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Advances in Brief

PMEPA1, an Androgen-regulated NEDD4-binding Protein, Exhibits Cell Growth Inhibitory Function and Decreased Expression during Prostate Cancer Progression[1]

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Abstract

***PMEPA1* was originally identified as a highly androgen-induced gene by serial analysis of gene expression in androgen-treated LNCaP prostate cancer (CaP) cells. *PMEPA1* expression is prostate abundant and restricted to prostatic epithelial cells. *PMEPA1*-encoded protein shows high sequence homology to a mouse *N4wbp4*-encoded protein that binds to Nedd4 protein, an E3 ubiquitin–protein ligase involved in ubiquitin-dependent, proteasome-mediated protein degradation. Studies from our and other laboratories have suggested the role of *PMEPA1* in cell growth regulation as noted by androgen induction of *PMEPA1* expression, elevated *PMEPA1* expression in nontumorigenic revertants of tumor cell lines after chromosome 8p transfer, and *PMEPA1* expression alterations (up- or down-regulation) in human tumors. Here, we demonstrate that PMEPA1 protein through its PY motifs interacts with WW domains of the human NEDD4 protein. Exogenous expression of *PMEPA1*, in widely used CaP cell lines DU145, PC3, LNCaP, and LNCaP sublines (C4, C4-2, and C4-2B), conferred cell growth inhibition, and at least one of the PY motifs of PMEPA1 may be involved in its cell growth inhibitory functions. Quantitative expression analysis of *PMEPA1* in paired normal and tumor cells of 62 patients with primary CaP revealed tumor cells associated decreased expression in 40 of 62 patients that were significantly associated with higher pathologic stage and serum prostate-specific antigen. Taken together, *PMEPA1* negatively regulates growth of androgen responsive or refractory CaP cells, and these functions may be mediated through the interaction of PMEPA1 with the NEDD4 protein involved in the ubiquitin–proteasome pathway. Loss or reduced *PMEPA1* expression in CaP further suggests for its role in prostate tumorigenesis.**

Introduction

Biological effects of androgens on target cells, *e.g.*, prostatic epithelial cell proliferation and differentiation, as well as androgen ablation-mediated cell death, involve AR-mediated[3] cell signaling (1). Systematic and comprehensive analysis of the ARGs should provide the biological reporters for androgen signaling in CaP. Our efforts to analyze ARGs by serial analysis of gene expression led to the discovery of *PMEPA1* (2, 3). *PMEPA1* expression was regulated by androgen in a dose- and time-dependent manner, and *PMEPA1* was highly expressed in the prostate in comparison with other organs.

PMEPA1 encodes a protein of 252 amino acids with a type Ib *trans*-membrane domain. PMEPA1 protein sequence homology search showed 83% identity to a recently reported mouse N4wbp4 protein, which was defined as one of the several proteins that bound to WW domain of the *Nedd4*-encoded protein (4). *Nedd4*, originally identified as a developmentally regulated gene in mice, now belongs to a family of ubiquitin–protein ligases characterized by two to four WW domains, a COOH-terminal homologous to E6-AP COOH terminus domain and an NH_2-terminal C2 domain (5–7). Additional studies implicated the roles of *Nedd4* in diverse cellular functions through the ubiquitin-dependent, proteasome-mediated protein degradation (8–12). The WW domain of the Nedd4 protein comprises of a module with two highly conserved tryptophan residues, which bind to target proteins that contain a PY motif, *e.g.*, PPxY (4, 13–17). The presence of two PY motifs in the predicted protein sequence of PMEPA1 and its similarity to the mouse Nedd4-binding protein, N4wbp4, suggest that PMEPA1 is a potential binding partner of the NEDD4, the human homologue of the Nedd4.

Studies of *PMEPA1* (2, 3, 18, 19) have suggested for its role in cell growth regulation as noted by the androgen induction of *PMEPA1* expression, elevated *PMEPA1* expression in nontumorigenic revertants of tumor cell lines after chromosome 8p transfer, and the *PMEPA1* expression alterations (up- or down-regulation) in human tumors. Furthermore, the induction of *PMEPA1* expression in nontumorigenic derivatives of multiple cancer cell lines, resulting from the introduction of chromosome 8p, suggested that *PMEPA1* might be the downstream target of the critical cell growth regulatory genes on chromosome 8, the most frequently altered chromosomes in CaP.

The androgen-regulated nature of *PMEPA1*, the potential of PMEPA1 protein as a NEDD4-binding partner and suggested cell growth regulatory functions of *PMEPA1*, have now provided the impetus to study biochemical and cell biological functions of *PMEPA1* and its CaP-associated alterations.

