Differential Regulation of Serum- and Glucocorticoid-Inducible Kinase 1 (SGK1) Splice Variants Based on Alternative Initiation of Transcription

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Key Words

SGK1 • Alternative splicing • Alternative promoter sites • Hypoxia • Cellular differentiation • Cancer

Abstract

The serum- and glucocorticoid-inducible kinase 1 (SGK1) is a key-regulator of transport, cell volume and cell survival. SGK1 transcription is under genomic control of a wide variety of hormones and cell stressors. Little is known, however, about sequence variation in SGK1 transcripts. Thus, we took an *in silico* approach to determine sequence variations in the N-terminal region of SGK1, which is considered particularly important for subcellular SGK1 localization. Expressed Sequence Tag analysis revealed two novel phylogenetically highly conserved SGK1 mRNAs with different promoter sites based on alternative initiation of transcription at -2981, -850 upstream of the transcription initiation site (+1) of the reference mRNA. RT-PCR in various human cell lines and tissues confirmed the expression of the 3 alternative splice variants, which differed exclusively in their first exons. The two novel variants were devoid of the

localization and degradation signal with otherwise unchanged and intact open reading frames. Spatial distribution of transcription factor binding sites among the three promoter sites indicated common responsiveness to glucocorticoids but different responsiveness to hypoxia and cellular differentiation. Differential expression under those conditions was confirmed for all variants in cultured myoblasts and myotubes. p53 and ETF-1 binding sites were overrepresented at the promoter site of the reference sequence variant SGK1(+1). Transcript levels were 4.1-fold [SGK1(+1)] and 3.1-fold [SGK1(-850)] higher in renal clear cell carcinoma than in remote tissue. The transcript levels were 42-fold [SGK1(+1)], 26-fold [SGK1(-850)] and 17-fold [SGK1(-2981)] higher in highly malignant human glioma cells than in non-neoplastic brain tissue. SGK1 transcript levels were differentially increased by differentiation or hypoxia (treatment with CoCl₂). In conclusion, the present observations disclose the transcription of three distinct SGK1 splice variants, which are all markedly upregulated in tumor tissue but differentially upregulated following differentiation or hypoxia.

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Introduction

The serum- and glucocorticoid-inducible kinase 1 (SGK1) was originally cloned as a gene transcriptionally stimulated by serum and glucocorticoids [1] but later shown to be similarly regulated by a variety of further hormones including mineralocorticoids [2-11], gonadotropins [12-16], 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) [17], transforming growth factor β (TGF β) [18-20], interleukin 6 [21], fibroblast and platelet-derived growth factor [22], thrombin [23], endothelin [24], and other cytokines [25-27].

Moreover, SGK1 transcription is regulated by cell volume [28, 29], and stimulated by excessive glucose concentrations [19, 30], heat shock, UV radiation and oxidative stress [31].

SGK1 is activated by insulin [32] through a signaling cascade involving PI3 kinase and PDK1 [32-34].

SGK1 participates in the regulation of a wide variety of functions including channel activity and transport [35]. Specifically, SGK1 stimulates the epithelial Na⁺ channel ENaC [4, 10, 19, 36-44], the K⁺ channels ROMK [45-47] and KCNE1/KCNQ1 [48, 49], the epithelial Ca²⁺ channel TRPV5 [50, 51], the Na⁺/H⁺ exchanger NHE3 [52, 53], the Na⁺, K⁺, 2Cl⁻ cotransporter NKCC2 [19] and the Na⁺/K⁺ATPase [54-56].

Moreover, SGK1 regulates a variety of nutrient transporters including the glucose transporters SGLT1 [57], GLUT1 [58], and GLUT4 [59], the amino acid transporters ASCT2 (SLC 1A5) [60], SN1 [61], and EAAT1 [62], EAAT2 [63], EAAT3 [64], EAAT4 [65] and EAAT5 [66], the Na⁺-coupled dicarboxylate transporter NaDC [67] and the Creatine transporter CreaT [68].

