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-asssociated 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...
would be of basic and clinical interest. However, there are as yet no small molecule modulators of LMTK2 kinase activity. Hence, 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 # CRI-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 4a-Phorbol-12,13-didecanoate or 4a-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 Biotechnology catalog # sc-2350), were obtained from Licor. All other reagents 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 # 926-4221) and β-tubulin (catalog # 926-4221) 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′-AACCTGTATCCCTGATGTAAGG-3′<br>R: 5′-CTGGCTGTTGTTGAACTCTA-3′ |
| PKC-α       | F: 5′-CGGAATGATGACAGATGAAAG-3′<br>R: 5′-ACCATAGGATGAAAGGGG-3′ |
| PKC-β       | F: 5′-TTCGGATCCATTTCCAGG-3′<br>R: 5′-GTCAATTCCTTAAATTTGATTCT-3′ |
| PKC-γ       | F: 5′-GGATGAGGAGGAGGAGG-3′<br>R: 5′-ACCATGATGTTTATAGGACG-3′ |
| PKC-δ       | F: 5′-AGATGGTACCTTTGTGTCACG-3′<br>R: 5′-GGCGGATAGTTTTGGG-3′ |
| PKC-ε       | F: 5′-ATGATGTTTTCAATCTGCTGTAAGG-3′<br>R: 5′-CTCCTCGGATACCCATCACTCTGTAAGGAGG-3′ |
| GAPDH       | F: 5′-GGGATGGAAGGGTATCTGGG-3′<br>R: 5′-GAGGGGAACTGTGTATCCT-3′ |
| α-TUBULIN   | F: 5′-TCTCAGAGTGCAGCATTCTG-3′<br>R: 5′-GGCCATCGGGCTGGCT-3′ |
| AP-1 Chop   | F: 5′-CTTTCATGGCATCATATGCTC-3′<br>R: 5′-CTGAGCAGTGATGAGAG-3′ |
| Human 18S   | F: 5′-GGCGGATAGTTTTGGG-3′<br>R: 5′-CCAGATCACTAAGG-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
2.8. Cellular fractionation and nuclear extraction

The cellular fractionation protocol was adapted from Suzuki et al. [16] with minor modifications. The pellet was lysed accordingly in 0.1% NP-40 lysis buffer, and the nucleus resuspended in Laemmli sample buffer. Cell lysates and nuclear extracts were sonicated at 20% output, 5 times for 5 s each on GE 50 T ultrasonic processor. The samples were then boiled at 95 °C for 1 min, and subjected to Western Blot analysis.

2.9. Immunofluorescence staining

Cells plated onto poly-L-lysine coated coverslips were fixed in 3.7% paraformaldehyde and stained for c-Jun or c-Fos. Binding of primary antibodies was detected using either bovine anti-goat or goat anti-mouse (Life Technologies catalog #A11005) secondary antibodies conjugated to fluorophores. Cells were mounted on glass slides and viewed using an Olympus FV10i confocal microscope.

2.10. Chromatin Immunoprecipitation

For chromatin immunoprecipitation, the protocols of Cold Spring Harbor (CSH) laboratory [17] and that of Nelson et. al [18] were combined. Typically, HEK293 were cells seeded in 100 mm tissue culture dishes, and treated with either DMSO or TPA. The cell lysis, immunoprecipitation, and washing steps were done following CSH protocol, while the elution was done with Chelex-100 resin using the protocol of Nelson et. al. The eluted DNA was amplified by real-time PCR described above using primers flanking the putative AP-1 binding site (listed in Table 1), and analyzed using the Viil7 software. Controls for Histone H3, beads only, non-template DNA, and IgG were included. In order to compare the recruitment of AP-1 complex proteins to the LMTK2 promoter in DMSO and TPA treated samples, the fold enrichment of the ChIP-ed DNA was calculated for each of the antibodies (c-Jun, c-Fos) compared to their respective IgG controls. Histone was used as the positive control.

2.11. Statistical analysis

The data for Western Blot, RT-PCR, Luciferase reporter gene assay, and ChIP are plotted as mean ± SEM. The control and test groups were compared using unpaired Students’ t-test. The criterion for statistical significance was set at p < 0.05.

3. Results

3.1. The lmtk2 gene has a putative TPA responsive promoter

DNA sequences corresponding to the 5’ flanking region of the human lmtk2 coding region were obtained using Switchgear genomics and confirmed using Ensembl and UCSC genome browser. Confirmed sequences were analyzed for the presence of potential conserved transcription factor binding sites using Consite (consite.genereg.net), which predicted a consensus TPA response element (TRE), with the sequence GGTGAGTGCAGTGG at position −440, upstream of the transcription initiation site (Fig. 1a,b).