Materials and Methods

Plasmids. Mammalian expression vectors encoding PMEPA1-V5 and PMEPA1-GFP fusion proteins were generated by PCR amplification of the *PMEPA1* open reading frame. For *PMEPA1*-V5-pcDNA3.1 vector, the primers 5′GCTGCTGGAGAACTGAAGGCG3′ and 5′GTGTCCTTTCTGTTTATCCTTC3′ were used. For *PMEPA1*-GFP-pEGFP vector, the primers used were 5′aagcttGCTGCTGGAGAACTGAAGG CG 3′ and 5′gaattcGGTGTCCTTTCTGTTTATC3′. The V5 tag or GFP protein was fused at the COOH terminus of the PMEPA1 protein. The PCR product for generating PMEPA1-V5 was inserted into pcDNA3.1-V5-His expression vector (Invitrogen, Carlsbad, CA). The PCR product for generating PMEPA1-GFP was

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[3] The abbreviations used are: AR, androgen receptor; ARG, androgen-regulated gene; GFP, green fluorescence protein; CaP, prostate cancer; TSG, tumor suppressor gene; cT, cycle threshold; EGFP, enhanced green fluorescence protein; GST, glutathione *S*-transferase; TGN, *trans*-Golgi network; GAPDH, glyceraldehyde phosphate dehydrogenase; LCM, laser-capture microdissection; QRT-PCR, quantitative reverse transcription-PCR; wt, wild type; PSA, prostate-specific antigen.

digested by *Hind*III and *Eco*RI and cloned into the same sites of pEGFP vector (Clontech, Palo Alto, CA). PMEPA1-PY motif mutants, in which the tyrosine residue (Y) was replaced with an alanine residue (A), were created by using QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) and using the *PMEPA1*-V5-pcDNA3.1 vector as a template. The plasmids of *PMEPA1-PY* motif mutants are as follows: (*a*) *PMEPA1-PY1m*-V5-pcDNA3.1, with the first PY motif mutation (Y126A); (*b*) *PMEPA1-PY2m*-V5-pcDNA3.1, with the second PY motif mutation (Y197A); and (*c*) *PMEPA1-PY1m/PY2m*-V5-pcDNA3.1, with both the PY motif mutations (Y126A and Y197A). The sequences of all of the inserts in expression vectors were verified by DNA sequencing.

A bacterial expression plasmid of human *NEDD4* gene (p*NEDD4WW*-GST-pGEX-2TK) encoding all four WW-domains (accession no. XM_046129) fused to GST (GST-WW fusion protein) was generated by PCR amplification of the coding region of the four WW-domains using the primers 5′GCAGGATCCCAACCAGATGCTGCTTGC3′ and 5′GCAGAATTCTTTTGTAATCCCTGGAGTA3′. Normal prostate tissue-derived cDNA was used as a PCR template, and the amplified fragment was cloned into the *Bam*HI/*Eco*RI sites of pGEX-2TK (Amersham Biotech, Piscataway, NJ). A mammalian expression vector (*NEDD4*-GFP-pEGFP) encoding NEDD4-GFP fusion protein was generated using the primers 5′GCAAAGCTTGTCCGG TTTGCTGGAAGC3′ and 5′GCAGAATTCCCTTTTTGTTCTTATTGGTGAC3′ to generate the *NEDD4* gene fragment by PCR.

PMEPA1 and NEDD4 Protein-binding Assays. The *in vitro* binding of PMEPA1 and NEDD4 was assessed by GST pull-down assays. GST-WW fusion protein was prepared and purified with glutathione-Sepharose beads per Amersham Biotech instructions. [^{35}S]methionine-labeled proteins representing PMEPA1 and its mutants were generated by *in vitro* transcription/translation (TNT T7 quick coupled transcription/translation system; Promega, Madison, WI). Briefly, the *PMEPA1*-V5-pcDNA3.1 or three mutants (2 μg) were incubated in 40 μl of reticulocyte lysate with 40 μCi of [^{35}S]methionine for 1.5 h at 30°C. [^{35}S]methionine incorporation into protein was measured, and samples were equalized on the basis of cpm. The GST-WW fusion protein bound to glutathione-Sepharose beads (5 μg) was incubated with the [^{35}S]methionine-labeled lysates (12 μl) in 0.4 ml of PBS (pH 7.4), 1 mM DTT, and protease inhibitors. The negative control for each [^{35}S]methionine-labeled lysate represented a reaction mixture with equivalent amounts of the lysate incubated with glutathione-Sepharose beads without GST-WW fusion protein. After 16 h of incubation at 4°C, the beads were washed six times with PBS, resuspended in SDS-PAGE sample buffer, and run on 12% SDS-PAGE gel under a reducing condition. The gels were dried and autoradiographed.

The interaction of PMEPA1 and NEDD4 proteins in cells was evaluated by a coimmunoprecipitation assay. 293 cells (human embryonal kidney cells) were cotransfected with *NEDD4*-GFP-pEGFP vector and one of the *PMEPA1*-V5 expression vectors encoding either wt PMEPA1-V5 or the PY mutants of *PMEPA1*. Thirty-six h later, the cells were collected and lysed, and the lysates were immunoprecipitated with anti-GFP antibody (Clontech) following the manufacturer's protocol. The immunoprecipitated proteins were subjected to immunoblotting with an anti-V5 tag antibody (Invitrogen).

