SGK1-dependent Intestinal Tumor Growth in APC-deficient Mice

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Key Words

Tumor growth • PI3-kinase • Serum and glucocorticoid inducible kinase • β -catenin • Cancerogenesis

Abstract

Adenomatous polyposis coli (APC) is inactivated in familial adenomatous polyposis and sporadic colorectal cancer. Mice carrying defective APC (*apc*^{Min/+}) spontaneously develop gastrointestinal tumors. APC binds GSK3 β , which phosphorylates β -catenin thus fostering its degradation. β -catenin upregulates the serum- and glucocorticoid-inducible kinase Sgk1, which inhibits GSK3 β . The present study explored the role of SGK1 in tumor growth of *apc*^{Min/+} mice. *apc*^{Min/+} mice were crossed with SGK1-knockout mice (*sgk1*^{-/-}) and their wild type littermates (*sgk1*^{+/+}) generating *apc*^{Min/+}/*sgk1*^{-/-} mice and *apc*^{Min/+}/*sgk1*^{+/+} mice. β -catenin abundance was determined by Western blotting and confocal microscopy. As a result *apc*^{Min/+}/*sgk1*^{+/+} mice developed significantly more intestinal tumors than *apc*^{Min/+}/*sgk1*^{-/-} mice. Following chemical cancerogenesis, colonic β -catenin protein abundance was significantly higher in *sgk1*^{+/+} mice than in *sgk1*^{-/-} mice.

β -catenin expression was significantly increased in HEK293 cells treated with dexamethasone for upregulation of Sgk1. In conclusion, SGK1 expression favors the development of intestinal tumors in APC-deficient mice, an effect at least partially due to enhanced β -catenin protein abundance.

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Introduction

The adenomatous polyposis coli (APC) tumor suppressor gene binds glycogen synthase kinase 3 β (GSK3 β), which phosphorylates β -catenin thus fostering its degradation [1-3]. APC participates in the triggering of cytosolic β -catenin degradation and thus interferes with the Wnt/ β -catenin pathway [4, 5]. Lack of APC is followed by accumulation of β -catenin, which enters the nucleus and stimulates the expression of several genes important for cell proliferation including Myc and Cyclin D1 [6, 7]. Accordingly, loss of function mutations of the *apc* gene cause multiple colonic adenomatous polyps eventually resulting in colon carcinoma [8, 9].

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Genes regulated by β -catenin and thus potentially sensitive to APC function include genes encoding for the serum- and glucocorticoid-inducible kinase SGK1 [10, 11]. SGK1 was originally cloned as serum- and glucocorticoid-regulated gene from rat mammary tumor cells [12]. The human isoform has been discovered as cell volume-regulated gene [13]. A wide variety of further stimuli of SGK1 expression have subsequently been identified including mineralocorticoids, gonadotropins, calcitriol, transforming growth factor β (TGF- β), interleukin 4, fibroblast- and platelet-derived growth factor [13, 14]. It is a substrate for the phosphoinositide-dependent kinase 1 (PDK1) and thus activated through the phosphatidylinositol (PI)-3-kinase pathway [14].

SGK1 [15–17] has been postulated to confer cell survival. SGK1 inhibits apoptosis in Madin-Darby canine kidney (MDCK) [16] and breast cancer cells [17] and mediates interleukin (IL)-6-dependent survival of cholangiocarcinoma cells [18]. SGK1 was, however, found to be downregulated in prostate cancer [19], ovarian tumors [20] and hepatocellular carcinoma [21]. SGK1 and SGK3 phosphorylate forkhead transcription factors, such as FKRL1 (FOXO3) [10, 15, 16, 22]. They phosphorylate Bad [23] leading to binding of phosphorylated Bad to the chaperone 14-3-3 thus preventing its translocation into mitochondria where Bad triggers apoptosis [24]. SGK1 stimulates NF- κ B [17, 25] and the voltage-gated K⁺ channel Kv1.3 [26], which is involved in the proliferative effect of IGF-I [26] and participates in the regulation of apoptosis [27, 28]. Furthermore, SGK1 phosphorylates and thus inhibits glycogen synthase kinase (GSK) 3 [29], thus presumably impeding the degradation of β -catenin [30].

