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Journal of Cellular Biochemistry 106:25–32 (2009)

Journal of Cellular Biochemistry

Calcium/Calmodulin-Dependent Kinase II Regulates Notch-1 Signaling In Prostate Cancer Cells

Olga A. Mamaeva,[1] Junghyun Kim,[1] Gong Feng,[1] and Jay M. McDonald[1,2*]

[1]*Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama*

[2]*Veterans Administration Medical Center, Birmingham, Alabama*

ABSTRACT

Notch signaling is associated with prostate osteoblastic bone metastases and calcium/calmodulin-dependent kinase II (CaMKII) is associated with osteoblastogenesis of human mesenchymal stem cells. Here we show that prostate cancer cell lines C4-2B and PC3, both derived from bone metastases and express Notch-1, have all four isoforms of CaMKII (α, β, γ, δ). In contrast, prostate cancer cell lines LNcaP and DU145, which are not derived from bone metastases and lack the Notch-1 receptor, both lack the alpha isoform of CaMKII. In addition, DU145 cells also lack the β-isoform. In C4-2B cells, inhibition of CaMKII by KN93 or γ-secretase by L-685,458 inhibited the formation of the cleaved form of Notch-1 thus inhibiting Notch signaling. KN93 inhibited down stream Notch-1 signaling including Hes-1 gene expression, Hes-1 promoter activity, and c-Myc expression. In addition, both KN93 and L-685,458 inhibited proliferation and Matrigel invasion by C4-2B cells. The activity of γ-secretase was unaffected by KN93 but markedly inhibited by L-685,458. Inhibition of the expression of α, β, or γ-isoform by siRNA did not affect Hes-1 gene expression, however when expression of one isoform was inhibited by siRNA, there were compensatory changes in the expression of the other isoforms. Over-expression of CaMKII-α increased Hes-1 expression, consistent with Notch-1 signaling being at least partially dependent upon CaMKII. This unique crosstalk between CaMKII and Notch-1 pathways provides new insight into Notch signaling and potentially provides new targets for pharmacotherapeutics. J. Cell. Biochem. 106: 25–32, 2009.

KEY WORDS: PROSTATE; CANCER; NOTCH-1; CALCIUM/CALMODULIN-DEPENDENT KINASE II; γ-SECRETASE; Hes-1; c-Myc

Prostate carcinoma is the most commonly diagnosed cancer and the second leading cause of cancer mortality in men in the United States [Jemal et al., 2007]. Metastases to bone represent very severe complications of prostate carcinoma; 85% of men who die of prostate cancer develop bone metastases. Unlike other tumors, like breast cancer or myeloma, that are associated with osteolytic bone metastases, prostate cancer bone metastases are predominantly osteoblastic, stimulate an increase in bone formation and an overall increase in bone volume [Mori et al., 2007]. The detailed intrinsic mechanisms that lead to bone metastases and the relationship between cancer cells and bone environment are largely still unknown.

It had been demonstrated that two prostate cancer cell lines (C4-2B and PC3) derived from bone metastases acquired osteomimetic properties after osteogenic induction in vitro: formed mineralized nodules and expressed osteoblast-specific genes, Runx-2, osteocalcin, and alkaline phosphatase [Lin et al., 2001; Zayzafoon et al., 2004]. The transformation of prostate cancer bone metastatic cells to osteoblast-like cells is dependent upon activation of the Notch and ERK pathways [Zayzafoon et al., 2004]. Furthermore, metastatic prostate cancer samples from bone of patients exhibit activated Notch-1 expression [Zayzafoon et al., 2004].

The Notch signaling pathway plays a pivotal role in various physiological developmental processes, including cell fate determination. Notch is a transmembrane receptor activated by binding with specific ligands presented by neighboring cells. There are four Notch receptors: Notch-1 to Notch-4 and six Notch ligands: Jagged 1 and 2 and Delta-like 1, 2, 3, 4 in mammalian cells. Upon binding with a ligand, the Notch receptor undergoes successive proteolytic cleavage events: cleavage at site S2 by ADAM metalloproteases followed by the transmembrane domain cleavage by γ-secretase that releases the Notch Intracellular Domain (NICD). The NICD translocates to the nucleus where it forms a complex with the ubiquitously expressed transcription factor, CSL that leads to activation of transcriptional repressors of the Hes and Hey families. Presently known gene targets for Notch signaling also include cell cycle regulators such as p21, cyclin D [Ronchini and Capobianco, 2001], transcription factors such as c-Myc, NF-kB2 [Oswald et al.,

Grant sponsor: United States Public Health Services; Grant number: CA 109119.

*Correspondence to: Jay M. McDonald, Department of Pathology, University of Alabama at Birmingham, 619 S. 19th Street, WP P220, Birmingham, AL 35249-7331. E-mail: mcdonald@uab.edu

Received 22 June 2008; Accepted 22 September 2008 • DOI 10.1002/jcb.21973 • 2008 Wiley-Liss, Inc.
Published online 19 November 2008 in Wiley InterScience (www.interscience.wiley.com).