Immunofluorescence Assays. These experiments were performed following the procedure described by Harvey *et al.* (17). Briefly, stable transfectants of LNCaP cells harboring *PMEPA1*-GFP-pEGFP (LNCaP-*PMEPA1*-GFP transfectant) were grown on coverslips for 2 days, fixed in 2% paraformaldehyde for 15 min, and permeabilized in 0.2% Triton X-100 for 2 min. Fixed and permeabilized cells were incubated with anti-GM130 (recognizes a *cis*-Golgi matrix protein) or anti-TGN38 (recognizes a protein localizing to TGN) monoclonal antibodies (BD Transduction Laboratory, San Diego, CA) at 6.25 μg/ml for 30 min at room temperature. Cells were then washed to remove excess or nonspecifically bound primary antibody followed by incubation with tetramethylrhodamine isothiocyanate-conjugated antimouse antibody (Sigma, St. Louis, MO) at 1:100 dilution for 30 min at room temperature. The sections were mounted with fluoromount (Southern Associates, Birmingham, AL), and the images were processed with a Leica fluoromicroscope and Open-Lab software (Improvision, Lexington, MA).

Colony Formation Assays and Cell Proliferation Analysis. Prostate cell lines LNCaP, PC3, and DU145 were purchased from American Type Culture Collection (Rockville, MD) and grown in the cell culture media as described by the supplier. The LNCaP sublines C4, C4-2, and C4-2B (20–22) were purchased from Urocor (Oklahoma, OK) and cultured in T medium (5% fetal bovine serum, 80% DMEM, 20% F12, 5 μg/ml insulin, 13.65 pg/ml Triiodo-Thyronine, 5 μg/ml apo-transferrin, 0.244 μg/ml biotin, and 25 μg/ml adenine). Three μg of plasmids (*PMEPA1*-V5-pcDNA3.1 or vector without *PMEPA1* insert) were transfected into the 50–70% confluent cells in triplicate in 60-mm Petri dishes with Lipofectamine (Invitrogen). TSG *p53* (wt) and mt p53 (R175H and G245D) were also used in parallel as controls. Approximately 36 h later, selection with G418 at 800 μg/ml (DU145 and PC3) or 400 μg/ml (LNCaP and its sublines) was initiated. Cells were maintained with G418-containing medium that was changed every 3–4 days. After 2–4 weeks of selection, the cells were rinsed with 1× PBS, fixed with 2% formaldehyde in 1× PBS for 15 min, stained with 0.5% crystal violet in 1× PBS for 15 min,

