Protein Kinase CK2 Controls the Stability of Prostate Derived ETS Factor

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Abstract: Prostate Derived ETS Factor (*PDEF*) is an ETS transcription factor expressed in prostate epithelial cells, and diminished PDEF protein accumulation is correlated with prostate cancer progression. PDEF interacts with the tumor suppressor NKX3.1, and this interaction abolishes the ability of PDEF to activate the *Prostate-Specific Antigen* promoter. NKX3.1 stability is known to be regulated by Protein Kinase CK2 and the E3 ubiquitin ligase TOPORS. To determine if PDEF and NKX3.1 are coordinately regulated in prostate cancer cells, the effect of CK2 inhibition on steady-state PDEF levels was explored. Inhibition of CK2 activity with apigenin or 4,5,6,7-tetrabromo-benzimidazole (TBB) reduced steady-state levels of PDEF in LNCaP cells, and this effect was reversed by inhibiting the 26S proteasome. siRNA-mediated knockdown of CK2α′ in LNCaP cells also reduced PDEF accumulation. Mass spectrometric analysis of phosphorylated recombinant PDEF revealed that Thr144, Ser151 and Ser187 are CK2 phosphoacceptor sites *in vitro*. PDEF was also robustly polyubiquitinated by TOPORS *in vitro*. These results suggest that PDEF and NKX3.1 are coordinately regulated by CK2 phosphorylation that inhibits their proteasomal degradation in prostate cancer cells.

Keywords: PDEF, CK2, TOPORS, TBB, NKX3.1, ETS, Ubiquitination.

INTRODUCTION

The ETS transcription factors influence a diverse array of cellular processes, including organogenesis, angiogenesis, cell cycle control, cell proliferation and motility, extracellular matrix remodeling, and hematopoietic differentiation. ETS factors have characteristics of both oncogenes and tumor suppressors [1]. The altered or aberrant expression of ETS factors in prostate epithelium can directly contribute to the development and progression of carcinoma [2]. ETS factors are controlled by posttranslational modification(s), particularly phosphorylation [3, 4]. A well-characterized example is the regulation of several subfamilies of ETS proteins by the Ras Mitogen-Activated Protein Kinase (MAPK) pathway where selective phosphorylation by MAPKs controls the ability to activate transcription of target genes, which in turn may contribute to the initiation, maintenance, or progression of neoplasia [3].

Prostate Derived ETS Factor (PDEF) is a member of the epithelial-specific ETS factor subfamily and is expressed in epithelial cells of the prostate, mammary, and salivary glands, colon, and ovary. Modulating PDEF expression in prostate, breast and colon cancer cells has revealed roles for this protein in controlling cell migration and invasion [5-10]. Constitutive or inducible PDEF expression inhibits migration and invasion in multiple breast cancer lines, and concomitant changes in focal adhesion complexes [8]. Consistent with these observations, knockdown of PDEF in prostate cancer cells results in increased migration and invasiveness and changes in expression of genes involved in these processes [5]. In contrast, however, PDEF overexpression in breast cancer cells results in an invasive and promigratory phenotype in the context of deregulated ERK/MAPK and constitutively active ErbB2/CSF-1R [6]. This apparent paradox may point to a context-dependent role of PDEF, and highlights the potential impact of altered signaling pathways.

PDEF protein expression in human breast and prostate cancers has been explored using a series of independently derived polyclonal antisera. Consistent with an anti-migration/invasion function, Feldman *et al*. demonstrated diminished PDEF accumulation in six of seven invasive ductal breast carcinoma cases [9]. In contrast, increased PDEF expression was observed in 90% of breast tumors in a tissue microarray [11]. Similarly, both diminished [12] and increased [11] PDEF accumulation has been reported in prostate cancer cases. Given the extent of morbidity and mortality associated with breast and prostate cancer, and the clear evidence of a role for this protein in controlling epithelial cells migration and invasion, it will be imperative to resolve these paradoxical observations.