1998; Weng et al., 2006], growth factor receptors, and regulators of apoptosis [Wu et al., 2007].

The role of the Notch pathway in cancer development was first identified in acute T cell lymphoblastic leukemias (T-ALL). Genetic rearrangements that led to constitutively over-expression of Notch-1 were found in more than 50% of human T-ALL [Pear et al., 1996]. It has also been reported that Notch signaling is involved in development of breast cancer [Wu et al., 2007], pancreatic cancer [Wang et al., 2006], albuminuria and podocyte dysfunction in diabetic nephropathy [Niranjan et al., 2008] and promotion of cell proliferation and resistance to apoptosis [Roy et al., 2007].

Calcium/calmodulin-dependent kinase II (CaMKII) is a multifunctional kinase that plays an important role in the regulation of cell growth, cytoskeletal organization, regulation of synaptic transmission, learning, and memory. It comprises a family of more than 30 isoforms derived from four closely related genes: α, β, γ, and δ [Srinivasan et al., 1994; Hudmon and Schulman, 2002; Zayzafoon, 2006]. It has been shown that CaMKII-α regulates osteoblast differentiation by controlling c-fos expression [Zayzafoon et al., 2005]. An important role of CaMKII in cancer pathogenesis has been reported. In human osteosarcoma cells, inhibition of the alpha-isoform of CaMKII led to cell cycle arrest by a p21-dependent mechanism. KN93, a CaMKII chemical inhibitor, decreases the in vivo growth of osteosarcoma cells injected intratibialy or subcutaneously in athymic mice [Yuan et al., 2007]. CaMKII is important for prostate cancer cell survival and promotes their progression to an androgen-independent state [Rokhlin et al., 2007].

Therefore, in this study, we investigate the role of CaMKII in the regulation of Notch-1 signaling in prostate cancer cell lines focusing mainly on the C4-2B cell line which is known to be able to mineralize in vitro after osteogenic induction and form osteoblastic lesions after injection in athymic mice.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL REAGENTS

The Human Prostate Cancer cell line C4-2B was purchased from UroCor, Inc. (Oklahoma City, OK). Cells were cultured in T-medium (Invitrogen, CA), supplemented with 5% fetal bovine serum (Sigma–Aldrich, St. Louis, MO), and 100 IU/100 μg/ml penicillin/streptomycin. LNCaP (CRL-1740), PC3 (CRL-1435), and DU145 (HTB-81) cell lines were purchased from American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium (Invitrogen), supplemented with 10% of fetal bovine serum (Sigma–Aldrich), and 100 IU/100 μg/ml penicillin/streptomycin.

REVERSE TRANSCRIPTION, RT-PCR, AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from cells using TRIZol Reagent (Invitrogen). First-strand cDNA was synthesized using 1 μg of RNA and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was performed on MyiQ Real-Time PCR Detection System (Bio-Rad Lab., Inc., CA), using SYBR Green JumpStart Taq Ready-Mix for quantitative PCR (Sigma–Aldrich). Primers sequences were as follows:

Notch-1 forward: 5′-CACTGTGGGCGGGTCC-3′;
Notch-1 reverse: 5′-GTTGTATTGGTTCGGCACCAT-3′;
Hes-1 forward: 5′-AGGCGGACATTCTGGAAATG-3′;
Hes-1 reverse: 5′-CGGTACTTCCCCAGCACACTT-3′;
18S forward: 5′-CGCCGCTAGAGGTGAAATTCT-3′;
18S reverse: 5′-CGAACCTCCGACTTTCGTTCT-3′;
α-CaMKII forward: 5′-ATCCCCACATCCACCTGAT-3′;
α-CaMKII reverse: 5′-CTGTGGAAGTGGACGATCTG-3′;
β-CaMKII forward: 5′-CTGTCAGCCAGAGATCACCA-3′;
β-CaMKII reverse: 5′-CTCTCGCCACAATGTCTTCA-3′;
γ-CaMKII forward: 5′-GGGACACGGTAACTCCTGAA-3′;
γ-CaMKII reverse: 5′-TGGCACCCTTCAGTTTTCTC-3′;
δ-CaMKII forward: 5′-CAGTACATGGATGGCAGTGG-3′;
δ-CaMKII reverse: 5′-GCAGAAGTGGCACTGTTGAA-3′.