SGK1 is further involved in the regulation of gene expression. The kinase associates with and phosphorylates the cAMP responsive element binding protein (CREB) and thus interferes with CREB-dependent gene transcription [69]. Moreover, SGK1 enhances the activity of the nuclear factor kappa B (NF κ B) [70] by association with and activation of I κ B kinase beta (IKK β) [70]. SGK1 enhances IKK β dependent phosphorylation I κ B α , leading to I κ B α degradation and thus activation of NF κ B [70] (Fig. 2). Beyond that SGK1 phosphorylates the forkhead transcription factor FKHR-L1 (FOXO3a) thus inhibiting FKHR-L1-dependent transcription, cell cycle arrest and apoptosis [71].

In view of the diverse functions of SGK1 the question arises, whether all functions are served by a unique protein or whether distinct splice variants of the SGK1 gene could be identified. Those splice variants may dif-

fer in subcellular localization and functional significance. Along those lines SGK1 has been localized in several subcellular compartments [72]. Moreover, stimulus dependent translocation of SGK1 from cytoplasm to nucleus has previously been observed [31, 34, 73, 74]. The mechanisms underlying targeting of the mature protein to mitochondria, endoplasmatic reticulum, cytoplasm or even plasma membrane [75] remain a matter of controversy. Most recent evidence points to a role of the N-terminus in subcellular SGK1 localization but remains conflicting as to the targeting of the mature protein, which may localize to the mitochondria [72, 76] or the endoplasmic reticulum [77, 78]. The very N-terminal region of SGK1 coded by the first exon was not only shown to be associated with localization but postulated to account for rapid turnover and degradation of the protein [76-78]. Moreover, the N-terminal region distinguishes between the isoforms SGK1, SGK2 and SGK3 and has just recently been implicated to be essential for isoform specificity of physiological functions [79].

The above mentioned studies dealing with the importance of the N-terminus for localization, protein instability or distinctiveness in physiological function are based on the assumption that there is just one N-terminal sequence of SGK1 that has been derived from the protein reference sequence (Swiss-Prot entry: O00141) and / or the related nucleotide reference sequence (GenBank: NM_005627.2).

In view of the existing evidence, we hypothesized that variation in the N-terminal sequence of SGK1 derived from alternative splicing or alternative initiation of transcription may contribute to a not yet understood versatility of localization, regulation of expression and physiological function. We took an *in silico* approach followed by experimental verification to study sequence variation in the N-terminal region of SGK1.

Material and Methods

Primary Expressed Sequence Tag analyses

Expressed Sequence tag (EST) analyses were done and graphic results were generated using the Genome Browser and its integrated bioinformatics software tools [80]. Alignment of ESTs at the SGK1 gene locus was done on the Human May. 2004 assembly and the ESTs released until 12/06 in GenBank [81] using the *BLAT*-algorithm [82]. Cross-validation of likelihood of existence and functional relevance of ESTs found in a region < 10kb upstream of the transcription starting site of the reference sequence of SGK1 (NM_005627.2) was done by the following criteria using the cited software, procedure or database information:

1. >1 EST can be found for a specific human gene locus [81]
2. Occurrence of typical splice donors and splice acceptors [82]
3. Sequence overlap with NM_005627.2 [82]
4. Significant transcriptional evidence based on the results of two tiling array studies [83, 84]
5. Conservation of EST expression across tissues [81]
6. Significant phylogenetic conservation of ESTs across 17 species [85]
7. Presence of eukariotically conserved transcription factor binding sites within 500bp around the 5' start of respective ESTs [86]
8. Presence of a CpG-island within 100bp of the ESTs [87]
9. Candidates fulfilling 7 of the 8 criteria were considered to possess a very high likelihood of being alternative splice variants that contribute to N-terminal sequence variation of SGK1.

Secondary in silico analysis of alternative splice candidates and their promoter region

EST candidates that emerged from primary EST analysis served to derive the potential SGK1 mRNA variants SGK1(-850) and SGK1(-2981) that contribute to N-terminal sequence variation in concert with the reference mRNA SGK1(+1). Alignment, test for open reading frame integrity and in silico translation of the three variants was done using GeneDoc 2.0.1 Multiple Sequence and Alignment Editor.