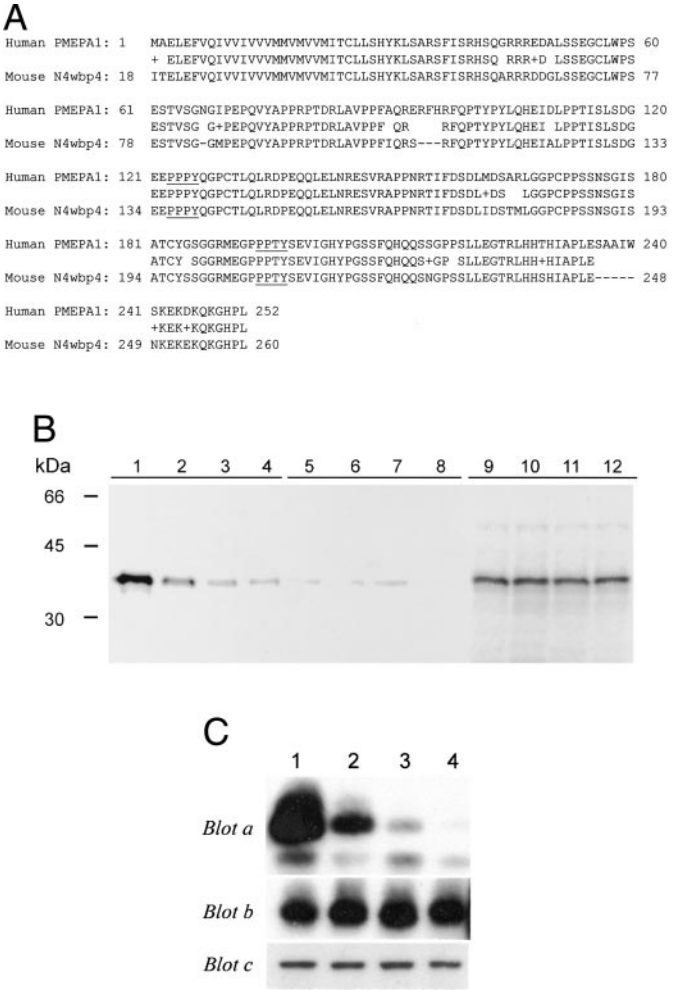

Fig. 1. Homology of human PMEPA1 and mouse N4wbp4 protein sequences and the interaction of PY motifs of PMEPA1 with the WW domains of NEDD4. In *A*, PMEPA1 and N4wbp4 (GenBank accession no. AK008976) exhibit 83% sequence identity (analyzed through the National Center for Biotechnology Information Web site).[4] The + denotes conservative substitution. The PY motifs are *underlined*. *B*, *in vitro* transcription/translation products ([^{35}S]Methionine-labeled lysates) derived from expression plasmids. *PMEPA1*-V5-pcDNA3.1 (*Lanes 1* and *5*), *PMEPA1-PY1m*-pcDNA3.1 (*Lanes 2* and *6*), *PMEPA1-PY2m*-pcDNA3.1 (*Lanes 3* and *7*), and *PMEPA1-PY1m/PY2m*-pcDNA3.1 (*Lanes 4* and *8*) were incubated with GST-NEDD4-WW-Sepharose beads (*Lanes 1–4*) or control GST beads (*Lanes 5–8*), and [^{35}S]Methionine-labeled proteins bound to GST-NEDD4-WW-Sepharose beads were solubilized in sample buffer and resolved by SDS-PAGE gel. Equal amounts of [^{35}S]Methionine lysates corresponding to samples in *Lanes 1–4* were run on SDS-PAGE gel without GST pull-down (*Lanes 9–12*). In *C*, 293 cells were cotransfected with expression vectors encoding NEDD4-GFP and one of the following fusion proteins: PMEPA1-V5 (*Lane 1*), PMEPA1-PY1m-V5 (*Lane 2*), PMEPA1-PY2m-V5 (*Lane 3*), or PMEPA1-PY1m/PY2m-V5 (*Lane 4*). The cell lysates from each group were immunoprecipitated with anti-GFP antibody and then subjected to immunoblotting (*Blot a*). Cell lysates from each group without immunoprecipitation were also processed for immunoblotting (*Blots b* and *c*) to serve as control. *Blots a* and *b* were detected by anti-V5 antibody, and *Blot c* was detected by anti-GFP antibody.

[4] Internet address: www.ncbi.nlm.nih.gov.

and rinsed one to two times with distilled H_2O. Colonies visible in each dish without magnification were counted by Open-Lab software. To assess the effects of PY motif mutations on the colony-forming ability of *PMEPA1*, LNCaP and PC3 cells were also transfected with *PMEPA1* mutants: *PMEPA1-PY1m*-pcDNA3.1, *PMEPA1-PY2m*-pcDNA3.1, or *PMEPA1-PY1m/PY2m*-pcDNA3.1. *PMEPA1*-V5-pcDNA3.1 and expression vector without insert served as positive and negative controls, respectively, for the *PMEPA1* mutants. Two independent colony-forming assays were performed as above.

To further evaluate the growth inhibitory effects of *PMEPA1* on CaP cells, a stable *PMEPA1*-GFP-Tet LNCaP transfectant was generated. Expression of PMEPA1-GFP fusion protein in these cells was negatively regulated by tetracycline in the medium (Clontech). For cell proliferation assays, 3000 *PMEPA1*-GFP-Tet LNCaP cells were seeded in 96-well plates with or without 1 μg/ml tetracycline in the medium. CellTiter 96 Aqueous One Solution kit (Promega) was used to measure the cell proliferation according to the manufacturer's instructions.

Prostate Tissue Specimens, LCM, and QRT-PCR Assay. Matched CaP and normal tissues were derived from radical prostatectomy specimens from 62 CaP patients treated at Walter Reed Army Medical Center (under an Institutional Review Board-approved protocol). The procedures for collecting specimens were described previously (23). Ten-μm frozen sections were prepared and archived at −70°C. Histologically normal prostate epithelial and prostate tumor cells from each patient were harvested by a pathologist (W. Z.) using LCM equipment according to the protocol provided by the manufacturer (Arcturus Engineering, Mountain View, CA). Total RNA was prepared from the harvested normal and tumor prostate epithelial cells as described previously (23) and quantified with Fluorometer (Bio-Rad, Hercules, CA). QRT-PCR was conducted using 0.1 ng of total RNA from paired normal and tumor cells. *PMEPA1* PCR primers were carefully designed that only amplify *PMEPA1* but not *STAG1*, an alternatively spliced form of *PMEPA1* (19). The PCR primers were 5′CATGATCCCCGAGCTGCT3′ and 5′TGATCTGAACAAACTCCAGCTCC3′, and the FAM-labeled probe was 5′AGGCGGACAGTCTCCTGCGAAAC3′. GAPDH gene expression was detected as the internal control (PE Applied Biosystems, Foster, CA). Paired triplicate samples (one lacking reverse transcriptase and duplicate with reverse transcriptase) were amplified in 50-μl volumes containing the manufacturer's recommended universal reagent, proper primers, and probe of *PMEPA1* or *GAPDH* using 7700 sequence detection system (PE Applied Biosystems). Results were plotted as average cT values for each duplicate sample minus the average duplicate cT values for *GAPDH*. Differences between matched tumor (T) and normal (N) samples were calculated using $2\exp(cT_{tumor} - cT_{normal})$ and expressed as fold changes in expression. The expression status of *PMEPA1* was further categorized as overexpression in tumor tissue (T > N), defined as 1+ (1.5–3-fold), 2+ (3.1–10-fold), 3+ (10.1–20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; reduced expression in tumor tissue (T < N), defined as 1− (1.5–3-fold), 2− (3.1–10-fold), 3− (10.1–20-fold), and 4− (>20-fold) decreased expression as compared with matched normal tissue; and no change (T = N) refers to the difference of *PMEPA1* expression between T and N as <1.5 folds (0). No detectable *PMEPA1* expression in one of the specimens of tumor/normal pairs was scored as 4+ for increased or 4− for decreased expression.

