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Activation of TPA-response element present in human Lemur Tyrosine Kinase 2 (*lmtk2*) gene increases its expression

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ABSTRACT

Regulatory elements present in the promoter of a gene drive the expression of the gene in response to various stimuli. Lemur Tyrosine Kinase 2 (LMTK2) is a membrane-anchored Serine/Threonine kinase involved in endosomal protein trafficking and androgen signaling amongst other processes. Previous studies have shown this protein to be of therapeutic importance in cystic fibrosis and prostate cancer. However, nothing is known about the endogenous expression of this protein and its regulation. In this study, we analyzed the gene encoding human LMTK2, to look for possible regulatory elements that could affect its expression. Interestingly, the human *lmtk2* gene contains a consensus TPA (12- O-Tetradecanoylphorbol-13-acetate)-responsive element (TRE) in the region preceding its start codon. The element with the sequence TGAGTCA modulates LMTK2 expression in response to treatment with TPA, a synthetic Protein Kinase C (PKC) activator. It serves as the binding site for c-Fos, a member of the Activator Protein −1 (AP-1) transcription factor complex, which is transactivated by PKC. We observed that TPA, at low concentrations, increases the promoter activity of LMTK2, which leads to a subsequent increase in the mRNA transcript and protein levels. This modulation occurs through binding of the AP-1 transcription factor complex to the *lmtk2* promoter. Thus, our current study has established LMTK2 as a TPA-responsive element-containing gene, which is upregulated downstream of PKC activation. Considering the involvement of LMTK2 in intracellular processes as well as pathological conditions, our findings demonstrate a way to modulate intracellular LMTK2 levels pharmacologically for potentially therapeutic purposes.

1. Introduction

Lemur Tyrosine Kinase 2 (LMTK2), also known as apoptosis-associated tyrosine kinase (AATYK-2), brain-enriched kinase (BREK) and cyclin dependent kinase 5 (cdk5)/p35-regulated kinase, is a 1503 amino acid protein encoded by the *lmtk2* gene, and belongs to the Serine/Threonine kinase family of membrane-anchored proteins [1,2]. Fluorescence protease protection studies have shown the protein to possess two short membrane spanning segments at the amino terminal region, tethering the kinase to lipid domains, with both amino and carboxyl termini exposed to the intracellular cytoplasm [3,4]. Although present predominantly in membranes of the endosomal recycling pathway [1,5,6], the protein can also be found associated with the nucleus [7]. Previous studies have shown that LMTK2 exits the endoplasmic reticulum (ER), en-route to intracellular membranes, using a diacidic ER export motif located within the cytosolic carboxyl domain [8]. Although the cellular role(s) of LMTK2 are still being elucidated, several functions have been ascribed to the kinase, including endosomal trafficking regulation and apoptosis [5,8,9]. LMTK2 is also associated with nerve growth factor (NGF) signaling, where it negatively regulates neuronal differentiation [1,10]. Of note, several human diseases are associated with LMTK2 activity, including cystic fibrosis and prostate cancer. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene give rise to the life-threatening clinical phenotype seen in patients with CF; intriguingly, CFTR is a major substrate for LMTK2 kinase activity [2]. Moreover, CFTR undergoes continual recycling between the plasma membrane and the endosome [11,12], a process regulated by LMTK2 [13,14]. Recent Genome-wide Association Study (GWAS) and functional studies have also implicated mutations in LMTK2 as a significant contributor to the development and progression of prostate cancer by negatively regulating androgen dependent signaling [7,15].