3.2. TPA regulates LMTK2 protein expression

TPA (Phorbol 12-myristate, 13-acetate) is a commonly used synthetic PKC activator. To evaluate whether TPA regulates endogenous LMTK2 expression, HEK293 cells were treated for 24 h with varying concentrations of TPA followed by evaluation of LMTK2 levels. The data obtained from densitometric analysis of protein levels on the Western blot were fit to a 3rd order polynomial curve fit using SigmaPlot software. The plotted line is a probability curve based on the analysis of the data. TPA exposure at low concentrations (10 nM and 50 nM) resulted in a significant increase (\(*p < 0.01\) for 10 nM and \(*p < 0.05\) for 50 nM) in LMTK2 protein levels, compared to DMSO control (Fig. 2a,b), with an EC50 of 5.6 nM. TPA concentrations higher than 100 nM did not result in further increases in LMTK2 protein levels but rather led to a biphasic dose-response with higher concentrations of TPA reversing the stimulatory effect (Fig. 2a,b) as seen with the highest TPA concentration tested (1000 nM). To confirm that the actions of TPA were mediated through activation of protein kinase C (PKC), rather than a direct impact of TPA on LMTK2 expression, we exposed HEK293 cells to varying concentrations of the biologically inactive TPA analogue 4α-phorbol 12,13-didecanoate (4α-TPA), which is unable to activate PKC (Fig. 2c,d). 4α-TPA (10 nM) did not elicit any increase in LMTK2 protein levels despite this concentration being effective for active TPA. 4α-TPA, at 50 nM, caused a slight decrease in LMTK2 protein levels, although this decrease was not statistically significant. Together, these data indicate that TPA modules LMTK2 expression at the protein level.

3.3. TPA response on LMTK2 protein expression in other cell lines

LMTK2 has been shown to regulate chloride conductance through CFTR in cystic fibrosis bronchial epithelial cells (CFBE) and endocytic trafficking and apoptosis in the cervical cancer cell line HeLa. Since these cells express endogenous LMTK2, we investigated the TPA response on LMTK2 protein expression in these cell lines as well. The cells were treated with the indicated concentrations of TPA for 24 h and LMTK2 protein levels quantified. The Western blots confirmed that CFBE and HeLa cells express basal level of LMTK2 endogenously. The data from the western blot were fit to a 3rd order polynomial curve fit using SigmaPlot software. In HeLa cells, TPA treatment resulted in significant increase in LMTK2 protein levels compared to vehicle control DMSO, after 24 h of treatment (Fig. 3a, b; \(*p < 0.05\) for difference from control). The EC50 was ~ 16 nM. Moreover, the response was biphasic, as seen in HEK293 cells. A similar response was also obtained for CFBE cells, where TPA significantly increased LMTK2 protein levels at 100 nM tested concentration (Fig. 3c, d; \(*p < 0.05\) for difference from control) with an EC50 value of 31.6 nM. There was no significant increase in LMTK2 protein levels beyond this concentration. In fact, at the highest concentration tested (1000 nM), LMTK2 protein levels were
20% lower than that of control. Taken together, the TPA response in both HeLa and CFBE cells followed the same pattern as seen in HEK293 cells. Thereafter, HEK293 cells were chosen to elucidate the mechanism of regulation of LMTK2 expression by TPA.