A recent proteomic analysis identified 121 potential protein partners of PDEF in breast cancer cells [13]. Proteins involved in a variety of cellular processes and in multiple subcelluar compartments were revealed, and interaction with Catenin gamma-1 and the serine/threonine kinase p62 were confirmed by immunoprecipitation/Western blot analyses [13]. PDEF has been demonstrated to interact with key regulators of prostate epithelial differentiation, including Androgen Receptor (AR) and the prostate tumor suppressor NKX3.1 [14, 15]. PDEF interaction with AR potentiates androgen-mediated activation of the Prostate Specific Antigen promoter, and NKX3.1 abolishes this activity [15]. NKX3.1 is known to be regulated by Protein Kinase CK2,

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which protects NKX3.1 from proteasome-dependent degradation [16].

CK2 is a ubiquitous, constitutively active serine/threonine kinase with over 300 known substrates. The CK2 consensus phosphorylation site is S/TXXD/E, where X is a non-basic residue, and the n+3 position relative to the target serine or threonine is most important [17]. CK2 is a member of the CMGC kinase subfamily whose members include Glycogen Synthase Kinase 3, cyclin-dependent kinases, and MAPKs [18]. Many transcription factors, for example tumor suppressor p53 and β-catenin, are targets of CK2 phosphorylation [19]. The CK2 holoenzyme exists as a heterotetramer, comprised of two β subunits complexed with catalytic α and/or α′ subunits. The free catalytic α and α′ subunits are also active independent of the holoenzyme and are believed to have distinct and separate functions [20]. Although the overall activity of CK2 is elevated in prostate tumor cells compared with normal prostate epithelial cells, the subcellular location and amount of free and complexed catalytic subunits of the kinase are relevant to the resulting biology [21]. The loss of the free α′ catalytic subunit from the nucleus could contribute to reduced or lost expression of NKX3.1 in prostate cancer [16].

In light of the fact that CK2 phosphorylation prevents ubiquitin-mediated degradation of NKX3.1 by the 26S proteasome, we postulated that CK2 could also potentially control PDEF stability [14, 16]. Here, we demonstrate that CK2 phosphorylates PDEF *in vitro* and identify three CK2 phosphoacceptor sites. We further demonstrate that pharmacologic inhibition of CK2 significantly reduces the steady-state levels of PDEF in prostate cancer cells, and show that PDEF can be polyubiquitinated by an E3 ubiquitin ligase that regulates NKX3.1 turnover. Together, these data indicate that PDEF and NKX3.1 are coordinately regulated at the post-translational level in prostate cancer cells.

MATERIALS AND METHODS

Development and Validation of a Rabbit Polyclonal Anti-PDEF Antibody

N-terminal amino acids 1-141 of human PDEF ($PDEF^{1-141}$) were cloned into pQE80L (Promega). Recombinant N-terminal 6xHis-PDEF protein was induced in *E. coli* strain BL21 at 37 °C with 0.6 mM isopropylthiogalactopyranoside. Cells were pelleted, resuspended in 300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 7.5, broken at 18,000 lb/in^2 in a French press and centrifuged at 20,000 x g for 30 minutes at 4 °C. The supernatant was collected and loaded to a nickel column (Ni-NTA (Qiagen)). PDEF was eluted with 300 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, pH 7.5. Fractions containing purified PDEF were used to gene-rate polyclonal antibodies in a New Zealand White rabbit. Serum and terminal bleeds were affinity purified against $PDEF^{1-141}$ and tested for reactivity by Western blot using recombinant PDEF protein and LNCaP cells transfected with full length human PDEF. Further determination of specificity and sensitivity was confirmed by Western blot comparisons with two commercially available anti-PDEF antibodies (Zymed and Santa Cruz), and Western blot analysis of protein signal in LNCaP cells treated with PDEF siRNA was compared with untreated LNCaP cells.

In Vitro Kinase Assay

In vitro kinase reactions were carried out in a buffer containing full-length human recombinant PDEF, CK2 holoenzyme (New England Biolabs) or recombinant CK2α, 200 μM ATP, and 1X CK2 reaction buffer (New England Biolabs) at room temperature for 60 minutes in a 100 μl volume.

