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Short Communication

Autoregulation of the human splice factor kinase CLK1 through exon skipping and intron retention

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ABSTRACT

Alternative splicing is a key process required for the regulation of gene expression in normal development and physiology. It is regulated by splice factors whose activities are in turn regulated by splice factor kinases and phosphatases. The CDC-like protein kinases are a widespread family of splice factor kinases involved in normal physiology and in several diseases including cancer. In humans they include the *CLK1, CLK2, CLK3* and *CLK4* genes. The expression of *CLK1* is regulated through alternative splicing producing both full-length catalytically active and truncated catalytically inactive isoforms, CLK^{T1} (arising from exon 4 skipping) and CLK^{T2} (arising from intron 4 retention). We examined *CLK1* alternative splicing in a range of cancer cell lines, and report widespread and highly variable rates of exon 4 skipping and intron 4 retention. We also examined the effect of severe environmental stress including heat shock, osmotic shock, and exposure to the alkaloid drug harmine on *CLK1* alternative splicing in DU145 prostate cancer cells. All treatments rapidly reduced exon 4 skipping and intron 4 retention, shifting the balance towards full-length CLK1 expression. We also found that the inhibition of CLK1 with the benzothiazole TG003 reduced exon 4 skipping and intron 4 retention suggesting an auto-regulatory mechanism. CLK1 inhibition with TG003 also resulted in modified alternative splicing of five cancer-associated genes.

1. Introduction

The CLK (CDC-2 like) protein kinases phosphorylate splice factors and contribute significantly to the regulation of alternative splicing. The *CLK1* gene was first identified in 1991 through a homology probing approach (Johnson and Smith, 1991). The full-length protein it encodes is 484 amino-acids long, of which the first 130 constitute a regulatory region required for the interaction of CLK1 with SR proteins (splice factors with a serine/arginine-rich domain). The remaining amino-acids form the catalytic domain containing the conserved signature EHLAMMERILG (Nayler et al., 1997; Menegay et al., 2000). There are four members of the *CLK* gene family in humans (*CLK1–4*); of these, *CLK1* and *CLK4* are most closely related. *CLK1*, *CLK2* and *CLK4* are ubiquitously expressed (Nayler et al., 1997), whereas *CLK3* expression is most prominent in spermatozoa (Menegay et al., 1999). The developmental and physiological functions of the four members of the *CLK* family are beginning to emerge. CLK2 is involved in the control of diet-induced thermogenesis in brown adipose tissue (Hatting et al., 2017) and is a suppressor of hepatic gluconeogenesis (Rodgers et al., 2010). CLK1 is required for cell cycle progression (Dominguez et al., 2016), and CLK1, CLK2 and CLK4 help regulate the aurora B-dependent abscission checkpoint during mitosis (Petsalaki and Zachos, 2016). CLK kinases phosphorylate the so-called 'SR protein' splice factors, facilitating their activation and release from nuclear speckles (Naro and Sette, 2013). However CLK kinases can also phosphorylate non-SR protein splice factors including the spliceosome-associated SPF45 (Liu et al., 2013).

CLK activity is increasingly associated with the development and progression of cancer (Naro and Sette, 2013; Corkery et al., 2015; Czubaty and Piekiełko-Witkowska, 2017). As a result there is considerable interest in developing selective CLK inhibitors that block tumour growth (Schmitt et al., 2014; ElHady et al., 2017; Murár et al., 2017; Sun et al., 2017; Riggs et al., 2017; Walter et al., 2017). CLK1 is also a potential target in the treatment of Alzheimer's (Jain et al., 2014) and has been earmarked for the treatment of Duchenne's muscular dystrophy as its inhibition causes the skipping of a mutated exon (Ogawa and Hagiwara, 2012; Sako et al., 2017). CLK targeting has also been proposed for the treatment of viral infection including HIV-1 (Wong et al., 2011) and influenza (Karlas et al., 2010; Zu et al., 2015).