3.4. TPA increases LMTK2 mRNA transcript level but has no effect on mRNA stability

Observing the effect of TPA on LMTK2 protein expression, we determined if such effect was mediated by modulation of endogenous LMTK2 mRNA levels. HEK293 cells were treated with the indicated concentrations of TPA or 4α-TPA for 24 h, and LMTK2 mRNA levels were quantified by real time qPCR. We observed that increases in protein levels were accompanied by a concomitant increase in the mRNA levels of LMTK2 upon 10 nM and 50 nM TPA exposure (*p < 0.05, **p < 0.001 for difference from control; ^p < 0.05, ^^p < 0.01 for difference from corresponding 4α-TPA treatment) (Fig. 4a). Treatment with inactive phorbol ester showed no change in mRNA levels compared to DMSO treatment. However, although the increase in mRNA levels at 50 nM TPA was significantly higher than DMSO or 50 nM 4α-TPA treatment, it was less than that at 10 nM TPA (although the difference was not statistically significant). This could be due to post-transcriptional changes in mRNA stability, but elucidating the reason was beyond the scope of our study. For all studies, GAPDH was used as an internal control. There was no change in the Ct value of GAPDH for any of the treatments. Considering the significant increase in the protein as well as mRNA levels of LMTK2 at 10 nM TPA treatment, this concentration was chosen for all the other experiments. Since gene expression can be regulated both at the transcriptional as well as post-transcriptional level, we wanted to assess whether TPA affected...
LMTK2 transcription or mRNA stability. Cells were treated with the transcription inhibitor Actinomycin D (ACD; 5 μg/ml) with or without 10 nM TPA. Samples were collected for measuring mRNA levels at 6 and 8 h post treatment. Beyond 8 h of Actinomycin D treatment, there was marked cell death. The non-coding 18 S rRNA was used as the reference gene in this case. As observed previously, 10 nM TPA alone was marked cell death. The non-coding 18 S rRNA was used as the reference gene. Data, obtained from at least 3 independent experiments done in duplicates. **p < 0.01 for difference from 4α-TPA treatment alone; ***p < 0.001 for difference from DMSO treatment; n.s. not significant. 18 S rRNA was used as the reference gene. Data represented are mean ± SEM for 2 independent experiments. ▲▲▲ p < 0.001 for difference from control; ^p < 0.01 for difference from corresponding 4α-TPA treatment, n.s. not significant. LMTK2 mRNA was quantified by real time qPCR. Cells exposed to TPA showed a significant increase in LMTK2 mRNA compared to control or 4α-TPA (**p < 0.05, ***p < 0.001 for difference from control; p < 0.05, *p < 0.01 for difference from corresponding 4α-TPA treatment, n.s. not significant) for at least 3 independent experiments. GAPDH was used as the internal control.

3.6. **TPA response on LMTK2 expression is mediated by Protein kinase C**

Classical and novel isoforms of protein kinase C (PKC) are the intracellular effector molecules acting downstream of TPA. Hence, we determined if the effect of TPA on LMTK2 expression was indeed mediated by PKC. For this, firstly, we identified which PKC isozymes were expressed in HEK293 cells. We found positive expression for α and δ isozymes, which PKC isozymes mediate the effect of TPA on LMTK2 expression. The LMTK2 promoter construct, driving the expression of a luciferase reporter gene (Gaussia luciferase), was obtained from Genecopoeia. As an expression control and for data normalization, the promoter plasmid also expressed secretory alkaline phosphatase (SEAP) under the constitutive control of a CMV promoter. This plasmid was expressed stably in HEK293 cells. We found that TPA caused a significant increase in LMTK2 promoter activity. The LMTK2 promoter activity, as measured by luciferase expression (Fig. 5a), within a similar TPA concentration range up to 72 h did not further increase luciferase expression. Peak luciferase expression driven by the lmtk2 promoter increased up to 24 h, and was decreasing luciferase expression (Fig. 5a). We then performed a time-course analysis of the effect of TPA on LMTK2 promoter activity. Cells were exposed to either DMSO vehicle control or TPA (10 nM) for increasing periods of time (0–72 h). We observed that TPA specific gene expression driven by the lmtk2 promoter increased up to 24 h, and was followed by a reduction, such that at 40 h of exposure to TPA, luciferase expression had returned to basal levels (Fig. 5b). Continuing TPA exposure up to 72 h did not further increase luciferase expression. Peak TPA dependent promoter activity was observed at 19 h following TPA exposure. The normalized luciferase signal data were fit to a 3rd order polynomial curve fit using SigmaPlot software. The graph is a probability curve based on the analysis of the data. Thus, consistent with previous data, TPA caused a concomitant increase in LMTK2 promoter activity as well.

3.6. **TPA response on LMTK2 expression is mediated by Protein kinase C**

Since TPA modulates LMTK2 protein and mRNA levels, and the lmtk2 gene contains a putative TPA response element, we wanted to assess the effect of TPA on LMTK2 promoter activity. The LMTK2 promoter construct, driving the expression of a luciferase reporter gene (Gaussia luciferase), was obtained from Genecopoeia. As an expression control and for data normalization, the promoter plasmid also expressed secretory alkaline phosphatase (SEAP) under the constitutive control of a CMV promoter. This plasmid was expressed stably in HEK293 cells. We found that TPA caused a significant increase in luciferase expression (Fig. 5a), within a similar TPA concentration range up to 72 h did not further increase luciferase expression. Peak luciferase expression driven by the lmtk2 promoter increased up to 24 h, and was followed by a reduction, such that at 40 h of exposure to TPA, luciferase expression had returned to basal levels (Fig. 5b). Continuing TPA exposure up to 72 h did not further increase luciferase expression. Peak TPA dependent promoter activity was observed at 19 h following TPA exposure. The normalized luciferase signal data were fit to a 3rd order polynomial curve fit using SigmaPlot software. The graph is a probability curve based on the analysis of the data. Thus, consistent with previous data, TPA caused a concomitant increase in LMTK2 promoter activity as well.