Cell Culture, Pharmacologic Inhibition of CK2, Western and Northern Blot Analysis

LNCaP cells were maintained in RPMI-1640 plus 10% fetal bovine serum in a humidified incubator with 5% CO_2. Culture media was supplemented with apigenin (75 μM) (Sigma), 4,5,6,7-tetrabromobenzotriazole (TBB) (50 μM) (Sigma and Calbiochem), or MG132 (10.5 μM) (Sigma) by dilution of 10 or 20 mM stock solutions in dimethylsulfoxide (DMSO). At specific time points, cells were harvested by trypsinization and lysed in 150 mM NaCl, 50mM Tris, 0.25% NP-40, pH 7.5, and protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad). Identical amounts of total protein were loaded on 12% SDS-PAGE gels and after electrophoresis, were transferred to polyvinylidene difluoride membranes. Rabbit anti-PDEF was added at 1:1,000 dilution at 4 °C overnight. In parallel, whole RNA was extracted from apigenin-treated cells and DMSO vehicle controls using an RNeasy Kit according to the manufacturer's instructions (Qiagen), and separated on a 1% glyoxal/DMSO/agarose gel (Ambion) and hybridized to a PDEF cDNA probe.

Mass Spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on CK2-phosphorylated recombinant PDEF that was resolved by 2D SDS-PAGE. Spots corresponding to phosphorylated PDEF were excised from a silver-stained gel. After in-gel trypsin digestion, eluted peptides were resolved on a C18 reverse phase column coupled on-line to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific). MS^3 was performed on precursor ions demonstrating neutral loss of a phosphate group. Data analysis was preformed using the Bioworks 3.3.1 software (Thermo Fisher Scientific).

Expression Constructs and GST Pull-Downs

Full-length recombinant PDEF was expressed and purified as described [22]. Recombinant GST-TOPORS was expressed and purified as described [23].

In Vitro Ubiquitination Assay

In vitro ubiquitination assays were carried out in 50 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 15 μM $ZnCl_2$, 4 mM ATP with the addition of 100 nM rabbit E1 (Boston Biochem), 200 nM human UbcH5a, and 250 μM human wild type recombinant ubiquitin (Boston Biochem) or no-lysine ubiquitin (Boston Biochem). Reactions were carried out at

37 °C for 60 minutes in a 10 µl volume. *In vivo* ubiquitination assays were performed as described [23].

RESULTS

Production and Characterization of an Anti-PDEF Polyclonal Antiserum

To reduce the likelihood of cross-reactivity with other ETS factors, the N-terminal 141 amino acids of human PDEF were chosen as the antigen (Fig. **1A**). $PDEF^{1-141}$ does not bear significant homology to other ETS factors or other human proteins, however, there is 89% homology in this region between mouse and human PDEF. This region was cloned into the bacterial expression vector pEQ80L, and expressed in BL21 cells. The protein was enriched from bacterial lysates by nickel affinity and anion exchange chromatography to ~90% purity. New Zealand White rabbits were immunized following a standard boost/bleed regimen, and post-immunization sera were screened by Western blot analysis of LNCaP cells transfected with exogenous PDEF (data not shown) and LNCaP cells treated with siRNA specific to PDEF (Fig. **1B**). A prominent 46 kDa species was observed, and was specifically diminished in the PDEF siRNA-treated cells. This apparent molecular weight is consistent with previous analyses of PDEF by SDS-PAGE [9, 10, 24]. Other molecular weight species were variably observed in Western blot analyses (Fig. **1B**) however these were not diminished by treatment with PDEF siRNA and represent cross-reactivity of the polyclonal sera. To further verify the specificity of the anti-$PDEF^{1-141}$, parallel Western blots with commercially available (Zymed, Santa Cruz Biotechnology), and existing anti-PDEF antibodies [8, 24] were performed (data not shown). This comparison indicated that our anti-PDEF antibody recognized a 46 kDa isoform PDEF indistinguishable from that recognized by extant antibodies.