Analysis of the preferential cellular localization of SGK1(+1), SGK1(-850) and SGK1(-2981) was done with pTarget [88] and Psort II in conjunction with iPSort [89]. The region 450bp up and 50bp downstream of the respective transcription initiation sites was screened for human transcription factor binding sites with PROMO [90] and the results cross-validated with pMATCH [91]. For each of the three promoter sites we determined a list of factors that were overrepresented at each of the sites in comparison to the other two sites. The TRANSFAC transcription factor accession numbers were converted into SwissProt IDs with TRANSFAC [86]. The three lists were tested for enrichment of Gene Ontology (GO) categories with the hypergeometric test against a reference list of all human transcription factors listed in PROMO in the WEB-based GENE SeT Analysis Toolkit (WebGestalt) [92].

Validation of differential expression and full length ORF expression of SGK1(+1), SGK1(-850) and SGK1(-2981) by RT-PCR

Full length ORF expression of SGK1(+1), SGK1(-850) and SGK1(-2981) was determined using the variant specific sense primers SGK54for, SGK93for, SGK-703for, SGK-718for, SGK-2830 and the common antisense primer SGK2314rev. The numbers of the respective primers indicate their 5' start base in relation to the reference sequence of the SGK1 mRNA (NM_005627.2) and the location of the primers relative to the new variants and the reference is shown in Fig. 2b.

Quantitative real-time RT-PCR

500 ng of every RNA sample was reverse transcribed with 100 U of Superscript II RNase H - Reverse (Invitrogen), using 500 ng random hexamer primer according to manufacturer's

instructions. The RNA isolated from tumour tissue specimens as well as the respective benign tissue samples were all reverse transcribed in the same run.

Both sense and antisense primers used for real-time RT-PCR were chosen to be specific for the respective mRNA variants SGK1(+1), SGK1(-850) and SGK1(-2981) by using intron spanning internal to the antisense primer sequences and specific sense primers for the first exon that differs between the three variants (see Fig. 3a for illustration).

All real-time RT-PCR assays were run using HotStarTaq-Polymerase (Qiagen) and SYBR-Green (Molecular Probes) in a 1/30.000 dilution as described previously [93]. Reactions were carried out on an iCycler (Biorad). Samples were run in triplicates with a final volume of 15 µl per single reaction. The cycling conditions comprised polymerase activation at 95°C for 15 min, and 40 cycles with denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s. Fluorescence was measured during the 72°C step. Formation of the expected PCR product was confirmed by melting curve analysis and agarose gel electrophoresis. Amplification efficiencies of corresponding primer sets were estimated by generating standard calibration curves with twofold dilution series using the Relative Expression Software Tool Rest-XL [94]. Fold differences of Mean Normalized Expression (MNE) values that reflect relative expression of a target to a house keeping gene were calculated as previously described [95].

Tissue culture experiments and RNA isolation for the analysis of stimulus dependent differential expression

Primary skeletal muscle cells were grown from satellite cells as described earlier [96]. All experiments were performed on the first-pass of subcultured cells that were plated at ~5 x 10⁴ cells in 60 cm² dishes in a 1:1 mixture of α-MEM and Ham's F-12 supplemented with 20% fetal bovine serum (FBS), 1 % chicken embryo extract and 0.2 % antibiotic antimycotic solution. When myoblasts reached 80 – 90 % confluence, the cells were fused for the indicated time points in α-MEM containing 5.5 mM glucose with 2 % FBS and 0.2 % antibiotic antimycotic solution. After 6 days, myotubes were stimulated with 0.2 mM CoCl₂ for the indicated time points. Total RNA was isolated with the RNeasy Kit (Qiagen, Hilden, Germany).

Expression of SGK1(+1), SGK1(-850) and SGK1(-2981) in normal control or neoplastic tissue

All 11 investigated human brain tumors were removed in the Department of Neurosurgery of the University of Tuebingen and neuropathologically diagnosed in the Institute of Brain Research according to the criteria of the WHO classification for human brain tumors. For quantitative analysis with RT-PCR 2 diffuse astrocytomas WHO grade II, 1 anaplastic astrocytoma WHO grade III and 4 glioblastomas WHO grade IV were included into the group of malignant astroglomas. Within 20 minutes after surgical removal, small fragments of the tumors remained unfixed and were frozen in liquid nitrogen. In addition, 6 non-neoplastic brain tissue specimens were obtained from autopsy cases which died of non-CNS diseases.