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increase the lmtk2 promoter activity (Fig. 6d), although it did not abolish the stimulatory effect completely. Since Calphostin C is a competitive inhibitor of PKC, increasing TPA concentration abrogated the inhibitory effect of Calphostin C. We attempted to completely block activation of PKC, but increasing Calphostin C concentration beyond 100 nM resulted in significant cell death (data not shown). Thus, these data taken together suggest that TPA response on LMTK2 expression is mediated by PKC.

3.7. TPA increases the cellular expression and nuclear localization of the AP-1 transcription factor complex proteins

To further investigate the molecular mechanisms underlying the ability of TPA to increase LMTK2 protein production, we investigated the putative TPA response element (TRE) in the lmtk2 promoter, GGTGAGTCAGTG, for possible binding sites for transcription factor(s). We noticed that the sequence TGAGTCA serves as the consensus binding site for c-Fos protein of the AP-1 (Activator Protein 1) transcription factor (TF) complex. This seemed reasonable, given that AP-1 is transactivated by activated PKC, and TREs have been shown to be probable binding sites for AP-1 complex. Since c-Fos partners with c-Jun to form the active AP-1 complex, we evaluated the expression and subcellular distribution of these proteins upon exposure of HEK293 cells to TPA. Confocal immunofluorescence microscopy revealed an increase in the nuclear localization of both c-Fos and c-Jun in cells treated with TPA for 24 h compared to cells treated with DMSO vehicle control (Fig. 7a,b). Cells treated with the inactive phorbol, 4α-TPA, failed to show a nuclear translocation of c-Fos and c-Jun in response to treatment (Fig. 7a,b). Parallel biochemical analysis, utilizing subcellular fractionation, further confirmed the nuclear localization. Moreover, we also observed an increase in the total cellular expression of c-Fos and c-Jun in response to active TPA, but not to either vehicle control alone, or the inactive phorbol 4-TPA (Fig. 7c,d). Thus, our data are consistent with a marked redistribution of the AP-1 transcription complex proteins to the nucleus in response to TPA treatment.

3.8. TPA response on LMTK2 expression occurs through binding of the AP-1 complex to LMTK2 promoter

All cells have a basal level activity of the AP-1 TF complex, as it is required for normal cell growth and division. However, our data showing an increased nuclear localization of AP-1 complex proteins in response to TPA, also reflected a binding of the AP-1 transcription complex to the lmtk2 promoter, as the promoter contains a putative c-Fos binding site. Hence, HEK293 cells were pretreated with T-5224, a selective inhibitor of c-Fos/AP-1 binding to its target DNA sequence, before TPA treatment. T-5224 on its own had a slight but insignificant effect on lmtk2 promoter activity. However, T-5224 caused a marked inhibition of the TPA response, causing a 60% inhibition in TPA stimulated promoter activity (Fig. 8a). Since T-5224 is a competitive inhibitor of AP-1 binding to DNA, T-5224 should have no effect on AP-1 nuclear trafficking and localization. We confirmed this by checking the nuclear levels of c-Jun and c-Fos upon T-5224 pretreatment. TPA alone caused a marked increase of the AP-1 components, c-Fos, and c-Jun to the nuclear compartment. However, pre-incubation of cells with T-5224 prior to TPA exposure did not diminish c-Fos or c-Jun nuclear translocation (Fig. 8b, c). To further verify that the consensus AP-1 binding sequence TGAGTCA in the lmtk2 promoter was responsible for driving the expression of luciferase in response to TPA, this sequence was mutated (TGAGTCA → TttaTCA) using site-directed mutagenesis. This mutation alone caused a significant decrease in promoter activity (Fig. 8d; p < 0.05). Moreover, it completely abolished any TPA dependent increase in luciferase activity (Fig. 8d; p < 0.05). Finally, to determine if the AP-1 transcription complex bound to the lmtk2 promoter in response to TPA exposure, we performed chromatin immunoprecipitation assay (ChIP) using c-Jun and c-Fos antibodies. Upon
exposure of cells to TPA, there was a significant increase in the recruitment of the AP-1 complex proteins c-Jun and c-Fos to the lmtk2 promoter compared to DMSO treatment (Fig. 8e). However, the recruitment of c-Fos to the promoter was 4 times higher than that of c-Jun. Thus, TPA response on LMTK2 expression required binding of the AP-1 complex to the lmtk2 promoter.