IN VITRO PEPTIDE CLEAVAGE ASSAY FOR *Γ*-SECRETASE ACTIVITY MEASUREMENT

This assay was performed as described by Kim et al. [2005]. Membranes were isolated from cells as follows: cells were washed two times with PBS (all procedures were carried out on ice or at 4°C), lysed in 150 μl buffer A {10 mM Tris–HCl (pH7.5), 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Sigma–Aldrich)}, and centrifuged at 13,400*g* for 20 min. The pellets were dissolved in 100 μl of buffer A with 0.2% CHAPSO and solubilized at 4°C for 40 min. with end-over-end rotation. The solubilized membranes were centrifuged at 13,400*g* for 20 min, and the supernatants were collected. Ten μl of supernatants were incubated with 20 μM of a fluorogenic γ-Secretase Substrate (NMA-GGVVIATVK (DNP)-DRDRDR-NH2, Calbiochem, CA) in Buffer A with 0.2% CHAPSO at 37°C overnight. The degree of substrate cleavage was measured using a Multi-Detection Microplate Reader Synergy2 (BioTec Instrument, Inc., VT) with excitation/emission wavelengths of 440/460 nm.

INVASION ASSAY

The invasive activity of C4-2B cells was examined using BD BioCoat Matrigel Invasion Chambers with 8 μm pore size membrane (BD Biosciences, Bedford, MA) according to the manufacturer's protocol. Cells (5×10^4) were seeded in serum-free medium in the upper chamber; the bottom wells were filled with complete medium. Inhibitors or DMSO were added both to the upper and bottom chambers. After 48 h of incubation, cells from the upper chamber were removed and cells that invaded through the Matrigel coated membrane were fixed with 4% paraformaldehyde and stained with 0.1% Crystal Violet. Invaded cells on the entire surface of the membrane were counted using a microscope at 100× magnification. Data represent the average of four independent experiments.

WESTERN BLOT ANALYSIS

Cells were lysed in RIPA buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCL, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor cocktail purchased from Sigma–Aldrich. Thirty μg protein was fractionated using SDS–PAGE and transferred to Immunobilon-P Transfer Membrane (Millipore, Billerica, MA). Primary antibodies

directed against cleaved Notch-1 (Val1744) and β-actin were from Cell Signaling (Beverly, MA), and c-Myc (9E10), Hes-1, phospho-CaMKII-α and HRP-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Amersham ECL Advance Western Blotting Detection Kit purchased from GE Healthcare (Buckinghamshire, UK) was used for detection.

GENE SILENCING BY siRNA

Cells were transfected with CaMKII siRNA specific to α, β, or γ-isoforms, or control non-functional siRNA (Santa Cruz Biotechnology) using Gene Eraser siRNA Transfection Reagent purchased from Stratagene (La Jolla, CA). Forty-eight hours after transfection cells were harvested for RNA or total protein extracted.

CELL PROLIFERATION ASSAY

Cells were seeded in 96-well plates at a density 1×10^4 cells/well and treated with KN93, or L-685,458, or DMSO. At the end of the experiment, cell proliferation was assessed using the CellTiter96 Aqueous One Solution Cell Proliferation Assay Reagent (Promega, Madison, WI) according to the protocol of the manufacturer.

TRANSIENT TRANSFECTION AND Hes-1 PROMOTER REPORTER ASSAY

Cells were plated at a density of 2×10^4 cells/cm^2 in 6-well plates and cultured for 24 h. Six hours before transfection, medium was replaced with fresh medium. Cells were co-transfected with 2 μg reporter plasmid pHes-1-Luc containing Hes-1 promoter fragment (−194 to +160) of the Hes-1 gene cloned upstream of the luciferase gene [Jarriault et al., 1995] and 10 ng pRL-TK (Renilla luciferase control plasmid) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Both plasmids were kindly provided by Dr. Jon Aster (Harvard University). Twenty-four hours after transfection, the medium was changed and the cells were treated with KN93 or DMSO as indicated for 16 h. At the end of incubation, cells were assayed for luciferase activity with a dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to an internal Renilla luciferase control. Data are expressed as fold difference relative to control in five independent experiments.

The expression vector containing the functional CaMKII-α gene was a generous gift from Dr. C.Hao (Emory University). C4-2B cells were transfected with CaMKII-a vector using Lipofectamin 2000 (Invitrogen) according to manufacturer's protocol. Cells were harvested for RNA and protein extracted at 72 h after transfection.

STATISTICAL ANALYSIS

For statistical analysis Microsoft Excel software was used. Values represent a mean ± SE. A Student's paired *t*-test was used to determine statistically significant differences: * used when $P \leq 0.05$, ** when $P \leq 0.01$.

RESULTS

CaMKII EXPRESSION IN PROSTATE CANCER CELLS

We examined the gene expression of the four isoforms of CaMKII: α, β, γ, and δ in prostate cancer cells C4-2B, PC3, LNCaP, and DU145.