Protein Kinase CK2 Phosphorylates PDEF

To determine if PDEF can be phosphorylated by CK2, *in vitro* kinase reactions were carried out with full-length recombinant PDEF or the N-terminal domain (amino acids 1-141) with CK2 holoenzyme or CK2α alone in the presence of γ-^{32}P-ATP. Robust phosphorylation was observed after gel electrophoresis followed by autoradiography (data not shown). To determine specific sites of CK2 phosphorylation in PDEF, *in vitro* kinase reactions were analyzed by LC-MS/MS. Products of kinase reactions were resolved by two-dimensional SDS-PAGE, silver stained, excised, and digested with trypsin. Mass spectra obtained from non-phosphorylated PDEF were compared with spectra generated from PDEF phosphorylated by CK2. These data revealed evidence for three CK2 phosphoacceptor sites in PDEF: Serine 187, in a fragment spanning amino acids 182-192, ELCAMS*EEQFR, with a mass of 1342.5766, and Threonine 144 and Serine 151, in a fragment spanning amino

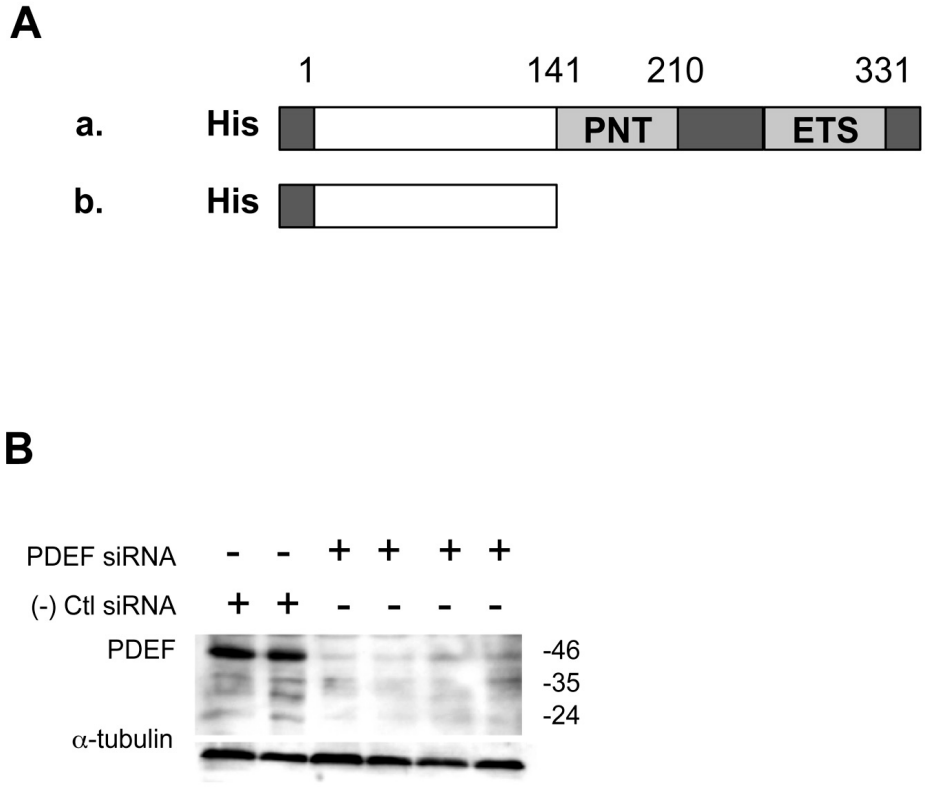

Fig. (1). Production of an anti-PDEF polyclonal antibody. A. The N-terminal region of PDEF (amino acids 1-141) was used to immunize rabbits to generate polyclonal anti-PDEF antibodies. **B.** Western blot detection of endogenous PDEF protein in LNCaP cells 24 hours after transfection with a PDEF siRNA or a negative control siRNA. The 46 kDa species is significantly diminished specifically in the PDEF siRNA-treated lanes. The blots were stripped and probed with an antibody against α-tubulin as a loading control.

Table 1. Peptides Identified by Mass Spectrometric Analysis of Recombinant PDEF Phosphorylated *in vitro* by CK2