Abbreviations list: ESE, exonic splice enhancer; ISS, intronic splice silencer

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The human *CLK1* gene is comprised of 13 exons, and is alternatively spliced expressing either a full length or truncated protein that lacks the catalytic domain (Duncan et al., 1997; Menegay et al., 2000). There are at least two alternative splicing mechanisms that result in the expression of truncated CLK1: skipping of exon 4 (Duncan et al., 1997) and intron retention, the latter reported in the mouse *Clk1* gene (Ninomiya et al., 2011). Most tissues express both full length and truncated CLK1, but their balance is altered following exposure to environmental stresses such as heat or osmotic shock (Menegay et al., 2000; Ninomiya et al., 2011; Corkery et al., 2015). There is a need to understand how *CLK1* expression, including its alternative splicing, changes and is regulated in normal development and disease. We examined the alternative splicing of human *CLK1* in a range of cancer cell lines, in response to environmental stress, and in response to CLK1 inhibition with TG003.

2. Materials and methods

2.1. Cell lines, cell culture and treatments

Prostate cancer cell lines (androgen independent PC3 and DU145, androgen dependent VCaP, and normal prostate epithelium PNT2) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5 mM glucose and L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml streptomycin and 100 μg/ml penicillin and 1% sodium pyruvate. Leukemic cell lines CMK (acute megakaryocytic leukemia); MOLT4 (acute lymphoblastic leukemia); RPKV8226 (myeloma); K562 (chronic myelogenous leukemia); HL60 (promyelocytic leukemia); and TK6 (hereditary spherocytosis) were cultured in RPMI medium with the same additives. All cell culture reagents were obtained from Sigma-Aldrich.

The alkaloid harmine and the CLK1 inhibitor benzothiazole TG003 were obtained from Sigma-Aldrich. TG003 stock solution was prepared in dimethylsulfoxide (DMSO) and harmine was dissolved in 50% ethanol. 1×10^6 DU145 PCa cells were treated with varying concentrations (10 nM–100 μM) of TG003 and 1–5 μM harmine for 48 h. For heat shock treatments, DU145 cells were incubated for one, three or 6 h at 42 °C in a tissue culture incubator (Orbital, S150 Stuart). Osmotic stress was induced by treatment with 200 mM or 400 mM sorbitol (Sigma-Aldrich) for 3 h.

2.2. RNA extraction and cDNA synthesis

RNA extraction was performed using the RNA miniprep kit from Agilent Technology Ltd., USA as per manufacturer's instructions. RNA quality was verified on 1% (w/v) agarose gels and the concentration and purity was determined using Nanodrop (Spectrophotometric). cDNA synthesis was performed using reverse transcriptase and a mixture of oligo (dT) and random primers as per manufacturer's instructions (Promega). 1 μg RNA was mixed with 0.5 μl of the oligo and 0.5 μl random primers provided by the kit in a volume of 5.0 μl with nuclease free water. The samples were placed in a heat block at 70 °C for 5 min, then chilled on ice for 5 min. To each 5 μl RNA sample, 15 μl of reverse transcription reaction mix was added. Primers were allowed to anneal at 25 °C for 5 min, cDNA synthesis proceeded at 42 °C for 1 h and finally the reverse transcriptase was inactivated at 70 °C for 15 min.

2.3. Polymerase chain reaction

cDNA was amplified with the GoTaq kit (Promega) as per manufacturer's instructions using the *CLK1* primers (*E3F1* CAAGGATGTGA ACCTGGACATCGC and *E4F1* GGAGGTCACCTGATCTGTCAG forward primers in exons 3 and 4 respectively, and reverse primers *E5R1* CTC CTTCACCTAAAGTATCAAC and *E5R2* CTGCTACATGTCTACCTCCCGC in exon 5, as well as *β-actin* forward TTAAGGAGAAGCTGT and reverse GTTGAAGGTAGTTTCGTGGAT to verify cDNA synthesis; all sequences shown are 5′-3′). PCR cycles were as follows: 95 °C for 2 min (initial denaturation), then 95 °C for 1 min. (denaturation), 59 °C for 1 min. (annealing), 72 °C for 1.5 min. (extension); cycles were repeated 34 times followed by a final extension step at 72 °C for 5 min.