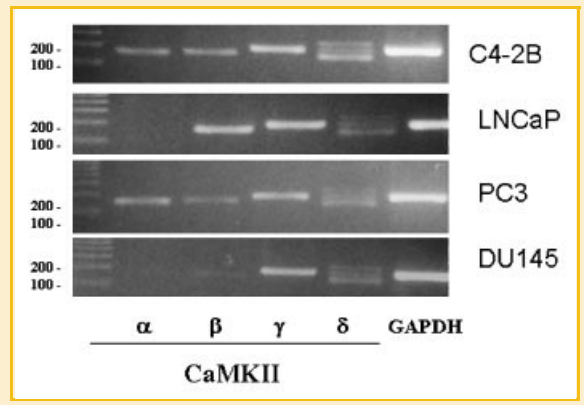

Fig. 1. CaMKII expression in prostate cancer cell lines. CaMKII gene expression was examined by RT-PCR using specific primers for α, β, γ, and δ isoforms of CaMKII gene and GAPGH primers as a control. Representative images from three independent experiments are shown.

Only C4-2B and PC-3 cells express all four isoforms of CaMKII (Fig. 1). LNCaP cells do not express the alpha-isoform of CaMKII and DU145 cells do not express α- or β-CaMKII. The pattern for the δ-isoform with multiple bands is commonly seen and most likely represents its splicing variants [Tombes and Krystal, 1997; Colbran, 2004]. Interestingly both C4-2B and PC3 cell lines express Notch-1 whereas LNCaP and DU 145 do not [Zayzafoon et al., 2004].

EFFECT OF CaMKII INHIBITION ON NOTCH-1 PATHWAY

We used a pharmacological inhibitor, KN93, to inhibit the CaMKII activity in C4-2B cells. KN93 binds directly to CaMKII and prevents it activation. We also used the γ-secretase inhibitor, L-685,458, that prevents cleavage of the Notch-1 receptor and thus inhibits the generation of the cleaved form of Notch-1 (NICD) and activation of its down-stream target genes, Hes-1 and c-Myc.

Western blot analysis revealed that KN93 inhibits expression of the cleaved Notch-1 protein. The effect of KN93 on NICD expression is similar to the effect of the γ-secretase inhibitor, L-685,458 (lane 3, Fig. 2A). NICD is expressed in untreated C4-2B cells (lane 1, Fig. 2A), which indicates that Notch-1 is constitutively active in these cells. Consistent with KN93 inhibition of the formation of NICD, it down-regulates Hes-1 mRNA expression in a dose-dependent manner (Fig. 2B) as determined by quantitative RT-PCR. Furthermore, Hes-1 promoter activity in cells treated with 10 μM KN93 for 16 h was significantly inhibited compare to control cells treated with DMSO (Fig. 2C). c-Myc protein expression was also inhibited by KN93 in a dose-dependent manner (Fig. 2D).

NOTCH-1 AND CaMKII REGULATE PROLIFERATION AND INVASION OF C4-2B CELLS

We examined the effect of the Notch-1 and CaMKII signaling pathways on C4-2B cell proliferation and invasion. Cells were seeded onto 96-well plates and incubated with KN93 or L-685,458 and proliferation was determined after 24, 48, and 72 h of incubation. As shown in Figure 3A.1, C4-2B cells treated with DMSO (control) exhibit exponential growth, and inhibition of CaMKII