Scans[1]	Peptide	MH+	DeltaM	z	Type	P(pro)/ P(pep)	Sf	Score Xc	Coverage Delta Cn	MW Sp
1 SAM pointed domain containing ets transcription factor [Homo sapiens]						2.30E-13	8.62	142.28		37493.9
4223	K.AAAGAVGLER.R	914.50543	0.46434	2	CID	3.84E-05	0.94	3.54	0.41	947.6
4305	-AFQELAGK-	863.46216	0.32945	2	CID	1.26E-03	0.80	2.44	0.15	638.1
4400	K.AFQELAGK.E	863.46216	0.47576	2	CID	1.20E-02	0.90	2.70	0.31	710.9
4929	K.ELCAMSEEQFR.Q	1342.57654	1.75798	2	CID	7.76E-07	0.88	3.05	0.36	434.2
5053	-ELCAMS#EEQFR-	1324.57660	0.40455	2	CID	1.96E-02	0.33	2.03	0.20	198.3
5304	-ELCAMSEEQFR-	1342.57654	-0.44455	2	CID	1.10E-06	0.18	1.97	0.05	179.5
5407	-MGSASPGLSSVSPSHLLLPPDTVSR-	2492.28125	1.69458	3	CID	1.87E-04	0.30	2.78	0.15	291.0
5416-5417	R.SPLGGDVLHAHLDIWK.S	1757.93335	1.19345	3	CID	2.90E-12	0.93	3.76	0.42	1257.1
5420	-WLLWTEHQYR-	1431.71680	-0.95431	3	CID	2.36E-05	0.32	1.69	0.13	395.9
5482	K.WLLWTEHQYR.L	1431.71680	0.57964	2	CID	6.20E-07	0.94	3.38	0.43	892.8
5588	-LLNITADPMDWSPSNVQK-	2029.00586	-0.40571	2	CID	8.92E-04	0.50	2.91	0.41	122.4
5596	-ELCAMS*EEQFR-	1422.57660	1.42003	2	CID	9.85E-01	0.01	1.32	0.32	115.3
5729	-LLNIT#ADPMDWSPSNVQK-	2011.00590	1.32618	2	CID	2.40E-07	0.54	3.16	0.28	174.0
5729	-LLNITADPMDWS#PSNVQK-	2011.00590	1.32618	2	CID	5.92E-04	0.05	2.28	0.37	116.2

[1]SEQUEST data identifying the three phosphoacceptor sites in the pointed domain of PDEF: Thr144, Ser151, and Ser187. highlighted in red are peptides containing the Ser187 phosphoacceptor site and in green are the Thr144, and Ser151 containing peptides. A +80 amu and/or -18 amu Shift in MH+ in the phospho-peptide over the non-phosphorylated peptide confirms phosphorylation at these sites. '*'Denotes phosphorylated site identified through gain of 80 amu and '#'denotes phosphorylated site identified through loss of 18 amu.

acids 140-157, LLNIT*ADPMWS*PSNVQK, with a mass of 2029.0059 (Table **1**).

Inhibition of CK2 Reduces the Steady State Level of PDEF in Prostate Cancer Cells

Apigenin (4',5,7,-trihydroxyflavone) is a common flavonoid distributed widely in fruits and vegetables [25], and is a potent inhibitor of CK2 and several other kinases [26]. To determine if apigenin exposure affects PDEF stability in prostate cancer cells, LNCaP cells were cultured in the presence or absence of apigenin and the steady-state level of PDEF was monitored by Western blot analysis after 4 hours of exposure. Significant down-regulation of PDEF was observed in LNCaP cells after 4 hours of apigenin exposure, raising the possibility that CK2 blockade altered PDEF accumulation (Fig. **2A**). However, while apigenin is a potent inhibitor of CK2, it has also been shown to block the activity of other kinases *in vitro* [26]. To further investigate the possibility that CK2 inhibition may affect PDEF level, LNCaP cells were treated with the highly CK2-selective inhibitor TBB [27]. After 4 hours of TBB treatment, the steady-state level of PDEF in LNCaP cells was reduced to an extent similar to that observed after apigenin treatment (Fig. **2C**). Parallel Northern blots conducted on apigenin (Fig. **2A**, bottom panel) and TBB-treated LNCaP cells (data not shown) revealed that the effect occurs post-transcriptionally, since *PDEF* mRNA accumulation was not affected by CK2 pharmacologic inhibition. In contrast, NKX3.1 has been shown to be affected at both the protein and mRNA level in similar CK2 pharmacologic inhibition studies [16]. Morphologic analysis by light microscopy revealed that approximately 10-30% of the cells lost contact with the cell culture vessel and were rounded and floating during the course of apigenin and TBB treatment. This effect was seen as early as 60 minutes after exposure to CK2 inhibitory agents.