Statistical Analysis. Statistical analysis was performed with the SPSS software package. The association between *PMEPA1* expression and clinicopathological features was analyzed using χ^2 and t tests. $P < 0.05$ was considered as statistically significant.

Results and Discussions

PMEPA1-PY Motifs Interact with the WW Domains of NEDD4. The WW domains of NEDD4 protein facilitate its binding to the target proteins via interaction with the PY motifs of NEDD4-binding proteins (4, 13–17). Predicted PMEPA1 protein sequence comprises of two PY motifs, PY1 (PPPY) and PY2 (PPTY). PY1 is in the central region of the PMEPA1 protein, and PY2 is close to the COOH terminus of the PMEPA1 protein (Fig. 1*A*). PMEPA1 shares 83% sequence identity with the protein encoded by *N4wbp4*, a gene expressed in mouse embryo (Ref. 4; Fig. 1*A*). *In vitro* translated [^{35}S]Methionine-labeled PMEPA1-V5 fusion protein, with the two intact PY motifs, showed binding to the GST-WW fusion protein (Fig. 1*B*, *Lane 1*). PMEPA1 with PY1 or PY2 mutations revealed significantly decreased binding to WW domains (Fig. 1*B*, *Lanes 2* and *3*). Furthermore, PMEPA1-V5 and NEDD4-GFP fusion proteins expressed in 293 cells showed strong association (Fig. 1*C*, *Lane 1*) and the mutant PMEPA1-V5 proteins having single mutation of PY1 or PY2 motif or double mutations of both PY1 and PY2 motifs exhibited significantly reduced binding to NEDD4 (Fig. 1*C*, *Lanes 2–4*). Thus, both *in vitro* and cell culture data support the prediction that PMEPA1 interacts with NEDD4, and these interactions are dependent on the binding of both PY motifs of PMEPA1 to WW domains. However, the PY2 motif mutation appeared to have a greater effect on binding of PMEPA1 to NEDD4 WW domain.

The high protein sequence identity of PMEPA1 with N4wbp4 suggests that *PMEPA1* is the human homologue of *N4wbp4*. On the basis of the suggested role of Nedd4 in mouse development (4–12), the homology of PMEPA1 to a Nedd4-binding protein, experimental documentation of binding of PMEPA1 to NEDD4, and the androgen regulation and prostate abundance of PMEPA1 warrant evaluations of the roles of *PMEPA1* in prostate development.

PMEPA1 Is a Golgi-associated Protein. To gain additional insights into PMEPA1 cellular functions, its subcellular localization was determined. PMEPA1-GFP fusion protein showed perinuclear localization with a Golgi-like appearance (Fig. 2*A*). We performed the immunofluorescence assay to test the hypothesis that PMEPA1 local-

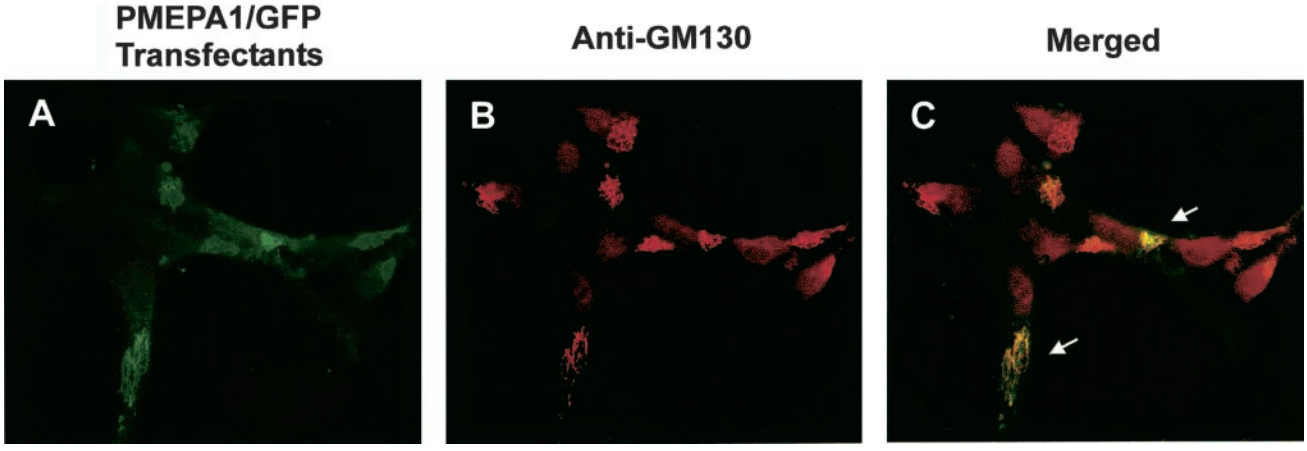

Fig. 2. PMEPA1 localizes to *cis*-Golgi matrix. *A*, LNCaP transfectants expressing PMEPA1-GFP fusion protein. In *B*, LNCaP transfectants stained with anti-Golgi structure protein GM130. *C*, superimposed images of *B* and *C*. *Arrows*, the colocalization of PMEPA1-GFP fusion protein and GM130. Magnification: ×100.