Given the importance of LMTK2 in normal cellular functions, as well as pathological conditions, regulators of LMTK2 activity or expression

Abbreviations: LMTK2, Lemur Tyrosine Kinase 2; PKC, Protein Kinase C; TPA, Phorbol 12-myristate 13-acetate; 4α-TPA, 4α-phorbol 12, 13-didecanoate; TRE, TPA-response element; ACD, Actinomycin D; Chx, Cycloheximide; AP-1, Activator Protein – 1; SEAP, Secretory Alkaline Phosphatase; GM-CSF, Granulocyte Macrophage Colony Stimulating Factor

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would be of basic and clinical interest. However, there are as yet no small molecule modulators of LMTK2 kinase activity. Hence, the aim of our study was to investigate whether the expression of endogenous human LMTK2 can be modulated. We investigated *lmtk2* promoter activity and expression in human embryonic kidney cells (HEK293), which expresses LMTK2 endogenously. Using this cell line, we wanted to determine if promoter sequences upstream from the *lmtk2* coding region contain elements, which can regulate downstream gene expression.

2. Materials and methods

2.1. Cell culture

HEK293 cells and HeLa cells were obtained from ATCC (Catalog # CRL-1573, Catalog # ATCC-CCL2), and cultured in DMEM medium (Thermo Fisher Scientific catalog # 10569-069) supplemented with 10% Fetal Bovine Serum (Hyclone catalog # SH30070.03) and 1% Pen-Strep (Thermo Fisher Scientific catalog # 15140-122). CFBE cells were a generous gift from Dr. Dieter Gruenert (UCSF, San Francisco, USA), and were cultured in EMEM medium (ATCC catalog # 30-2003) supplemented with 10% FBS and 1% Pen-Strep.

2.2. Reagents and antibodies

PKC activator Phorbol-12-myristate-13-acetate or TPA (EMD Millipore catalog # 524400), its biologically inactive analogue 4α-Phorbol-12,13-didecanoate or 4α-TPA (EMD Millipore catalog # 524394) and Calphostin C (EMD Millipore catalog # 208725) were dissolved in DMSO for making stock solutions. Actinomycin D, Cycloheximide and LMTK2 antibodies were from Sigma Aldrich. AP-1 binding inhibitor T-5224 was from Apexbio (catalog # B4664). All secondary antibodies, except bovine anti-goat (Santa Cruz Biotech catalog # sc-2350), were obtained from Licor. All other reagents were from Sigma Aldrich and were of reagent grade quality. Plasmids containing luciferase driven by the LMTK2 promoter were obtained from Genecopoeia (HPRM18222-PG04). LMTK2 antibody (catalog # HPA010657) was from Sigma-Aldrich. Antibodies directed against c-Jun (catalog # sc-74543x), c-Fos (catalog # sc-52x, catalog # sc-166940), GAPDH (catalog # sc-25778) and Lamin A (catalog # sc-206820) were from Santa Cruz; antibodies directed against β-actin (catalog # 926-42212) and β-tubulin (catalog # 926-42211) were from Licor Biosciences. The concentrations used for the primary and secondary antibodies for specific experiments are mentioned in the respective figure legends. For experimental analysis, cells were treated with either vehicle alone (DMSO, 0.001%) or reagents at the specified concentrations. Treatments were done for 24 h, unless mentioned otherwise.

2.3. Immunoblot analysis

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) with 1X Protease Inhibitor Cocktail (Thermo Fisher Scientific catalog # 1861278) and proteins were resolved by SDS-PAGE as previously described [3]. Appropriate loading controls were used. Detection of primary antibodies was performed either by using IRDye-conjugated secondary antibodies (LiCor Biosciences), followed by visualization on an Odyssey SA infrared imaging system (LiCor Biosciences), or by using SuperSignal West Femto Maximum Sensitivity Substrate (Invitrogen catalog # 34095).

2.4. Real-time PCR quantitative analysis

Cells were lysed in Trizol reagent (Thermo Fisher Scientific catalog # 15596) according to the manufacturer's instructions. RNA was extracted using Phenol-Chloroform extraction method and cDNA was made using ThermoScript RT-PCR kit (Thermo Fisher Scientific catalog # 11146). qPCR was done using PowerUp SYBR Green mastermix (Thermo Fisher Scientific catalog # A25741) and the amplification was performed on ViiA7 (Applied Biosystems). Data were analyzed using the ViiA7 software. GAPDH or 18 S rRNA was used as reference gene where applicable. The primer sequences are provided in Table 1. All primers were obtained through Integrated DNA technologies.