To determine the role of both CK2 catalytic subunits in maintaining the steady-state level of PDEF, specific siRNAs were used to knock down either CK2α or CK2α′. In LNCaP cells transfected with a CK2α′-specific siRNA, PDEF accumulation was diminished after 24 hours (Fig. **2D**). In contrast, knockdown of CK2α did not affect the protein level of PDEF (Fig. **2D**).

Blocking the 26S Proteasome Reverses the Effect of CK2 Inhibition on PDEF Accumulation

Based on the results of pharmacologic and siRNA-mediated CK2 inhibition, we hypothesized that phosphorylation by CK2 stabilized PDEF, and that the abrogation of CK2 activity resulted in PDEF degradation, possibly by the 26S proteasome pathway. To determine if this was the case, LNCaP cells were treated with apigenin in the presence or absence of the proteasome inhibitor MG132. The effect of apigenin on PDEF accumulation was reversed in the presence of MG132 (Fig. **2B**), suggesting that PDEF is degraded by the 26S proteasome in prostate cells and that phosphorylation by CK2 prevents this degradation.

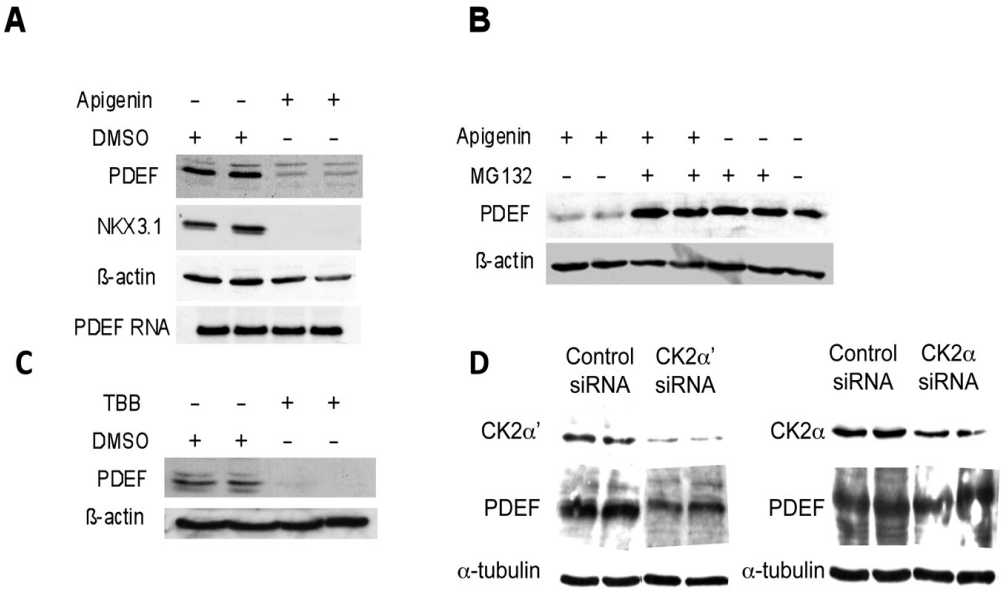

Fig. (2). Inhibiting Protein Kinase CK2 Reduces Endogenous PDEF. A. Western blot analyses (top 3 panels) to detect PDEF and NKX3.1 in LNCaP cells treated with apigenin for 4 hours. The same membrane was probed to detect β-actin as a loading control. Northern blot analysis (bottom panel) to detect PDEF mRNA. **B.** Western blot analysis to detect PDEF in LNCaP cells cultured for 4 hours in the presence or absence of MG132 and apigenin. MG132 treatment reverses the effect of apigenin on PDEF. **C.** Western blot analysis to detect PDEF in LNCaP cells treated with TBB for 4 hours. **D.** Western blot analysis to detect PDEF in LNCaP cells transfected with siRNA to knock down expression of individual CK2 catalytic subunits. PDEF is reduced in LNCaP cells treated with siRNA to CK2α′**.** The blots were stripped and probed with an antibody against β-actin as a loading control.