Tumour tissue obtained from 10 renal clear cell cancer patients was pairwise compared with corresponding

Table 1. Distribution of the most frequent human transcription factor binding sites among the SGK1 promotor loci.

Transcription Factor	Entry	+1	-850	-2981	Remark
Glucocorticoid receptor (GR)	P04150	29	51	45	Known to regulate SGK1
General transcription factor II-I	P78347	21	26	26	General transcription factor
CCAAT/enhancer binding protein beta (C/EBP beta)	P17676	4	18	17	Important for immune and inflammatory response
Transcription factor E2F1	Q01094	26	8	1	Active in mid-to-late G1 phase
Cellular tumor antigen (p53)	P04637	18	11	1	Antiapoptotic
Forkhead box protein P3 (FOXOP3)	Q9BZS1	6	10	14	Known to be phosphorylated by SGK1 -> cell survival
C-ets-1 protein (p54)	P14921	3	14	10	Protooncogene
Suppressor of cytokine signaling 6	Q14765	3	14	10	Upregulated in cancers
X box binding protein -1 (XBP-1)	P17861	2	7	14	Activates unfolded protein response genes during ER stress
Transcription factor AP-2 alpha (AP2-alpha)	P05549	9	4	4	Developmentally regulated activator of transcription

histologically benign peritumoral renal parenchyma from the same patients, respectively (remote). Additionally, cells from the commercially available glioma cell lines LN229, LN308, A172 and a SV40 immortalized fetal human astrocyte cell line (FHAS-SV40) were analysed in RT-PCR experiments. Total RNA was isolated from 50 mg of tissue or ~105 tissue culture cells by Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. All samples were subjected to treatment with RNase free DNase I (Boehringer, Mannheim, Germany). The integrity of the purified RNA was verified by agarose gel electrophoresis, followed by ethidium bromide staining. OD260/OD280 nm absorption ratio was >1.95.

Quantitative real time RT- PCR

The sequences of the PCR primers used in this study are listed in table 1. Primer design was done with the software Primer357 and Primer Premier 5 (PREMIER Biosoft International). All primers were selected to reveal an equal annealing temperature of about 60°C. They were synthesized by MWG (Ebersberg, Germany). For quantitative analysis by RT-PCR ubiquitin B precursor (UBB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as housekeeping genes using the primers (all given 5' to 3') UBB_for TTC AGT CAT GGC ATT CGC AG, UBB_rev CTT AAG TTG GGG CAA ATG GC, GAPDH_for TCA ACA GCG ACA CCC ACT CC, GAPDH_rev TGA GGT CCA CCA CCC TGT TG. As a positive control for effective stimulation of differentiation and for the induction of hypoxia the following primers served to measure the expression of the immediate early response gene EGR1: EGR1_for GGC TTA TAA ACA CAT TGA ATG CG, EGR1_rev ACA CCA CAT ATC CCA TGG GC. Specificity of all primer pairs was verified by controlling amplicon size as determined by gel electrophoresis and melting curve analysis.

Real-time PCR data processing and statistical analysis

For quantitative expression analysis Mean Normalized Expressions (MNEs) of the target genes relative to UBB or GAPDH as Housekeeping genes were calculated. MNEs are linear expression values of a target gene relative to a reference gene, calculated from the average CT value of the three repli-

cates for the target and for the reference gene. Despite of the fact that the MNEs were calculated from the logarithmic scaled raw data they offer the possibility to calculate standard errors and confidence intervals (CIs) for the MNE as well as for derived fold-difference values, which reflect measuring variability [95]. For the tissue culture experiments the expression data are presented as means and 99% CIs for three measuring repetitions. All tissue culture experiments were verified a second time by repeating the procedure starting with the cell isolation from the patient up to the expression analysis. For the tissue culture experiments GAPDH proved to be a reliable house-keeping gene with no evidence of expression variation under the respective experimental conditions, while a relatively higher expression of GAPDH in tumour than in benign tissue was observed. Therefore UBB had to be used for calculating MNE values in the quantitative tissue expression experiments.

