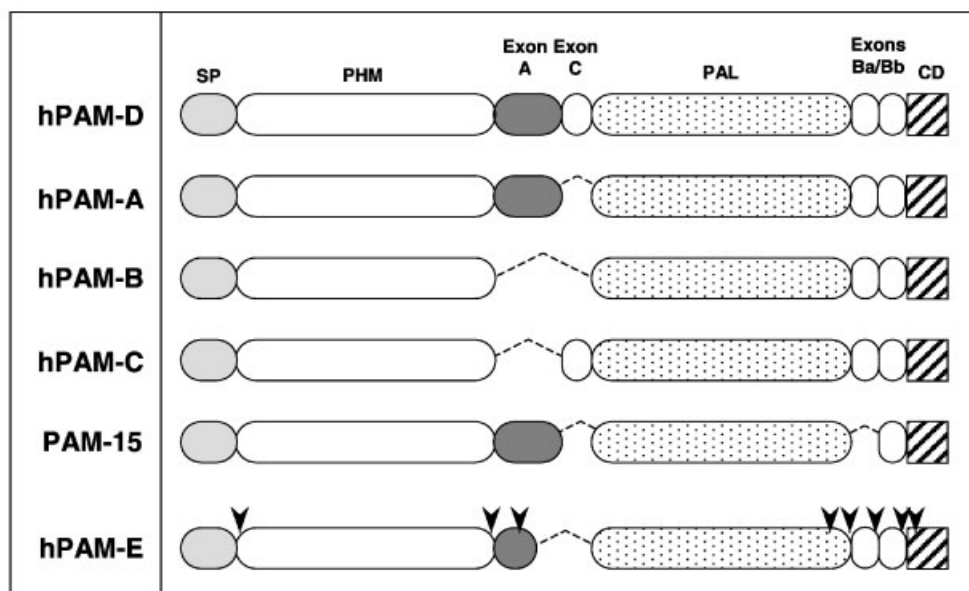


Figure 6. Schematic representation for RT-PCR and sequencing results. (A) Amplified region by RT-PCR. The exons encoding for each protein region are named and drawn to scale in thick lines. Note that for PAM E, the splicing phenomenon occurs among an intermediary sequence inside exon A and the first exon coding for PAL (PAL₁). (B) The complete amplified nucleotide sequence for PAM E is included. Underlined nucleotides indicate primer localization. Highlighted

nucleotides are changed with respect to previously published sequences. Dotted gaps indicate the region where the novel alternative splicing occurs. (C) PAM proteins with the hypothetical structure of the newly described PAM E. Signal peptide and cytoplasmic domain (CD) are included. Localization of paired basic amino acid residues representing potential cleavage sites (arrow heads) are indicated only in PAM D and PAM E proteins.

[32,33]. This is also true for the human prostate PC-3 cells [16]. This finding correlates with the very low peptide/mRNA ratio found in many cell systems, which indicates a rapid secretion of AM [34,35]. The second possibility, which has also been suggested by several authors, claims that those larger peptides are

secreted and have a physiological relevance. This last option is based on several observations: (1) Western blot analysis of extracts obtained from specific areas of some organs (i.e., rat prostate [13] and brain [36]) only show AM precursors while in other zones of those organs, mature AM is also found;

(2) AM-immunoreactive molecules larger than the fully processed AM are secreted in certain systems, like plasma [37], milk [38], and culture-conditioned media of certain cancer cell lines [9]; and (3) finally, it has also been described that either mature AM or precursors can be secreted by astrocytes depending on the presence of cytokines in the culture medium [39].

Northern blot analysis has demonstrated that all the PC cell lines studied produce the *preproAM* mRNA. However, PCR amplification has shown that together with the classical mRNA, another recently identified alternatively-spliced mRNA [29] derived from *preproAM* gene is also expressed. This mRNA (that has been detected by in situ hybridization in normal neuroendocrine prostatic cells) retains an intron that includes an early stop codon and thus, encodes for a precursor containing PAMP but not AM [29]. Our previous work shows that PAMP immunoreactivity in prostate is detected in neuroendocrine cells but not in the non-neuroendocrine cell population [13,14]. The presence of mRNA encoding specifically for PAMP in the PC cell lines we have studied is an unexpected finding and needs further clarification, since none of these cell lines exhibit neuroendocrine phenotype under normal culture conditions.

The variety of PAM mRNAs and proteins found in the prostatic material is likely related to the presence of different substrates susceptible to amidation. We previously reported that PAM is expressed in PC neuroendocrine and non-neuroendocrine neoplastic cells [14]. Our present study shows the presence of different PAM mRNAs and PHM/PAM proteins in extracts of the non-neuroendocrine cells DU 145, PC-3, LNCaP, and PC-346C. Alternative splicing of the *PAM* gene renders several mRNA forms [40]. There are five human PAM transcripts described in the study so far: hPAM-A, hPAM-B, hPAM-C, hPAM-D, and PAM-15 [30,31,41]. They differ in the presence or absence of the linker regions between PHM and PAL sequences (exons A and/or C) and/or the transmembrane domain (Figure 6). We have found that neoplastic prostate cell lines express hPAM-A, hPAM-B, and hPAM-C mRNA. However, the 657-bp band could also represent PAM-15 mRNA, because the two proteins do not differ in the region amplified by our specific PAM primers.