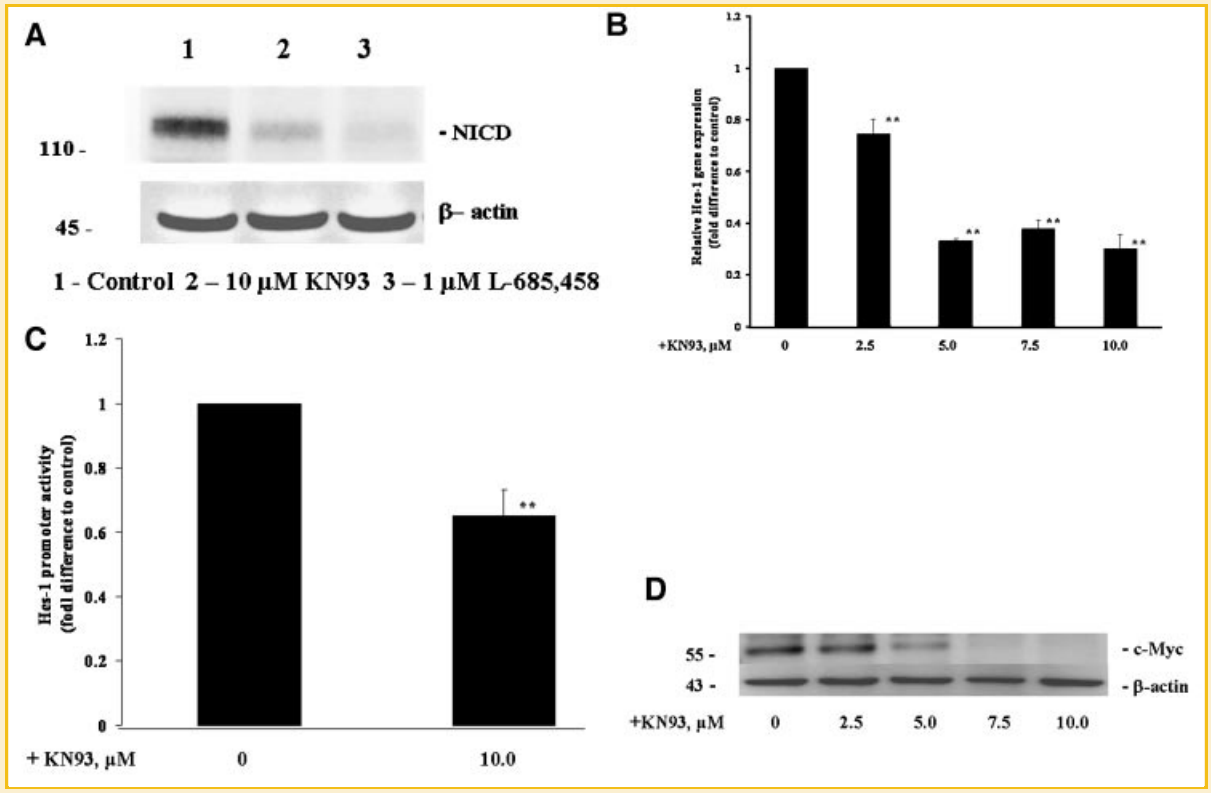

Fig. 2. KN93 inhibits the expression of cleaved Notch-1 protein, Hes-1 gene expression, Hes-1 promoter activity, and c-Myc protein expression. A: C4-2B cells were treated with 10 μM of KN93, or 1 μM L-685,458, or DMSO (control) for 24 h. Total cell lysates were used for Western blot with cleaved Notch-1 antibody (Val 1744); membrane was re-probed with β-actin antibody for loading control. Representative image from three independent experiments is shown. B: C4-2B cells were treated with indicated concentrations of KN93 or DMSO (control) for 16 h. Quantitative real-time PCR was performed with Hes-1 and GAPDH specific primers. Data were obtained from three independent experiments and results expressed as mean ± SE; $^{**}P \leq 0.01$. C: Hes-1 promoter activity was determined in C4-2B cells co-transfected with pHes-1 and pRL-TK as indicated in Materials and Methods Section. Twenty-four hours after transfection cells were treated with 10 μM KN93 or DMSO (control) for 16 h. Luciferase activity was assessed using dual luciferase reporter assay. Firefly luciferase activity was normalized by Renilla luciferase activity. Data expressed as n-fold difference relative to control and were obtained in five independent experiments; $^{**}P \leq 0.01$. D: Protein expression of c-Myc in C4-2B cells treated with indicated concentration of KN93 for 24 h. β-actin used as loading control. Representative images from four independent experiments are shown.

activity by KN93 led to significant inhibition of cell proliferation. After 72 h of incubation with 5 μM KN93 proliferation was inhibited by 61%, and 10 μM KN93 completely inhibited cell proliferation. The γ-secretase inhibitor, L-685,458 (2.5 μM led to 50% inhibition of cell growth after 72 h (Fig. 3A). One μM L-685,458 yielded identical results (data not shown).

We tested the effect of KN93 and L-685,458 on the ability of C4-2B cells to invade through Matrigel. Cells were treated with KN93 or L-685,458 as described in Materials and Methods Section. After 48 h of incubation with 5 μM KN93 or 2.5 μM L-685,458, the number of cells invaded through Matrigel was significantly less than in the control cells treated with DMSO (Fig. 3B).

EFFECT OF KN93 ON γ-SECRETASE ACTIVITY IN C4-2B CELLS

To determine if inhibition of CaMKII activity by KN93 has a direct effect on γ-secretase activity in C4-2B cells, we used an in vitro peptide cleavage assay. The membrane fractions of cells treated with KN93 or L-685,458 were assayed for γ-secretase activity. There were no differences in γ-secretase activity in cells treated with 5 μM or 10 μM KN93 compared to control cells, whereas 1 μM L-685,458 decreased γ-secretase activity by 40% and 2.5 μM L-685,458 by approximately 50% (Fig. 4).

EFFECT OF SPECIFIC INHIBITION OF CaMKII ISOFORMS ON NOTCH-1 PATHWAY

In order to further investigate the role of different isoforms of CaMKII on the regulation of Notch-1 signaling, we used specific siRNAs. We determined Hes-1 gene expression in C4-2B cells transfected with each of the siRNAs specific to α, β, γ, or δ-isoforms of CaMKII, or control non-functional siRNA. Quantitative real-time PCR analysis revealed no significant alteration in Hes-1 gene expression by any one of the tested siRNAs (Fig. 5). We then ascertained the effect of the siRNAs on the gene expression of CaMKII isoforms by quantitative real-time PCR. In cells transfected with α-CaMKII siRNA, CaMKII-α gene expression was down-regulated by 70%. However, in the same cells, gene expression of the β-isoform was up-regulated approximately twofold (Fig. 6). Similarly in cells where γ-CaMKII was down-regulated by 80%, the