MNE values nested in the groups of non-malignant and malignant tissues were log-normalized. The log-normalized renal clear cell carcinoma expression values were pair wise compared to the benign remote tissue of the same patient by Student t-tests and the 95% CIs and the related mean differences were transformed back into fold-difference values (benign to tumour). The same procedure but using a non-pair wise comparison by Student t-tests was used for the comparison of benign brain tissue to astrocytomas.

Results

In silico analysis suggests expression of two novel SGK1 mRNA variants

As shown in Fig. 1a, EST analysis disclosed 17 human and 45 non-human ESTs suggesting the expression of an SGK1 mRNA variant SGK1(-850) with a transcription initiation side 850bp upstream of the transcription initiation site +1 of the reference sequence mRNA SGK1(+1). 19 human and 10 non-human ESTs additionally support the existence of an SGK1 mRNA variant

Fig. 1. An *in silico* analysis of the gene locus of SGK1 on Chr. 6 was done using the Genome Browser and its integrated software tools as described in the method section (a). Line 1 shows the reference mRNA of SGK1 aligned to its gene locus followed by lines with all human and vertebrate ESTs that do not align properly to this reference sequence within their 5' region but share sequence homology in their 3' region. Two new potential alternative transcription initiation sides emerge with highly conserved splice sites among species and tissues and indicate the existence of two novel alternative SGK1 mRNA variants SGK1(-850) and SGK1(-2981) that have a different first exon but may otherwise share sequence homology. The "TARs" data indicate expression of the more 5' located variant SGK1(-2981) by presence of a green box and the "A375 Txn" data indicate expression of both variants by presence of blue vertical lines. Interestingly the phylogenetic conservation among vertebrate species as illustrated by the height of the blue vertical lines in the "conservation" track is much higher for the cds and the promoter region of the novel candidates (red boxes) than for the reference. An alignment of the translated sequences belonging to the novel variants SGK1(-850) and SGK1(-2981) as well as the reference SGK1(+1) is given in b). The presence of a different first exon did not lead to a loss of open reading frame (ORF). In fact three putative proteins derive for which their known domains are shaded and explained in the legend. The alternative first exon leads to a loss of the hydrophobic proteasomal degradation signal and may additionally affect localization of the novel variants.

SGK1(-2981). Even though the 91 sequences are derived from 7 different species (Macaca, Bos, Ovis, Sus, Mus, Rattus, Homo) and more than 25 different tissues the splice sites between the two alternative first exon and the second exon of SGK1(+1) were completely conserved among all ESTs. Tissue independent expression of at least SGK1(-850) was additionally supported by the presence

of two CpG islands in proximity (< 100bp) of the putative transcription start side. The tiling array data from the TARs project and the Affymetrix Transcriptome Project Phase 2 (AffyTXN2) both support the expression of SGK1(-2981). Expression of SGK1(-850) is supported by (AffyTXN2) only (see Fig. 1).

Phylogenetic conservation across 17 vertebrate spe-

Fig. 2. RT-PCR covering almost the complete sequence of the respective 3 mRNA variants by using specific sense primers (labelling above the picture in a) for the first exons against a common reverse primer in the 3' UTR of SGK1. All expected full length sequence sizes were detected and additional minor bands were visible. ** represents the 2 kb and * the 1.5 kb lane of the 250 bp marker. B An overview about the location of all SGK1 related primers used in this study. C. Expression of the respective variants in various tissues and cell lines.

cies was not only high for the two novel alternative first exons but also for the respective putative promoter regions of these variants which showed transcription factor binding sites that are conserved across humans, mice and rats. In fact, the phylogenetic conservation in the region up to 500bp upstream of SGK1(+1) was much lower than for both of the new variants. This suggests a pivotal role for the transcriptional regulation of SGK1(-850) and SGK1(-2981).