3.9. Effect of cytokine GM-CSF on LMTK2 expression

GM-CSF or granulocyte macrophage colony stimulating factor is a cytokine well-characterized mostly in hematopoietic cells. It has been shown to stimulate diacylglycerol generation, leading to increases in PKC activity in leukemia cells [19]. Moreover, GM-CSF has been shown to increase c-Jun mRNA expression in leukemia cells [20]. Interestingly, HEK293 cells express GM-CSF receptor, and GM-CSF has been shown to promote cell survival in these cells [21]. Taking these observations together, we investigated whether GM-CSF mimics TPA response on LMTK2 expression. HEK293 cells were treated with increasing concentrations of GM-CSF for 24 h, and LMTK2 protein levels were quantified. Interestingly, there was no increase in LMTK2 expression upon GM-CSF treatment. In fact, at the highest concentration tested (10 nM), GM-CSF reduced LMTK2 protein levels by ~25% compared to control (Fig. 9a, b). There was no change in the expression of β-actin. From our previous experiments, we observed that there was increased nuclear localization of c-Jun and c-Fos upon TPA treatment, which ultimately led to an increase in LMTK2 expression. So, we checked the nuclear levels of c-Jun and c-Fos upon GM-CSF treatment. Interestingly, there was no significant change in the total or nuclear c-Jun levels in GM-CSF treated samples, compared to control treatment. Moreover, GM-CSF failed to induce c-Fos expression. Nuclear c-Fos was present only in TPA treated cells (Fig. 9c). This further confirms that transactivation and nuclear localization of c-Fos is a prerequisite for the increase in LMTK2 expression by TPA.

4. Discussion

The aim of our study was to determine if endogenous LMTK2 levels could be manipulated through transcriptional changes modulating the lmtk2 promoter. Phorbol esters are known to alter the levels of gene expression by activating protein kinase C [22]. We undertook an analysis of the upstream 5′ DNA sequence for the lmtk2 gene and identified a potential phorbol ester activation site. This 12-nucleotide sequence conformed to the consensus sequence for a TPA (PMA or Phorbol 12-myristate 13-acetate) response element, TRE [23–25]. Exposure of cells to the phorbol ester TPA resulted in an increase in LMTK2 promoter activity, with a concomitant increase in the mRNA and protein levels. The dose response to TPA was, however, biphasic, with higher TPA concentrations or prolonged exposure attenuating the increased expression of LMTK2. This likely reflects the fact that prolonged exposure of cells to high concentrations of phorbol esters leads to a down regulation of PKC activity [26,27]. That activation of PKC by TPA was required for the increased expression of LMTK2 was demonstrated by the observation that 4α-TPA, a TPA analogue unable to activate PKC, was without effect in increasing LMTK2 mRNA and protein levels. In fact, at 50 nM, 4α-TPA lightly decreased the protein and mRNA levels. This can be attributed to the fact that, being a structural analogue of diacylglycerol, the natural activator of PKC, both TPA and 4α-TPA compete for binding to the same domain on PKC. So, 4α-TPA at higher
concentrations slightly decreased the endogenous PKC activation.

The broad specificity PKC inhibitor Calphostin C also blocked the stimulatory actions of TPA on LMTK2 expression, consistent with PKC being the immediate target of TPA activity. However, multiple PKC isoforms are expressed in HEK293 cells, including classical, novel and atypical isoforms; but, in the present study, we were unable to determine which isoform(s) were efficacious in mediating the TPA response, due to the poor selectivity of PKC isoform inhibitors. Future studies utilizing PKC isoform specific shRNA constructs will likely resolve this issue.

To confirm that the actions of TPA on LMTK2 protein and mRNA levels were indeed mediated by the lmtk2 promoter, a construct containing the lmtk2 promoter driving a luciferase gene was obtained. A time-course study of luciferase expression also revealed a biphasic
response, with peak expression occurring around 19 h after TPA exposure. Again, this may reflect PKC downregulation due to prolonged exposure to TPA. Similar biphasic time-courses for TRE have also been reported for the neuropilin-1 promoter [28].

The downstream effector of TPA on promoter activity is the AP-1 transcriptional complex [25,29–31]. The AP-1 transcription factor is a dimeric complex that contains members of the Fos, Jun, ATF and MAF protein families. T-5224 is a selective inhibitor of c-Fos/AP-1 binding to its target DNA [32], and blocked the effects of TPA in increasing LMTK2 promoter activity, without changing the nuclear levels of the AP-1 complex proteins. This is consistent with TPA/PKC stimulation working on downstream binding of AP-1 to the TPA response element (TRE) in the lmtk2 promoter. Moreover, mutation of the TRE blocked the TPA response, again consistent with the notion that a TPA, PKC, AP-1 signaling cascade ultimately results in AP-1 binding to the TRE on lmtk2 promoter. AP-1 complex components normally traffic to the nucleus to bind to TRE sequences in response to PKC activation [29,30]. Our findings showed similar results. In addition, using ChIP assay, we demonstrated that c-Jun and c-Fos also bound to the lmtk2 promoter TRE.