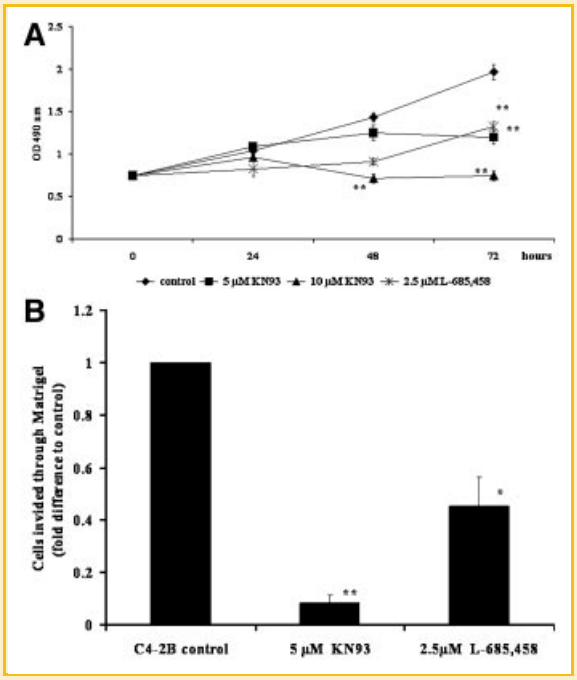

Fig. 3. Notch-1 and CaMKII are essential for proliferation and invasion of prostate cancer cells. A: Effect of KN93 or L-685,458 on the proliferation of C4-2B cells. Cells were plated into 96-well plates at 10×10^3 cell/well (5–7 wells for each treatment), and treated with indicated concentrations of KN93, L-685,458, or DMSO. MTS proliferation assay was performed immediately after plating and than at 24, 48, and 72 h. Values were obtained in three independent experiments and represent mean ± SE; $^{**}P \leq 0.01$. B: Effect of KN93 or L-685,458 on cell invasion through Matrigel. 5×10^4 cells were seeded into Matrigel Invasion Chambers in serum-free medium. Bottom wells were filled with complete medium. Inhibitors or DMSO were added to the upper and bottom wells. After 48 h incubation, invaded cells were stained with crystal violet and counted on the entire surface of the membrane under a microscope using 100× magnification. Values were obtained in four independent experiments and represent fold differences compared to control C4-2B cells (mean ± SE; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$).

β-isoform was up-regulated over twofold compared to control. Likewise the siRNA inhibition of β-CaMKII resulted in substantial, albeit reduced, expression alpha and γ-CaMKII isoforms. Thus down-regulation of one of the CaMKII isoforms led to compensatory changes in the expression of one or more of the others isoforms (Fig. 6). Note that although the data shown in Figure 6 represent one experiment, when α-CaMKII was suppressed in the three identical experiments, both β and γ isoforms were increased by a mean of 2.4- and 2.3-fold compared to control, confirming that when one isoform of CaMKII is suppressed, compensatory but variable changes in the expression of the other isoforms occurs.

EFFECT OF OVER-EXPRESSION OF CaMKII ON NOTCH-1 PATHWAY

We examined Hes-1 gene expression in C4-2B cells transfected with plasmid DNA carrying the functional α-CaMKII gene which resulted in up-regulation of Hes-1 gene expression compared to control cell (Fig. 7A). This was accompanied by an increase in Hes-1 protein expression as determined by Western blot (data not shown). Expression of the Notch-1 gene was unchanged (data not shown).

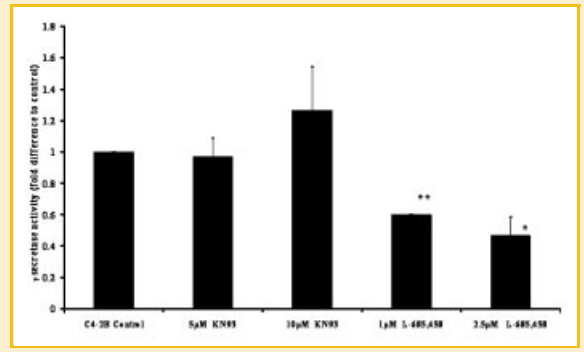

Fig. 4. γ-Secretase activity in C4-2B cells. C4-2B cells were plated at 1×10^5 cells/ml into 12-well plates, in triplicate for every treatment. Cells were treated with KN93, L-684,458, or DMSO (control) for 24 h. At the end of the experiment cells were lysed and processed as described in Materials and Methods Section. Every sample was probed twice. Protein concentration in the samples was determined with Coomassie Plus Protein Assay Reagent (Pierce). Data are presented as relative fluorescent units normalized to proteins concentration in the sample compare to values from control C4-2B cells and were obtained in three independent experiments.

Up-regulation of CaMKII-α in transfected cells was confirmed by Western blot with antibody specific for the phosphorylated form of CaMKII-α (Fig. 7B).