Sequence analysis of SGK1(+1), SGK1(-850) and SGK1(-2981)

EST analysis indicated the expression of three SGK1 mRNA variants that only differ in their first exons. RT-PCR analysis confirmed the expression of all three full length variants of expected size with the three sense prim-

ers specific for the different first exons against a common antisense primer at the end of the 3'UTR (Fig. 2a). In myotubes and the glioblastoma cell line LN229 we found full-length RT-PCR products of the expected size for the novel and the reference mRNA variants (Fig. 2a). Some smaller bands were also visible and it can not be excluded that additional splice variants of SGK1 exist. A further analysis of the expression of the three variants in different tissues and cell lines revealed widespread expression of SGK1(+1) and SGK1(-850) and a more tissue specific expression of SGK1(-2981) which is principally in line with an absence of a CpG island in this variant (Fig. 2c). However, any conclusions that compare the variants with conventional RT-PCR should be drawn with care given that the antisense primer in Fig. 2a and 2c had to be non-specific, which hampers the synthesis

Fig. 3. Course of expression of Egr1 and the different SGK1 mRNAs in myoblasts differentiating into myotubes (a) and following treatment with CoCl_2 (b). Means and 99% CIs for three measurement repetitions are given and results were verified a second time in a different cell line.

of the variants that are underrepresented relative to the more abundant SGK1(+1). For quantitative analysis of differential gene expression we therefore used the variant specific reverse primers SGK141rev, SGKEx0-2rev, SGKEx1-2rev (intron spanning internal to the primer sequence) in conjunction with variant specific sense primers as described in the next paragraph.

In silico translation of the three related putative mRNA sequences SGK1(+1), SGK1(-850) and SGK1(-2981) revealed that all of them have an intact open reading frame and are translated into 3 proteins of almost identical molecular weight that differ in the N-terminal region. An alignment of the sequences is shown in Fig. 1b. The “GMVAIL” sequence can only be found in SGK1(+1).

Transcription factor binding site analysis of the promoter regions of SGK1(+1), SGK1(-850) and SGK1(-2981) suggest differential regulation of expression

As shown in Fig. 1a, the regions 50bp down- and 450bp upstream of the respective new mRNAs revealed a very high level of phylogenetic conservation. This indicated that regulation pathways of the novel candidates might be central and common to vertebrate species. Analysis revealed an interesting distribution pattern of human transcription factor binding sites among the three promoter regions (Table 1).

The most frequently found binding site in all promoter sites of the serum- and glucocorticoid-induced kinase 1 variants were for the glucocorticoid receptor fol-

Fig. 4. Expression of the detectable variants SGK1(-850) and SGK1(+1) in the renal cell carcinoma (rcc) tissue and the related histological benign “remote” tissue of 10 patients is shown in a). b) shows a comparison of astroglia tissue (n=7) with benign brain tissue (n=6). In a) pair wise comparison using the students T-test was applied and measurements of samples from the same patient are connected. The horizontal line represents the mean expression level of each mRNA variant in all samples. In b) Arithmetic means and 95 % CIs of transcript levels in normal brain and astroglia.

lowed by binding sites for the general transcription factor (GTFII-1). Surprisingly, no serum response factor binding site was found. Instead many binding sites pointed towards the known physiological importance of this kinase for the regulation of apoptosis and towards a function related to immune response. Especially the transcription factors related to those functions showed an asymmetric distribution pattern (Table 1). Similar to overweight of binding sites for E2F1, p53 and AP2-alpha at the SGK1(+1) promoter there were 6 SP1 binding sites for SGK1(+1) and only 2 for SGK1(-850) and none for SGK1(-2981). GO-analysis done with the transcription factors that were overweighed at each of the three sides was done against the pool of transcription factors underlying the PROMO analysis. The transcription factors dominating at SGK1(+1) were significantly associated with “positive regulation of transcription” (NFKB1, RXRA, NFATC2, SP1; $p < 0.02$) and “anti-apoptosis” (NFKB1, NFKB3, $p < 0.04$), while “cell organization and biogenesis” (ESR1, STAT1 and AR; 0.037) emerged for SGK1(-850) and “immune system process” (STAT5A, CEBPA, XBP1, FOXP3) for SGK1(-2981).

Promotor site analysis additionally revealed one potential hypoxia response element (HRE) with the consensus sequence A/GCGTG located up- and three HREs

downstream of SGK1(-2981) which suggests a regulation of particularly this most 5’ located variant by hypoxia.

Differential regulation of SGK1(+1), SGK1(-850) and SGK1(-2981) expression during differentiation and treatment with CoCl₂

The asymmetric distribution of transcription factor binding sites related to cellular differentiation focussing close to the transcription initiation start of SGK1(+1) led to the hypothesis, that the three variants may show different stimulators of expression during differentiation.