The present study was performed to elucidate the role of SGK1 in APC-dependent colonic tumor growth *in vivo*. To this end, APC-deficient Sgk1-expressing and Sgk1-knockout mice were compared. Furthermore, chemical cancerogenesis was induced by 1,2-dimethylhydrazine/dextran sulfate treatment in gene targeted mice lacking functional SGK1 (*sgk1*^{-/-}) and their wild type littermates (*sgk1*^{+/+}).

Materials and Methods

Animals

Mice with mutated *apc* (*apc*^{Min/+}) and their wild type littermates (*apc*^{+/+}) with a mixed (C57/Bl-6-Sv129J) background were generated by breeding of male *apc*^{Min/+} mice initially obtained from the Jackson Laboratory [31]. Where indicated, additional mice were generated by crossbreeding *apc*^{Min/+} mice

with gene targeted mice lacking functional SGK1 (*sgk1*^{-/-}) [32] to generate mice carrying the mutant APC and either expressing (*apc*^{Min/+}/*sgk1*^{+/+}) or lacking (*apc*^{Min/+}/*sgk1*^{-/-}) SGK1. *apc*^{Min/+}/*sgk1*^{+/+} or *apc*^{Min/+}/*sgk1*^{-/-} mice were sacrificed at the age of 73 to 126 days by CO₂ asphyxiation or cervical dislocation. The intestinal tract was removed and divided into four segments consisting of three equal lengths of small intestine (proximal, middle, and distal) and the whole of the large intestine. Each segment was flushed with PBS, opened longitudinally, and laid out on filter paper. The samples were fixed in neutral buffered formalin (NBF) overnight and stored in 70% EtOH. Before analysis, preparations were stained with 0.2% methylene blue for 3 minutes and were washed in PBS for 20 minutes. Tumors were counted with a dissecting microscope at x3 magnification. All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local authorities.

Tumor induction in *sgk*^{+/+} and *sgk*^{-/-} mice

Colonic tumors were generated as described previously [33]. At the age of 8 weeks the animals were treated with intraperitoneal injection of 20 mg/kg 1,2-dimethylhydrazine (DMH; Sigma) and subsequently with three cycles of alternating administration of distilled water containing 30 g/L synthetic dextran sulfate sodium (DSS; mol mass 5000; Wako Pure Chemical Industries, Ltd. Japan) for 7 days followed by distilled water for subsequent 14 days. All mice were anesthetized with ether and sacrificed at the age of 20 weeks. After death, the entire colorectum from the colocolic junction to the anal verge was examined. Fresh specimens were placed in liquid nitrogen and subsequently stored at -80°C for further analysis. The colon was then opened longitudinally, washed with PBS, and divided into three portions (proximal, middle and distal). After macroscopic inspection the colon was fixed in a 40% g/L formaldehyde buffer solution (pH 7.4).

HEK 293 cells

Human embryonic kidney (HEK) 293 cells were grown in DMEM medium containing 10% fetal calf serum under standard culture conditions (37°C, 5% CO₂). Three million cells were incubated for 72 hours in the presence or absence of 1 μ M dexamethasone (Sigma, Schnelldorf, Germany).

Western blotting

Mice were sacrificed by cervical dislocation under ether anaesthesia, and the abdomen was opened. The colon was then longitudinally cut, and the lumen was cleaned with PBS. A piece of 0.5 g of colon containing tumor tissue was added to 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM Na₃VO₄, 0.4% β -mercaptoethanol) containing protease inhibitor cocktail (Sigma). Three million HEK 293 cells were lysed in 100 μ l lysis buffer. The tissue was then homogenized with a Dounce homogenizer on ice for 30 min while the cells were homogenized by pipetting on ice. Samples were centrifuged at 17,000 rpm for 20 min, and supernatants were collected. After measurement of the total protein concentration (Bradford assay), 80 μ g of protein from colonic tissue and 40 μ g of protein from HEK 293

Fig. 1. Tumor incidence in APC-deficient SGK1 knockout and SGK1-expressing mice. Arithmetic means $\pm$ SEM ($n = 7$ -8) of the number of intestinal tumors in 70-120 day-old *apc*^{Min/+}/*sgk1*^{+/+} (white bar) and *apc*^{Min/+}/*sgk1*^{-/-} (black bar) mice. * indicates significant difference between the genotypes ($p < 0.05$).