However, there was less pulldown of bound chromatin with c-Jun than with c-Fos. Possible reasons for the difference between the amount of c-Jun and c-Fos pulled down could be differences in accessibility of epitopes to antibodies in an AP-1 complex, or differences in binding affinities between the two antibodies.

GMCSF is a known endogenous stimulator of PKC activity [19]. In contrast to TPA activation of PKC which elicited an increase in lmtk2 gene expression, GMCSF was unable to enhance lmtk2 transcription and translation. Indeed, at high concentrations, GMCSF slightly reduced LMTK2 protein levels. While the molecular mechanisms underlying the discrepancy between TPA and GMCSF actions on LMTK2 expression are not fully elucidated, it likely involves differences in activation of the AP-1 transcription complex. Thus, whereas TPA causes an increase in nuclear localization of both c-Jun and c-Fos, GMCSF only increases nuclear c-Jun, with little or no effect on c-Fos. It is, therefore, possible that GMCSF causes an incomplete activation of transcriptional complexes necessary for lmtk2 transcription. High concentrations of GMCSF may preferentially cause the nuclear translocation of monomeric c-Jun to the nucleus, which may well impede the ability of c-Jun in AP-1

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Fig. 8. TPA response on LMTK2 expression occurs through binding of AP-1 complex to LMTK2 promoter: a, LMTK2 promoter activity (normalized to CMV promoter activity) as observed in HEK293 cells treated with AP-1 binding inhibitor T-5224 in presence or absence or TPA. Data have been obtained from 3 independent experiments. *p < 0.05 for difference from TPA-treated cells by unpaired Students’ unpaired t-test. b, nuclear expression of c-Fos and c-Jun were determined upon T-5224 treatment in presence or absence of TPA. Lamin A was used as the nuclear marker. c, the protein expression from panel b are normalized to Lamin A, quantified and represented as mean ± SEM. d, TPA response on LMTK2 promoter activity in HEK293 cells expressing LMTK2 promoter construct with the putative TRE mutated (described in Methods). The LMTK2 promoter activity was normalized to CMV promoter activity. Data from 3 independent experiments are represented as mean ± SEM (*p < 0.05 for difference from TPA treatment, ^p < 0.05 for difference from DMSO treatment, and ^^p < 0.01 for difference from DMSO treatment by Students’ unpaired t-test). e, quantification of ChIP signal from qPCR analysis as a measure of the recruitment of c-Jun and c-Fos to LMTK2 promoter. The values are represented as fold enrichment over the respective IgG. Data from 3 independent experiments are represented as mean ± SEM. *p < 0.05 for difference from control (DMSO treated cells) by Students’ unpaired t-test.
complexes to activate gene transcription. GMCSF has been shown to be associated with cell survival and differentiation [19–21,33], whereas LMTK2 knockdown has been associated with increased cellular proliferation as well as apoptosis in different cell lines [7,9]. Future studies elucidating the precise physiological role of LMTK2 in cells and signaling mechanisms involved in downregulating endogenous LMTK2 levels will provide an explanation for the change in its expression in response to different external stimuli. Interestingly, TPA increased LMTK2 expression in CFBE cells. Previous studies have shown that LMTK2 negatively regulates chloride conductance mediated by CFTR in these cells [13]. Our preliminary finding in this regard would throw some light on the correlation between PKC activation and CFTR function in these cells. Moreover, LMTK2 downregulation has been shown to reduce the levels of pro-apoptotic Bcl-2 levels in HeLa cells [9]. We have shown that TPA increases LMTK2 levels in HeLa cells. Considering the fact that TPA is associated with cell cycle delay and antiproliferative activity in HeLa cells [34–36], our preliminary data suggest that LMTK2 might be involved in apoptotic pathway in HeLa cells. Further studies into the intracellular mechanisms regarding this would provide a more confirmatory result.

5. Conclusions

In summary, our present study has established lmtk2 as a TPA-response element containing gene, which is transcriptionally upregulated downstream of PKC activation in certain cell types. Moreover, it has also shown that the TPA response on LMTK2 expression occurs through transactivation of the AP-1 transcription factor complex, and binding of the transcription factor complex to the lmtk2 promoter to drive its expression. Thus, it is formally possible that levels of LMTK2 activity within a cell could be altered through manipulation of LMTK2 transcription in a therapeutically beneficial manner. Future studies involving the development of small molecule modulators of LMTK2 would evaluate the feasibility of pharmacological modulation of LMTK2 activity.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable

Availability of data and material
The data generated and analyzed during the current study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions

NAB and ID designed the experiments. ID performed the experiments. NAB and ID analyzed and interpreted the data. ID wrote the paper. NAB approved the final draft of the manuscript.