We found a typical up-regulation of the early growth response gene (EGR1) during the course of skeletal muscle cell differentiation which reached a maximum of 2.92-fold (99% CI: 2.24 – 3.59) with subsequent decline (Fig 3a). Of the SGK1 variants SGK1(+1) showed the most prominent differential regulation with an almost steady increase reaching 7.76-fold (99% CI: 5.43-10.10) of basal expression at 144 h.

Since putative hypoxia response elements are located in the promoter of SGK1(-2981) we treated the myotubes with 0.2 mM CoCl₂ to induce hypoxia. The expression of the SGK1 variants (+1) and (-850) was modestly increased after 60 min of CoCl₂ treatment.

SGK1(+1)	SGK1(-850)	SGK1(-2981)
39.1 %: cytoplasmic		
30.4 %: nuclear	52.2 %: nuclear	56.5 %: nuclear
13.0 %: mitochondrial	21.7 %: mitochondrial	26.1 %: cytoplasmic
4.3 %: cytoskeletal	13.0 %: cytoplasmic	8.7 %: cytoskeletal
4.3 %: vacuolar	8.7 %: secretory vesicles	4.3 %: Golgi
4.3 %: plasma membrane	4.3 %: vacuolar	4.3 %: mitochondrial
4.3 %: secretory vesicles		

Table 2. shows the 10 most frequent binding sites for the listed transcription factors (column 1) and its SwissProt Entry Number (column 2). The number of binding sites is given in column 3-5.

In contrast, the SGK1(-2981) transcript was 119-fold (99%CI: 63-175) up-regulated by the induction of hypoxia, pointing to the regulation of this variant by functionally active hypoxia response elements. After 20 h of CoCl₂ treatment, the expression of all variants was clearly decreased, suggesting a down-regulation of SGK1 expression by prolonged hypoxia.

Up-regulation of SGK1 mRNA in renal cell carcinoma and astrocytomas is most prominent for the reference mRNA SGK1(+1)

Analysis of the promoter region of the SGK1 variants and the functional relevance of SGK1 for apoptosis and cell survival mechanisms raise the question, whether this kinase is up-regulated in tumors. In GO analysis of the promoter region and the focus of p53 binding sites around the transcription start side of SGK1(+1) suggested a more prominent role for this variant in neoplasm compared to the novel candidates. This hypothesis is also supported by the fact that this variant seems to be more prominently up-regulated during differentiation. With astrocytoma and renal cell carcinoma we choose two tumour cell types that are known to frequently show deletions of p53.

In renal cell carcinoma expression of the variant SGK1(+1) was 4.1-fold (CI 2.0-8.5, $p = 1.54 \times 10^{-3}$) and of SGK1(-850) 3.1-fold (CI 1.3-7.5; $p = 1.78 \times 10^{-2}$) higher compared to remote tissue (Fig. 4a). In high grade human malignant gliomas all three variants were expressed and SGK1(+1) was 42-fold ($p < 7 \times 10^{-6}$), SGK1(-850) 26-fold ($p < 5 \times 10^{-3}$) and SGK1(-2981) 17-fold ($p < 5 \times 10^{-3}$) elevated compared to normal brain tissue (Fig. 4a).

Discussion

The present observations disclose the existence of at least three SGK1 splice variants, an observation pointing to the involvement of different SGK1 protein variants in distinct cellular functions. The three variants are based on the use of alternative first exons, show integrity of the ORF and therefore code for three proteins that only differ in the very N-terminal region. As a consequence the variants differ in the sequence parts important for protein stability and localization [72, 76-78], while sequence parts important for the well known kinase activity including an ATP binding domain, but also for ENaC-dependent Na⁺ transport and Forkhead-dependent gene transcription [79] are not affected (Fig 1b). The observation may help to resolve the controversial discussion on the major localization site for the mature SGK1 protein [72]. At least in theory different antibodies generated against different N-terminal epitopes of SGK1 may detect different protein variants, leading to controversial results on protein localization and stability. Those results may trigger divergent considerations about SGK1 function by virtue of the spatial dependent availability of different factors interacting with SGK1.