izes to the Golgi complex. The images of subcellular location of GM130, a *cis*-Golgi protein, showed a similar pattern as PMEPA1-GFP fusion protein (Fig. 2*B*). Superimposition of the images of PMEPA1-GFP fusion protein and GM130 in LNCaP-PMEPA1-GFP transfectants confirmed the localization of PMEPA1-GFP fusion protein on *cis*-Golgi structure (Fig. 2*C*). We did not see the colocalization of PMEPA1-GFP and TGN-38, which localizes to TGN (data not shown). In this regard, the subcellular localization of *PMEPA1* is similar to other two newly identified Nedd4 WW domain-binding proteins, N4wbp5 and N4wbp5a, which were also localized to the Golgi complex (12, 17). N4wbp5a was observed to sequester the trafficking of Nedd4/Nedd4–2, thereby increasing the activity of the epithelial sodium channel, a known target down-regulated by *NEDD4* (12). As a highly ARG and NEDD4-binding protein, the localization of PMEPA1 on Golgi apparatus may suggest for the role of *PMEPA1* in the regulation of protein turnover of AR targets.

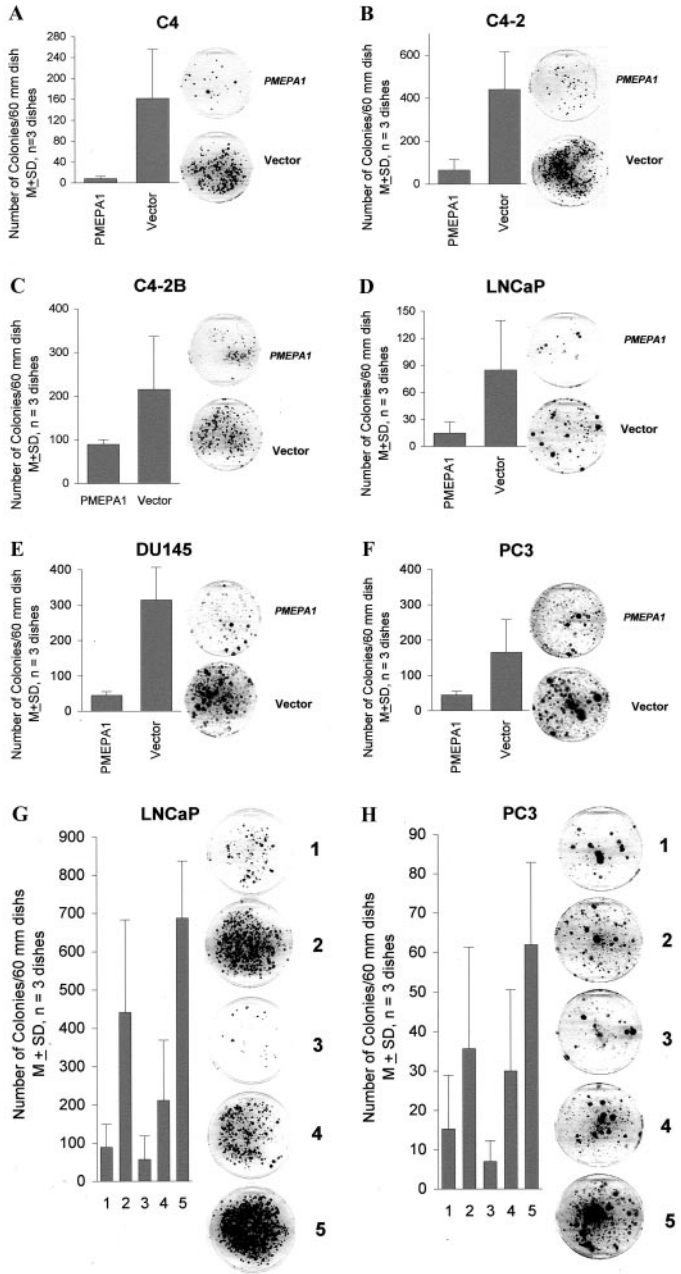

Fig. 3. Effect of *PMEPA1* on colony formation. Prostate tumor cell lines C4 (*A*), C4-2 (*B*), C4-2B (*C*), LNCaP (panel *D*), DU145 (*E*), and PC3 (*F*) were transfected with 3 μg each of *PMEPA1*-V5-pcDNA3.1 (*PMEPA1*) and pcDNA3.1 vector (Vector) in triplicate sets. In separate experiments, LNCaP (*G*) and PC3 (*H*) cells were transfected with control vector or expression vectors encoding wt-PMEPA1 or PMEPA1-PY mutants (*1, PMEPA1*-V5-pcDNA3.1; *2, PMEPA1-PY1m*-pcDNA3.1; *3, PMEPA1-PY2m*-pcDNA3.1; *4, PMEPA1-PY1m/PY2m*-pcDNA3.1; *5*, pcDNA3.1). Transfected cells were selected for plasmid-containing cells with G418 for 3 weeks, and surviving cells were fixed and stained with crystal violet. Colonies were counted and displayed as histograms. For each cell line, a photograph of one dish of cells treated with 3 μg of each plasmid is also shown.