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Appendix A. Transparency document

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References

[1] S. Kawa, J. Fujimoto, T. Tezuka, T. Nakazawa, T. Yamamoto, Involvement of BREK, a serine/threonine kinase enriched in brain, in NGF signaling, Genes Cells 9 (2004) 219–232.
[2] H. Wang, D.L. Brautigan, Peptide microarray analysis of substrate specificity of the transmembrane Ser/Thr kinase KPI-2 reveals reactivity with cystic fibrosis transmembrane conductance regulator and phosphorylase, Mol. Cell Proteom. 5 (2006) 2124–2130.
[3] A. Nixon, Y. Jia, C. White, N.A. Bradbury, Determination of the membrane topology of lemur tyrosine kinase 2 (LMTK2) by fluorescence protease protection, American journal of physiology, Cell Physiol. 304 (2013) C164–C169.
[4] C. White, A. Nixon, N.A. Bradbury, Determining membrane protein topology using fluorescence protease protection (FPPI), J. Vis. Exp. (2015) e52509.
[5] M.V. Chibalina, M.N. Seaman, C.C. Miller, J. Kendrick-Jones, F. Buss, Myosin VI, and its interacting protein LMTK2 regulate tubule formation and transport to the endocytic recycling compartment, J. Cell Sci. 120 (2007) 4278–4288.
[6] T. Inoue, T. Kon, R. Okhura, H. Yamakawa, O. Ohara, J. Yokota, K. Sutoh, BREK/LMTK2 is a myosin VI-binding protein involved in endosomal membrane trafficking, Genes Cells 13 (2008) 483–495.
[7] K. Shah, N.A. Bradbury, Lemur Tyrosine Kinase 2, a novel target in prostate cancer therapy, Oncotarget 6 (2015) 14233–14246.
[8] E.C. Butler, N.A. Bradbury, Signal dependent ER export of lemur tyrosine kinase 2, BMC Cell Biol. 16 (2015) 26.
[9] A. Conti, M.T. Majorini, E. Fontanella, A. Bardelli, M. Giacca, D. Delia, M. Mano, D. Lecis, Lemur tyrosine kinase 2 (LMTK2) is a determinant of cell sensitivity to therapy, Oncotarget 6 (2015) 14279–14289.
[10] A. Conti, M.T. Majorini, E. Fontanella, A. Bardelli, M. Giacca, D. Delia, M. Mano, D. Lecis, Lemur tyrosine kinase 2 (LMTK2) is a determinant of cell sensitivity to therapy, J. Neurochem. 121 (2012) 343–348.
[11] J.A. Picciano, N. Ameen, B.D. Grant, N.A. Bradbury, Eme-1 regulates the recycling of the cystic fibrosis transmembrane conductance regulator, Am. J. Physiol. Cell Physiol. 285 (2003) C1009–C1018.
[12] M.R. Silvius, Bertrand, C.A. Ameen, N. Golin-Bisello, F. Butterworth, M.B. Frizzell, R.A. Bradbury, N.A. Bradbury, Rab11h regulates the apical recycling of the cystic fibrosis transmembrane conductance regulator in polarized intestinal epithelial cells, Mol. Biol. Cell 20 (2009) 2373–2350.
[13] S. Luz, R.M. Cihl, D.L. Brautigan, M.D. Amaral, C.M. Farinha, A. Sviatecka-Urban, LMTK2-mediated phosphorylation regulates CFTR endocytosis in human airway epithelial cells, J. Biol. Chem. 289 (2014) 15080–15090.
[14] N.A. Bradbury, R.J. Bridges, Y. Jia, Dependence of CFTR trafficking on BREK/LMTK2 kinase in human colon and mouse airway epithelial cells, Pediatr. Pulmonol. (2011).
[15] L.W. Harries, J.R. Perry, P. McCullagh, M. Crundwell, Alterations in LMTK2, MSMB and HNF1ßb gene expression are associated with the development of prostate cancer, BMC Cancer 10 (2010) 315.
[16] K. Suzuki, P. Bose, R.Y. Leong-Quong, D.J. Fujita, K. Riabowol, REAP: a two minute cell fractionation method, BMC Res Notes 3 (2010) 294.
[17] M.F. Carey, C.L. Peterson, S.T. Smale, Chromatin immunoprecipitation (ChIP), Cold Spring Harb. Protoc. 2009 (2009) pdb prot5279.
[18] J.D. Nelson, O. Denisenko, K. Bomszytk, Protocol for the fast chromatin immunoprecipitation (ChIP) method, Nat. Protoc. 1 (2006) 179–185.