Together with these known PAM mRNAs, we have identified a new variant that has probably been originated by mRNA alternative splicing. We have called it hPAM-E, and differs from the other described forms in the linker region between PHM and PAL, known as region A. The region A is codified by a single exon in chromosome 5. This implies that the alternative splicing found in hPAM-E occurs between the end of the intron preceding the PAL domain and an internal sequence of exon A. Indeed, the two initial nucleotides in the missing region of the exon

A are GT. A protein translated from hPAM-E would lack 46 aminoacids from the COOH-terminus of the region A, which is a 107-aminoacid sequence located between the enzymatic domains PHM and PAL. hPAM-E protein would maintain the paired basic residues (KK) necessary for the endoproteolytic cleavage that separates PHM from PAL. The existence of a protein with such a modified linker region could have important physiological implications. Region A exhibits pH-dependent conformational changes that determine whether the dibasic aminoacids are accessible to endoproteolysis [42]. Therefore, structural changes in hPAM-E could involve a way of regulating the proteolytic separation of the PAM enzymatic domains through the secretory pathway. Besides, it is worth noting that changes in tyrosine and histidines in region A have been described as important for a rapid trans-Golgi network traffic and for Cu-ion binding, necessary in the PHM catalytic reaction, respectively [43,44]. The relevance of the novel alternative splicing mechanism we hypothesize here, as well as the significance of the two aminoacid changes, should be further studied.

Translation of human PAM mRNAs renders a bifunctional enzyme (containing PHM and PAL activities). They are all transmembrane precursors, except for PAM-15, which is soluble. Through endoproteolytic cleavage, PAM can produce monofunctional enzymes (PHM or PAL) [12]. By Western blot analysis, we have detected several PHM/PAM-immunoreactive proteins in PC and in H69 SCLC cell lines. A PAM bifunctional enzyme should have a minimal relative molecular mass of 75 kDa [45]. In our case, the band of 105 kDa could correspond to any of the bifunctional PAM (including PHM and PAL domains), most likely to hPAM-A [41]. Bands below 75 kDa probably correspond to monofunctional PHM proteins. These PHM molecules may be biologically active. It has been demonstrated that the smallest PHM fragment maintaining enzymatic activity weighs 34 kDa [45]. This hypothesis is further supported by the finding that DU 145, PC-3, and LNCaP cells exhibit PHM activity [15].

We have found that PC samples show AM and PHM/PAM immunostaining independently of being androgen-dependent (PC-310 xenograft model, PC-346C and LNCaP cells) or -independent (DU 145 and PC-3 cell lines). AM-related peptides are produced by neoplastic cells in the PC-310 model, even after host mice castration. In addition, LNCaP expresses the *preproAM* gene after steroid hormone-depletion. It has been observed that *preproAM* mRNA expression in rat ventral prostate is downregulated after castration [46]. However, our present results show that this does not seem to be the case for the human prostate. There exist important ontogenic and biological differences between human and rat prostate. The divergence is larger in the case of rat prostatic ventral lobes, whose embryological origin is different from

that of the human prostate [47]. Although all the rat lobes express androgen receptor, a distinct androgen regulation of certain proteins occurs in each particular type of lobe. For example, the expression of metalloproteases [48] or adhesion molecules, such as C-CAM [49] is controlled by androgens in the rat ventral prostate, but not in the dorsal prostate. Therefore, the fact that AM expression is androgen-dependent in the ventral lobes of the rat prostate does not imply a similar regulation in the dorsolateral lobes or in the human prostate.

In a previous work we reported that androgen-independent PC xenograft models showed a stronger PHM/PAM-immunostaining than androgen-dependent tumors [14]. Consistently with those data, our Western blot analysis shows that androgen-independent DU 145 and PC-3 cells have more intense PHM/PAM-immunoreactive bands than androgen-dependent (LNCaP or PC-346C) cell lines. Moreover, the intensity of the bands derived from LNCaP extracts seem to increase after hormone deprivation, showing that androgen removal induces the expression of PAM protein. This leads most likely to an increase in the amidation rate (and therefore, bioactivation) of peptides in androgen-independent conditions. Active amidated peptides can have different roles in prostate cancer cells. Some peptides promote proliferation, whereas others exert cell growth inhibition. The final effect will probably depend on the balance of both types of peptides. Regarding AM, we have recently shown that cells overexpressing this peptide have decreased cell proliferation rates in vitro and tumorigenicity in vivo [16].

In summary, we demonstrate in the present work that the expression of preproAM and the amidating enzyme PAM is androgen-independent in PC. We also show that *preproAM* is widely expressed in PC, independently of the tumor grade, and levels of circulating androgens. Thus, AM-related peptides and especially PHM/PAM, whose expression tends to increase in hormone-deficient conditions, could play a role in the local control of prostate tumor growth after long-term androgen-withdrawal therapy. Finally, we report the presence in PC of a novel transcript derived from the human PAM gene, which lacks a part of exon A region, i.e., hPAM-E.

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