Table 1
Primers used in the study.

Primer name	Sequence
LMTK2	F: 5′-AACTGTGTATCCTGCTGTAAGG-3′ R: 5′-CTGCTGGTGGTGTGAAATCTA-3′
PKC-α	F: 5′-CGGAATGGATCACACTGAGAAG-3′ R: 5′-ACATAAGGATCTGAAAGCCCG-3′
PKC-β	F: 5′-TTCCCGATCCCAAAAGTGAG-3′ R: GTCAAATCCCAATCCCAAATCTC-3′
PKC-γ	F: 5′-GGAGGGCGAGTATTACAATGTG-3′ R: 5′-GGGATGGGAGAGGAAGAGG-3′
PKC-δ	F: 5′-ACCATGAGTTTATCGCCACC-3′ R: GCATTTCTTGTGGATGGCAG-3′
PKC-θ	F: 5′-ACAATTACAAGAGCCCGACC-3′ R: 5′-GGTTTATGCCACAAAGGTTGG-3′
PKC-η	F: 5′-GTAAATGCGGTGGAACTTGC-3′ R: 5′-ACCCCAATCCCATTTCCTTC-3′
PKC-ε	F: 5′-CCTACCTTCTGCGATCACTG-3′ R: 5′-TACTTTGGCGATTCCTCTGG-3′
PKC-ζ	F: 5′-TGCTTACATTTCCTCATCCCG-3′ R: 5′-CGCCCGATGACTCTGATTAG-3′
PKC-ι	F: 5′-ATGTGTTCCCTTGTGTACCAG-3′ R: 5′-CGCCTGTTGAAACGCTTG-3′
AP-1 Binding mutant	F: 5′-TTGAGCTCAGGAGGTTTATCAGTGGGTGATGCCGAGAGAG-3′ R: 5′- CTCTCTCGGCATCACCCACTGATAAACCTCCTGAGCTCAA-3′
GAPDH	F: 5′- GGAAGGTGAAGGTCGGAGTC-3′ R: 5′- CTGGAAGATGGTGATGGGATTTC-3′
α-TUBULIN	F: 5′-TCCAGATTGGCAATGCCTG-3′ R: 5′- GGCCATCGGGCTGGCT-3′
AP-1 ChIP	F: 5′- GTCTGCCAGGTGGACAAGAG-3′ R: 5′- CCTCTCAGCCATCAAACAGC-3′
Human 18S rRNA	F: 5′-GGCCCTGTAATTGGAATGAGTC-3′ R: 5′-CCAAGATCCAACTACGAGCTT-3′

2.5. LMTK2 promoter activity

HEK293 cells were transfected stably with a Luciferase reporter gene driven by the LMTK2 promoter (Genecopoeia); the plasmid also contained a secreted alkaline phosphatase gene (SEAP) driven by a constitutive CMV promoter. Cells were treated as described in **Results**. The luciferase assay was done using the Secrete-pair Dual Luminescence assay kit (Genecopoeia catalog # SPDA-D010) following the manufacturer's instructions, and the luminescence was recorded using the Synergy Biotek multi-mode plate reader.

2.6. PKC isoforms

Total RNA from resting HEK293 cells was extracted and cDNA was prepared as mentioned above. The control samples lacked reverse transcriptase during cDNA synthesis to eliminate genomic DNA contamination. The primers for testing the PKC isoforms are listed in Table 1. PCR amplification was done using Taq polymerase (New England Biolabs catalog # M0273) in Eppendorf Mastercycler gradient, and the samples were then run on 2% agarose gel.

2.7. Mutation of the AP-1 binding site in LMTK2 promoter

The consensus AP-1 binding site (TGAGTCA) in the LMTK2-Luciferase promoter clone was mutated using the primers listed in