[19] M. Nishimura, K. Kaku, V. Azano, K. Okatui, Y. Inoue, T. Kaneko, Stimulation of phosphoinositid turnover and protein kinase C activation by granulocyte-macrophage colony-stimulating factor in HL-60 cells, Blood 80 (1992) 1045–1051.
[20] S.E. Adunyah, T.M. Unlap, F. Wagner, A.S. Kraft, Regulation of e-cadherin expression and AP-1 enhancer activity by granulocyte-macrophage colony-stimulating factor, J. Biol. Chem. 266 (1991) 5670–5675.
[21] A. Zambrano, E. Jara, P. Murgas, C. Jara, M.A. Castro, C. Angulo, I. Concha, Cytokine stimulation promotes increased glucose uptake via translocation at the plasma membrane of GLUT1 in HEK293 cells, J. Cell Biochem. 110 (2010) 1471–1480.
[22] M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, Y. Nishizuka, Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters, J. Biol. Chem. 257 (1982) 7847–7851.
[23] K.K. Park, E. Jung, S.K. Chon, M. Seo, H.W. Kim, T. Park, Finding of TRE (TPA responsive element) in the sequence of human tauine transporter promoter, Adv. Exp. Med Biol. 526 (2003) 159–166.
[24] H. Knudsen, T. Olesen, A. Riccio, P. Ungaro, L. Christensen, P.A. Andreasen, A common response element mediates differential effects of phorbol esters and forskolin on type-1 plasmogen activator inhibitor gene expression in human breast carcinoma cells, Eur. J. Biochem. 220 (1994) 63–74.
[25] P. Angel, M. Imagawa, R. Chiu, B. Stein, R.J. Imbra, H.J. Rahmshdorf, C. Jonat, P. Hertzlich, M. Karin, Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor, Cell 49 (1987) 729–739.
[26] J.L. Sanders, P.H. Stern, Expression and phorbol ester-induced down-regulation of protein kinase C isozymes in osteoblasts, J. Bone Miner. Res 11 (1996) 1862–1872.
[27] B.L. Webb, S.J. Hirst, M.A. Giembycz, Protein kinase C isoforms: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis, Br. J. Pharmacol. 130 (2000) 1433–1452.
[28] M. Rossignol, J. Pouyssegur, M. Klagsbrun, Characterization of the neuropilin-1 promoter; gene expression is mediated by the transcription factor Sp1, J. Cell Biochem. 88 (2003) 77–87.
[29] R. Eferl, E.F. Wagner, AP-1: a double-edged sword in tumorigenesis, Nat. Rev. Cancer 3 (2003) 859–868.
[30] E. Shaulian, M. Karin, AP-1 as a regulator of cell life and death, Nat. Cell Biol. 4 (2002) E131–E136.
[31] W. Lee, A. Haslinger, M. Karin, R. Tjian, Activation of transcription by two factors that bind promoter and enhance sequences of the human metallothionein gene and SV40, Nature 325 (1987) 368–372.
[32] K. Tsujihara, H. Chaki, T. Takahara, H. Kobota, T. Tanaka, Y. Aikawa, S. Shizawa, S. Hirose, Discovery of nonpeptidic small-molecule AP-1 inhibitors: lead hopping based on a three-dimensional pharmacophore model, J. Med Chem. 49 (2006) 80–91.
[33] C.S. Park, Y.S. Chi, S.Y. Kii, S.H. Moon, S.W. Jeong, S.T. Uh, Y.H. Kim, Granulocyte macrophage colony-stimulating factor is the main cytokine enhancing survival of eosinophils in asthmatic airways, Eur. Respir. J. 12 (1998) 872–878.
[34] A. Banu, B.A. Teicher, J.S. Lazo, Involvement of protein kinase C in phorbol ester-induced sensitization of HeLa cells to cis-diaminedichloroplatinum(II), J. Biol. Chem. 265 (1990) 8451–8457.
[35] V. Kinzel, J. Richards, M. Storh, Tumor promoter TPA mimics irradiation effects on the cell cycle of HeLa cells, Science 210 (1980) 429–431.
[36] V. Kinzel, G. Bonheim, J. Richards, Phorbol ester-induced G2 delay in HeLa cells analyzed by time lapse photography, Cancer Res. 48 (1988) 1759–1762.