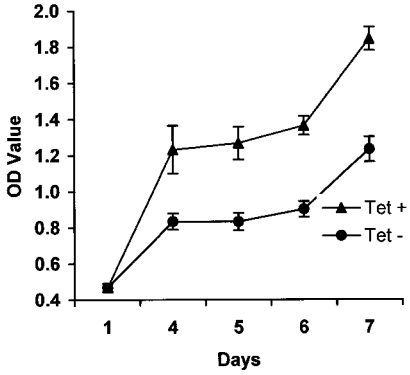

Fig. 4. Effect of *PMEPA1* on cell proliferation. Stable *PMEPA1*-GFP-Tet-LNCaP transfectants (described in "Materials and Methods") were seeded in 96-well plates with or without 1 μg/ml tetracycline in the medium. The cell proliferation was measured using the CellTiter 96 Aqueous One Solution kit at the indicated time. *Tet* + and *Tet* −, the cell culture medium with or without tetracycline, respectively. The absorbance values which reflected the cell numbers are significantly different ($P < 0.01$) between the two groups, except on day 1.

Table 1 *PMEPA1 expression status in primary prostate cancers*

PMEPA1 gene expression in 62 matched LCM-derived prostatic cancer cells (T) and normal cells (N) was analyzed by QRT-PCR. The results were represented as differential expression in T and N as described in "Materials and Methods." T < N, reduced expression in tumor tissue, defined as 1− (1.5–3-fold), 2− (3.1–10-fold), 3− (10.1–20-fold), and 4− (>20-fold) decreased expression as compared with matched normal tissue; T > N, overexpression in tumor tissue, defined as 1+ (1.5–3-fold), 2+ (3.1–10-fold), 3+ (10.1–20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; and T = N, similar expression between T and N, refers to the difference of *PMEPA1* expression between T and N as <1.5-fold (0).

PMEPA1 expression	No. of patients/group (%)	Degree of *PMEPA1* expression	
		Quantity	No. (%)
T < N	40 (64.5)	1−	11 (27.5)
		2−	17 (42.5)
		3−	5 (12.5)
		4−	7 (17.5)
T > N	10 (16.1)	1+	6 (60.0)
		2+	4 (40.0)
		3+	
		4+	
T = N	12 (19.4)	0	

***PMEPA1* Inhibits Growth of CaP Cells.** To investigate the biological effects of *PMEPA1* expression in the regulation of cell growth and contribution of PY motifs for such functions, we performed the colony formation assay by transfection expression vectors of the wt-*PMEPA1* and *PMEPA1-PY* mutants. As shown in Fig. 3, *A–F*, colony-forming abilities of CaP cell lines DU145, PC3, LNCaP, and LNCaP sublines were significantly suppressed by transfection of the sense version of wt-*PMEPA1* expression vector. Under these conditions, wt-p53 showed similar cell growth inhibition (data not shown). In two independent experiments, mutations of PY1 motif appear to abolish the inhibition of colony formation by wt-*PMEPA1*, emphasizing the role of PY1 motif in PMEPA1 and NEDD4 interactions and the biological functions of *PMEPA1* (Fig. 3, *G* and *H*). It is intriguing that both PY1 and PY2 mutations diminished the *in vitro* binding of PMEPA1 to NEDD4; however, in the context of biological function of *PMEPA1* in the colony-forming assay, only mutation of PY1 motif has functional consequence. The growth inhibitory effect of *PMEPA1* appears to be linked to the interactions of PY1 motif to NEDD4 WW

Table 2 *PMEPA1 expression and clinicopathological features*

The patients were grouped into two groups: T < N ($n = 40$), *PMEPA1* expression reduced ≥1.5-fold in tumor cells in comparison with matched normal cells, and T ≥ N, *PMEPA1* expression was similar between tumor and normal cells ($n = 12$) or increased in tumor cells ($n = 10$).