DISCUSSION

It had been well established that calcium/calmodulin-dependent kinase II (CaMKII) plays an important role in regulating diverse physiological processes in cells including proliferation, and is important for osteoblast and osteoclast differentiation [Hudmon and Schulman, 2002; Seales et al., 2006; Zayzafoon, 2006; Ang et al.,

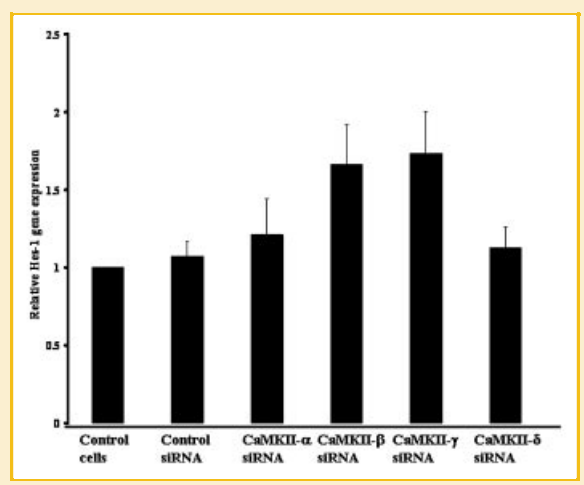

Fig. 5. Effect of specific inhibition of CaMKII isoforms on Hes-1 gene expression. C4-2B cells were transfected with siRNA specific to a α, β, γ, or δ-isoforms of CaMKII using Gene Eraser Transfection Reagent (Stratagene). 48 h after transfection cells were harvested for RNA extraction and relative Hes-1 gene expression was determine using quantitative real-time PCR. Values were normalized to 18S RNA values and represent the mean ± SE of three independent experiments.

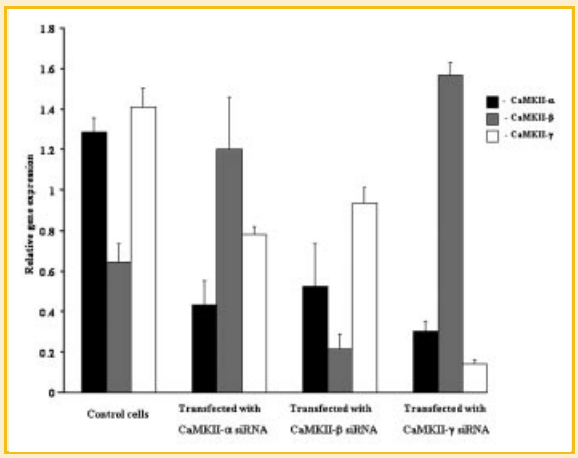

Fig. 6. Effect of inhibition of CaMKII by siRNAs on the expression level of others CaMKII isoforms. The relative gene expression of CaMKII isoforms was determined using quantitative real-time PCR and normalized to 18S RNA values. Values represent the mean ± SE of one experiment performed in triplicate.

2007]. For example, CaMKII promotes proliferation of human osteosarcoma cells [Yuan et al., 2007], promotes the resistance to oxidative stress of MCF-7 breast cancer cells [Rodriguez-Mora et al., 2006], and plays an important role in prostate cancer cell survival and progression to an androgen-independent state [Rokhlin et al., 2007]. The purpose of these studies was to determine the role of

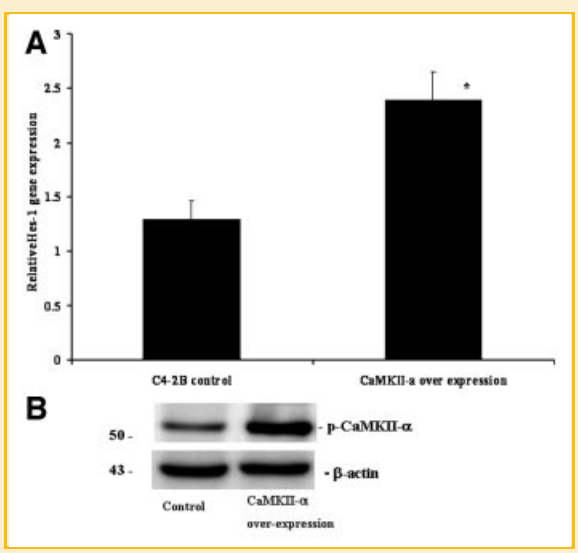

Fig. 7. Over-expression of CaMKII-α leads to activation of Notch-1 signaling. A: C4-2B cells were transfected with expression vector containing functional CaMKII-α gene. Relative Notch-1 or Hes-1 gene expression was determined in 72 h post-transfection in quantitative real-time PCR; values were normalized to 18S RNA and represent the mean ± SE from three independent experiments; $^{*}P \leq 0.05$. B: Activation of CaMKII in transfected cells was confirmed using Western blot with antibody specific to phosphorylated CaMKII; membrane was re-probed with β-actin antibody for loading control. Representative image from three independent experiments is shown.