2.4. Agarose gel electrophoresis

20 μl aliquots of amplified PCR products were run in 2% (w/v) agarose gel at 95 V and 400AMP for 1 h. Agarose gels were stained in 0.2 mg/ml ethidium bromide (Sigma-Aldrich). Gel images were obtained using UV transluminator (Fisher Scientific).

2.5. Statistical analysis

All statistical tests were performed using GraphPad Prism 7.03. Mann-Whitney *t*-tests were used to compare significance between two groups. For multiple comparisons, one-way ANOVAs with Kruskal-Wallis test were performed. For all graphs, n.s. denotes not significant, $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$, $^{***} = p \leq 0.001$.

3. Results

3.1. Features of CLK1's exon 4 and intron 4

We began by examining the exon/intron structure of the human *CLK1* gene, focusing on exon 4 and surrounding intronic sequence (Fig. 1). The structure of the human *CLK1* gene mirrors closely that of the corresponding mouse gene, with a short intron separating exons 4 and 5 in both species and very high sequence conservation in exon 4 and flanking intron. Skipping of exon 4 has previously been reported in the human *CLK1* gene (Duncan et al., 1997), and both exon 4 skipping and intron 4 retention has been reported in the mouse gene (Ninomiya et al., 2011). Exon 4 skipping results in a premature stop codon interrupting the amino-terminal regulatory domain, resulting in a truncated 120 amino-acid previously named $CLK1^{T}$ (Duncan et al., 1997). Intron 4 retention also creates a premature stop codon and a truncated CLK1, albeit a little longer (160 amino-acids). We propose to call the former CLK^{T1} (arising from exon 4 skipping) and the latter $CLK1^{T2}$ (due to intron 4 retention, Fig. 1A and B).

We analysed the exon 4 sequence on the RBPmap and SROOGLE servers (Paz et al., 2014; Schwartz et al., 2009) and noted a number of interesting features (Fig. 1C). Firstly, there is a weak pyrimidine tract with three purines preceding exon 4 (…*CUUGACGUUUCCAG*, consensus Y_{11}NCAG, where Y is U or C). The 5′ splice site at the end of exon 4 is also not especially strong, GAT*guauag* compared to the consensus MAG*guragu* where M is A or C and R is A or G. This suggests that the baseline efficiency of exon 4 recognition and splicing is likely to be low. In contrast the end of intron 4 possesses a better pyrimidine tract (…*UUCUCUCUUUUAG*). This suggests that more efficient exon 4 inclusion may require the action of specific splice factors acting through exonic splice enhancers (ESEs). Using the SROOGLE server (sroogle.tau.ac.il) we identified the presence of putative ESEs particularly at the beginning of exon 4 (Zhang and Chasin, 2004); these correspond to potential binding sites for splice factors SRSF1 and SRSF2, (Schwartz et al., 2008). Additional potential splice factor binding sites were identified using RBPmap (Paz et al., 2014), ESE finder (Cartegni et al., 2003) and SpliceAid (Piva et al., 2009). Exon 4 appears particularly dense with potential SRSF1 binding sites (Fig. 1C). Through SROOGLE we also noted the presence of a potential intronic splice silencer (ISS) immediately following the 5′ splice site at the end of exon 4.

3.2. Alternative splicing of CLK1 in cancer cell lines

Based on the reported skipping of exon 4 and retention of intron 4 in mouse embryo fibroblast-like NIH-3 T3 cells (Ninomiya et al., 2011) we designed human-specific primers for RT-PCR including a forward

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