TOPORS Interacts with and Ubiquitinates PDEF

Based on the fact that TOPORS ubiquitinates NKX3.1 in prostate cancer cells [28], we hypothesized that PDEF might also be a TOPORS substrate. To determine if TOPORS can ubiquitinate PDEF, *in vitro* ubiquitination assays were performed in the presence of ubiquitin, E1 and UbcH5a E2 enzymes, and TOPORS (Fig. **3A**). Higher molecular weight forms of PDEF were observed that were not present in the

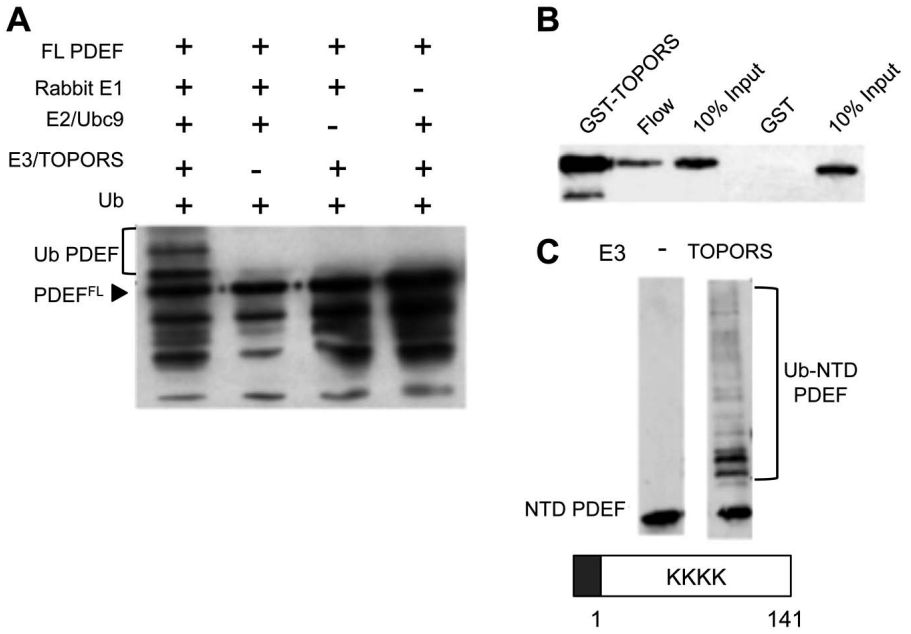

Fig. (3). PDEF interacts with and is ubiquitinated by the E3 ligase TOPORS. A. TOPORS ubiquitinates PDEF *in vitro. In vitro* ubiquitination assays were conducted using recombinant E1, E2 (UbcH5a), E3 (TOPORS), Ub, and PDEF. A Western blot using anti-PDEF antibodies is shown. **B.** Western blot of a GST pull-down assay using anti-PDEF antibodies. Equimolar amounts of GST and GST-tagged TOPORS immobilized on glutathione beads were incubated with purified His-tagged recombinant PDEF, washed, and eluted with SDS-PAGE buffer. **C.** The N-terminal region of PDEF ($PDEF^{1-141}$, NTD-PDEF) is ubiquitinated *in vitro* by TOPORS. A Western blot using anti-PDEF antibodies is shown. $PDEF^{FL}$, full-length PDEF.

control reactions, suggesting that TOPORS ubiquitinates PDEF *in vitro*. To determine if TOPORS could interact with PDEF, GST pull-down assays were performed. Recombinant GST-tagged TOPORS was purified from bacterial lysates and immobilized on glutathione beads as bait, and recombinant His-tagged PDEF was captured by TOPORS in pull-down assays (Fig. **3B**).

The N Terminal Region of PDEF is Ubiquitinated by TOPORS

PDEF is a 335 amino acid protein that contains 19 lysine residues, four reside in the N terminal region, four reside in the Pointed domain, one resides in the Pointed/ETS inter-domain region, and ten reside in the ETS domain. The high molecular weight forms of PDEF observed in Fig. (**3A**) may represent multiply mono-ubiquitinated or poly-ubiquitinated PDEF. Additionally, TOPORS robustly ubiquitinates the N-terminal region of PDEF (amino acids 1-141), which contains four lysine residues (Fig. **3C**).