CaMKII in the regulation of Notch-1 signaling pathway in human prostate cancer cells. It has previously been demonstrated that Notch-1 pathway is activated in prostate cancer cells and is important in the development of osteomimetic properties by prostate cancer bone metastatic cell lines [Zayzafoon et al., 2004].

We first examined the expression of α, β, γ, and δ-isoforms of CaMKII in different prostate cancer cell lines and found that only C4-2B and PC3 cell lines express all four CaMKII isoforms. Interestingly these cell lines are derived from bone metastases and are able to develop bone metastases after inoculation in mice and express high levels of Notch-1 [Kaighn et al., 1979; Thalmann et al., 1994; Zayzafoon et al., 2004]. LNCaP cells that originated from a lymph node metastatic lesion of human prostatic adenocarcinoma [Horoszewicz et al., 1983] do not express α-CaMKII or Notch-1 [Zayzafoon et al., 2004]. DU145 cells derived from a lesion in the brain [Stone et al., 1978] also do not express α or β-CaMKII or Notch-1 [Zayzafoon et al., 2004]. These findings are possibly related to our previous data demonstrating that C4-2B cells but not LNCaP cells are able to differentiate into osteoblast-like cells and α-CaMKII plays an important role in osteoblast differentiation [Zayzafoon et al., 2004, 2005]. It has also been reported that the expression profile of CaMKII isoforms is tissue-specific and may change upon transformation of cells to malignancy [Srinivasan et al., 1994; Tombes and Krystal, 1997].

The canonical Notch1 pathway functions as a signal transduction pathway for transmitting extracellular signals from the cell surface directly to the nucleus. It is constitutively activated in many cancer cells [Roy et al., 2007]. Recently obtained data revealed direct interaction between Notch-1 and c-Myc. Using ChIP-on-chip assay and gene expression analysis it was demonstrated that Notch1 and c-Myc directly bind and up-regulate many of the same target genes [Palomer et al., 2006]. Clinical samples from patients with prostate cancer and from prostate cancer cell lines had up-regulated c-Myc expression levels compared to normal cells [Latil et al., 2000]. In our studies, c-Myc expression in C4-2B cells was inhibited dose dependently by the CaMKII inhibitor, KN93.

Our results suggest that CaMKII regulates Notch-1 signaling in C4-2B cells. Inhibition of CaMKII activity by KN93 leads to a decrease of NICD protein expression and down-regulates the expression of Notch-1 target gene Hes-1 and its promoter activity. Our previous data demonstrated that γ-secretase inhibitor L-685,458 inhibited Hes-1 gene expression in C4-2B cells [Zayzafoon et al., 2004]. Consistent with these results, over-expression of functional α-CaMKII in C4-2B cells leads to up-regulation of Hes-1 gene expression. However, inhibition of CaMKII by KN93 has no effect on γ-secretase activity. These data demonstrate that CaMKII regulates the Notch-1 signaling pathway and suggest that the mechanism of CaMKII regulation of NICD is independent of γ-secretase activity.

Notch-1 promotes growth and invasion of C4-2B cells: both KN93 and γ-secretase inhibitor, L-685,458, significantly inhibit cell growth and invasion through Matrigel. Both CaMKII and Notch-1 have similar effects on growth and invasion and are consistent with the concept that CaMKII and Notch signaling pathways may overlap or be co-regulatory. These effects of Notch-1 signaling on growth and invasion are consistent with results obtained with MDA-MB231 (ERα^{-}) breast cancer cells where Notch-1 knockdown or γ-secretase

inhibition led to decreased cyclins A and B1 and G_2 arrest [Rizzo et al., 2008].

In order to further investigate the role of CaMKII isoforms in the regulation of the Notch-1 pathway we used the individual siRNAs specific for the α, β, γ, or δ isoforms and evaluated the gene expression of Notch-1 target gene, Hes-1. We observed a very interesting phenomenon: the down regulation of one of the CaMKII isoforms in C4-2B cells leads to compensatory changes in gene expression of others isoforms. This may be a mechanism for C4-2B cells to maintain sufficient levels of CaMKII activity for optimal proliferation activity and survival. It also likely explains why inhibition of CaMKII isoforms by isoform-specific siRNAs did not inhibit Hes-1 expression, whereas chemical inhibition by KN93, which inhibits the activity of all isoforms, did inhibit Hes-1 expression. Consistent with this, over-expression of CaMKII-α increased Hes-1 expression.

In conclusion, here we demonstrated that C4-2B prostate cancer cells express four isoforms of CaMKII: α, β, γ, and δ. CaMKII regulates the Notch-1 pathway. Inhibition of CaMKII activity leads to down-regulation of the Notch-1 and its target genes and disrupts cell growth and invasion activity, whereas over-expression of CaMKII-α increases Notch-1 signaling. The concept that CaMKII and Notch-1 signaling pathways overlap may be utilized in designing cancer chemotherapeutic agents.

ACKNOWLEDGMENTS

Supported by a United States Public Health Services grant to J.M.M. (CA 109119).

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