PMEPA1 expression	Pathologic stage (%)		PSA range (%)			PSA recurrence (%)		Time to recurrence after surgery (month)
	T2	T3	≤4 ng/ml	4.1–10 ng/ml	10.1–20 ng/ml	No	Yes	Mean ± SE
T < N	11 (27.5)	29 (72.5)	1 (2.5)	30 (75.0)	9 (22.5)	29 (72.5)	11 (27.5)	8.2 ± 3.4
T ≥ N	12 (54.5)	10 (45.5)	5 (22.7)	15 (68.2)	2 (9.1)	19 (86.4)	3 (13.6)	18.4 ± 6.3
P	0.035			0.023		0.211		0.18

domain. This interpretation is based on the striking observations showing distinctively more colonies with PY1 motif mutant in comparison with wt-*PMEPA1*. Moreover, the growth inhibitory effect of *PMEPA1* has been confirmed by the cell proliferation characteristics of stable *PMEPA1*-GFP-Tet-LNCaP cells, where exogenous *PMEPA1* is up-regulated in the absence of tetracycline. The growth of the *PMEPA1*-GFP-Tet LNCaP cells in tetracycline-negative medium is significantly slower than that of PMEPA1-tet LNCaP transfectant in tetracycline-positive medium (Fig. 4). LNCaP cells with *PMEPA1* overexpression also revealed increased retinoblastoma phosphorylation, further confirming cell growth inhibitory effect of *PMEPA1* (data not shown).

PMEPA1 is expressed in AR-positive CaP cell lines: LNCaP and its sublines (C4, C4-2, and C4-2B). LNCaP cells are androgen dependent for growth. Although the growth of LNCaP sublines is androgen independent, AR is critical for their proliferation (24). We observed that overexpression of *PMEPA1* by transfecting the *PMEPA1* expression vector into LNCaP and its sublines significantly inhibited the cell proliferation. Because our preliminary observations showed that *PMEPA1* overexpression in LNCaP cells resulted in altered expression of AR downstream genes,[5] we hypothesized that the growth inhibitory effect of *PMEPA1* on LNCaP and its sublines may be mediated through directly or indirectly affecting AR functions. Despite the growth inhibitory effect on AR-positive CaP cell lines, *PMEPA1* was also found to inhibit the growth of AR-negative prostate tumor cells, DU145 and PC3, suggesting that the growth inhibitory effects of *PMEPA1* on DU145 and PC3 could be mediated through alternative mechanisms, *e.g.*, regulation of other nuclear steroid receptors by *PMEPA1*. Nonetheless, inhibition of CaP cell growth by *PMEPA1* suggested that *PMEPA1* might be involved in CaP development.

Decreased *PMEPA1* Expression in Prostate Tumor Tissues. CaP cell growth inhibitory functions of *PMEPA1* led us to carefully evaluate the relationship of *PMEPA1* expression alterations to the clinicopathologic features of CaP. The overall expression pattern of the *PMEPA1* is shown in Table 1. Comparison of *PMEPA1* expression between tumor and normal cells revealed tumor cell-associated decreased expression (T < N) in 64.5% tumor specimens (40 of 62), increased expression (T > N) in 16.1% specimens (10 of 62), and no change (T = N) in 19.4% specimens (12 of 62). When these expression patterns were stratified by organ-confined (pT2) and nonorgan-confined (pT3) disease, a higher percentage of *PMEPA1* reduction was seen in pT3 (74%) *versus* pT2 (48%). Because the T > N group has a few cases, we combined the T > N and T = N groups (T ≥ N group). Comparison of the clinicopathologic parameters between the *PMEPA1*-T < N and *PMEPA1*-T ≥ N groups revealed that the *PMEPA1*-T < N group had a significantly higher percentage of patients with pT3 tumors ($P = 0.035$), and more patients in this group had a higher level of preoperative serum PSA ($P = 0.023$; Table 2). Of 62 patients whose tumors were analyzed for *PMEPA1* expression, 14 patients showed CaP recurrence as defined by serum PSA level ≥ 0.2 ng/ml after prostatectomy. Of the 14 patients, 11 showed reduced tumor-associated *PMEPA1* expression (78.5%). Reduced *PMEPA1* expression seems to associate with a higher recurrence rate and shorter duration to recurrence after surgery, although the statistical analysis did not reveal a significant difference which might be attributable to the small number of patients (Table 2). It is worth noting these observations in the context of the report showing consistent high levels of induction of *PMEPA1* in nontumorigenic revertants of tumor cell lines after the transfer of human chromosome 8p (18), which harbors putative TSGs (25, 26). Among chromosomal "hot spots" in CaP and other cancers, 8p loss is particularly frequent, occurring in ~80% of prostate tumors (27–29). Transfer of single human chromosome 8 has been shown to result in suppression of the malignant phenotype or the metastatic ability in a variety of cell lines (18, 30, 31). Therefore, it is possible that the reduced expression of *PMEPA1* in prostate tumors might result from the loss of the putative TSGs or cell growth inhibitory genes on chromosome 8p in prostate tumor cells. Two very recent interesting reports have described *PMEPA1* as a transforming growth factor-β-induced gene and marker of terminal colonocyte differentiation (32, 33).

In summary, we have demonstrated that PMEPA1 interacts with NEDD4 through its PY motifs. *PMEPA1* has cell growth inhibitory effects when overexpressed in androgen-dependent or -independent CaP cells. PY1 motif of PMEPA1 appears to modulate its cell growth inhibitory function. *PMEPA1* may function in the regulation of protein turnover via ubiquitination/proteasome pathways in cells. The biological effects of *PMEPA1* in CaP cells and expression pattern of *PMEPA1* in CaP indicate that *PMEPA1* may function as the cell growth inhibitor regulated by androgen.

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