DISCUSSION

The reduced expression of PDEF protein in prostate cancer cells results in increased migration and invasion and the transcriptional activation of multiple genes involved in epithelial-to-mesenchymal transition [5]. Given the potential role of this protein in regulating processes central to neoplastic progression, it is important to elucidate regulatory mechanisms that operate to maintain its steady-state level and functional activity. Evidence that PDEF may be post-transcriptionally regulated in prostate epithelial cells has been reported [12]. Translation of PDEF mRNA was shown to be inhibited specifically in several prostate cancer cases, and transfection analyses implicated the 5' and 3' untranslated regions of the PDEF mRNA in translational control. A recent analysis of PDEF mRNA and protein expression in breast cancer cell lines and patient samples has clearly elucidated a post-transcriptional control mechanism that acts regulate PDEF [29]. In this study, two miRNAs capable of targeting PDEF mRNA were identified and functionally characterized. Both miR-204 and miR-510 were shown to repress PDEF translation, and both were found to be elevated in breast cancer, providing a highly plausible mechanism whereby PDEF levels can be down-regulated in patients [29].

To date, post-translational regulatory mechanisms that regulate PDEF have not been systematically addressed, although mutation of a potential MAPK phosphoacceptor site at Threonine 50 was shown to abolish the ability of PDEF to induce migration or invasion of MCF-10A cells in which ErbB2, CSF-1R/CSF-1 or MEK2DD were co-expressed [6]. Since PDEF and NKX3.1 functionally associate in prostate cancer cells, we sought to determine whether PDEF was regulated by two known regulators of NKX3.1, Protein Kinase CK2 and the E3 ubiquitin ligase TOPORS. CK2 phosphorylation stabilizes NKX3.1, and prevents its degradation by the 26S proteasome [16]. The data reported here demonstrate that blocking CK2 activity in prostate cancer cells also results in a sharp and sustained decrease in the steady-state level of PDEF, strongly suggesting that both proteins are coordinately regulated by CK2 signaling. One known function of NKX3.1 is to down-regulate PDEF-mediated activation on the PSA promoter. Hence, it is possible that the coordinate regulation of these two transcriptional regulatory proteins may serve to keep PDEF activity in check through a negative feedback loop.

Unlike NKX3.1, the mRNA level of PDEF was not diminished in response to CK2 blockade, suggesting that the effect occurs exclusively at the post-translational level. It is also interesting to note that knocking down CK2α′, but not CK2α, affected the level of PDEF. NKX3.1 also appears to be preferentially regulated by CK2α′ in LNCaP cells [16]. Although the overall activity of CK2 is elevated in prostate tumor cells compared with normal prostate epithelial cells, the subcellular location and amount of free and complexed catalytic subunits of the kinase are relevant to the resulting biology [21]. The loss of the free CK2α′ catalytic subunit from the nucleus could contribute to reduced expression of PDEF and NKX3.1 in prostate cancer.

In light of our observation that PDEF is readily phosphorylated *in vitro* by CK2, it is plausible that the CK2 stabilizing effect is direct. Mass spectrometric analyses provided evidence for three CK2 phosphoacceptor sites within the PDEF Pointed domain and the function of CK2 phosphorylation at these sites is currently under investigation. Given the importance of the Pointed domain in mediating protein-protein interactions, it will be interesting to determine how mutation of these sites affects the PDEF interactome that has recently been described [13].

Our observation that treatment with MG132 prevents the loss of PDEF when CK2 activity is blocked provides strong evidence that PDEF turnover is largely proteasome-dependent, and that at least one role of CK2 phosphorylation may be to prevent proteasome-mediated degradation. Proteasome blockade is a promising new approach to treat prostate and other forms of cancer [30] either alone or in concert with other chemotherapeutics. Although stabilizing Nuclear Factor kappa-B appears to be an important functional outcome of proteasome inhibition in cancer cells, it is possible that stabilization of PDEF and the tumor suppressor NKX3.1 may also be physiologically relevant effects of these drugs in prostate cancer. Our demonstration that PDEF is a robust target of the E3 ubiquitin ligase TOPORS raises the possibility that therapies directed at TOPORS inhibition may also stabilize PDEF in prostate cancer cells. However, it is important to note that TOPORS also appears to have a tumor suppressor role in some contexts [31].

The data reported here establish CK2 as a major regulator of PDEF accumulation in prostate epithelial cells. It will be important to determine other functional consequences of CK2 phosphorylation in PDEF, and to identify other kinases that modulate the function of this ETS protein in prostate and other cancers.

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