Discrimination of the putative proteins is compromised by the similar predicted molecular mass (SGK1(+1) ~ 49.0 kD, SGK1(-850) ~ 50.6 kD, and SGK1(-2981) ~ 52.1 kD). Moreover, the localization of the epitopes recognized by the antibodies used to detect SGK1 is unknown. The protein analysis is further compromised by the different expression levels of the 3 different variants. In all our experiments the reference SGK1 mRNA SGK1(+1) was highly expressed. However, our mRNA

data suggest that for myotubes, brain and glioma tissue the difference in expression between the reference SGK1(+1) and SGK1(-850) is about more than 20-fold and between SGK1(+1) and SGK1(-2981) even more than 500-fold. Accordingly, only in renal cell carcinoma and kidney the difference between SGK1(+1) and SGK1(-850) was less than 10-fold (see Fig. 4a and b for log scaled relative expression values). Thus, the present observations cannot predict, whether the transcripts are indeed translated into the respective proteins.

All three SGK1 variants appear to be expressed in a variety of tissues and of the variants only SGK1(-2981) lacked a CpG-island and seems to be expressed in a more tissue specific manner (Fig. 2c).

The two new variants arise from alternative initiation of transcription at -2981, -850 upstream of the transcription initiation site (+1) of the reference mRNA. All three alternative splicing variants are therefore separated by more than 500 bp from each other which indicates the use of alternative promoters for generation of the different transcripts [97]. This assumption is verified by computational promoter site analysis and by differential regulation of the respective three transcripts. Differential response of the variants to hypoxia and differentiation were successfully predicted by the asymmetric distribution of the relevant transcription factor binding sites across the promoter regions of the candidates.

Bioinformatic approaches are also more or less capable to predict the localization of proteins based on sequence analysis [88]. The program iPsort found the (GM)VAIL-IAF sequence of SGK1(+1) and the LARARL sequence of SGK1(-850) as potential N-terminal signal motives that target the proteins to the mitochondria. Psort II suggested a preferential localization of the three candidates based on their complete sequence (Table 2). Since there is up to date no reference signal for a localization to the ER, we additionally performed a localization prediction by pTarget that works independent of the existence of reference localization sig-

nals by using a neural network approach. According to this analysis the sequence SGK1(-2981) has a likelihood of 81 % to be retained in the ER. However, these findings have to be considered preliminary until they can be verified experimentally.

The sensitivity of SGK1 transcription to hypoxia presumably contributes to its upregulation during ischemia [98]. Several SGK1 dependent functions may contribute to the regulation of cell survival during energy shortage, such as stimulation of the Na⁺ coupled creatine transporter CreaT [68] or the glucose transporter GLUT1 [58]. Moreover, SGK1 may stimulate matrix deposition and thus contribute to the replacement of cellular tissue with connective tissue [35, 99]. Thus, during sustained hypoxia energy consuming cells are replaced by extracellular matrix and thus energy consumption is cut down.

The high frequency of cancer associated transcription factor binding sites (see "Remark" column in table 2) indicates a more prominent role of all SGK1 variants in cancer cells of different origin. The transcript levels of all three splice variants are enhanced in astrocytoma and renal clear cell carcinoma, indicating that SGK1 may participate in the regulation of cell proliferation and/or cell survival. SGK1 has indeed been postulated to confer cell survival [70, 100, 101], an effect partially attributed to phosphorylation of forkhead transcription factors [100, 102, 103]. Along those lines, SGK1 has been shown to inhibit apoptosis of breast cancer cells [70], an effect attributed to stimulation of NFκB [70]. Moreover, SGK1 has been shown to enhance the activity of Kv1.3, a K⁺ channel implicated in the regulation of cell proliferation [104-107]. Activation of K⁺ channels hyperpolarizes the cell membrane, and thus provides the driving force for Ca²⁺ entry through the Ca²⁺ release-activated Ca²⁺ channel ICRAC [108]. Ca²⁺ entry is one prerequisite for the stimulation of cell proliferation [109]. Conversely, Kv1.3 channels [110, 111] and ICRAC [112] are inhibited by stimulation of CD95, a receptor, which triggers apoptosis of lymphocytes [113, 114].

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