Fig. 2. Western Blotting of β -catenin expression in colon tumors from APC-expressing and APC-deficient SGK1 knockout and SGK1-expressing mice. A. Original Western Blots illustrating the β -catenin (upper panel) and GAPDH (lower panel) protein abundance in chemically-induced colonic tumors from APC-expressing (*apc*^{+/+}) and APC-deficient (*apc*^{+/+}) SGK1 knockout (*sgk1*^{-/-}) and wild type (*sgk1*^{+/+}) mice. B. Arithmetic means $\pm$ SEM ($n = 3$ -5) of the relative densities of β -catenin protein expression in chemically-induced colonic tumors from APC-expressing (*apc*^{+/+}) and APC-deficient (*apc*^{+/+}) SGK1 knockout (*sgk1*^{-/-}) and wild type (*sgk1*^{+/+}) mice; * indicates significant difference between the genotypes (paired t-test; $p < 0.05$).

cells were solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 1 h. The membrane was then incubated with rabbit anti β -catenin antibody (1:1000; 92 kDa, Cell Signaling, Germany) at 4°C overnight. After washing (TBST) and subsequent blocking the blot was incubated with secondary anti rabbit antibody (1:2000, Cell Signaling) for 1 h at room temperature. After washing antibody-binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). For loading control the blot was stripped in stripping buffer (Roth, Karlsruhe, Germany) at 56°C for 30 min. After washing with PBST the blot was blocked with TBST + 5% milk for 1 h at room temperature. The blot was then incubated with an anti GAPDH antibody (1:1000, 37 kDa, Cell Signaling) at 4°C overnight. After washing with PBST and incubation with anti rabbit antibody (GAPDH, 1:2000, Cell Signaling), antibody-binding was detected. Bands were quantified with Quantity One Software (Biorad, München, Germany).

Confocal microscopy

To localize β -catenin protein expression, 5 μ m-thick frozen tissue sections from the colon were incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature. After washing twice with PBS the slides were incubated

overnight at 4°C with rabbit anti- β -catenin (1:200, Cell Signaling). The slides were rinsed three times with PBS and incubated with secondary FITC goat anti-rabbit antibody (1:400, Invitrogen) for 1.5 h at room temperature. After three washing steps the nuclei were stained with DRAQ-5 dye (1:1000, Biostatus, Leicestershire, UK) for 10 min at room temperature. The slides were washed again three times and then mounted with ProLong Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 40/1.3 NA DIC.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. Data were tested for significance using paired or unpaired student's t-test, as appropriate. Differences were considered statistically significant if p -values were < 0.05 .

Results

To determine the impact of SGK1 on APC-sensitive tumor growth, mice with mutated *apc* (*apc*^{Min/+}) were

Fig. 3. Confocal microscopy of β -catenin expression in colonic tumors from SGK1 knockout and SGK1-expressing mice. Confocal microscopy of β -catenin expression (green) and nuclear staining (red) in colonic tumor tissue from SGK1 knockout ($sgk^{-/-}$) and wild type ($sgk^{+/+}$) mice. The upper panels depict the representative tumor size.

crossbred with gene targeted mice lacking functional SGK1 ($sgk^{-/-}$) to generate mice carrying the mutant APC and either expressing ($apc^{Min/+}/sgk^{+/+}$) or lacking ($apc^{Min/+}/sgk^{-/-}$) SGK1. The mice were grown until the age of 70–120 days, and sacrificed. Subsequent inspection of the intestine revealed uniform tumor development in wild type mice. As illustrated in Fig. 1, the number of intestinal tumors was significantly lower in $apc^{Min/+}/sgk^{-/-}$ mice than in $apc^{Min/+}/sgk^{+/+}$.

As lack of APC is known to favour the accumulation of β -catenin, Western Blotting was performed to determine the β -catenin abundance in chemically-induced colon tumor tissue from $sgk^{-/-}$ and $sgk^{+/+}$ mice. As illustrated in Fig. 2, colonic tissue from $sgk^{-/-}$ mice

Fig. 4. Western Blotting of β -catenin expression in HEK 293 cells incubated in the presence or absence of dexamethasone. A. Original Western Blots illustrating the β -catenin (upper panel) and GAPDH (lower panel) protein abundance in human embryonic kidney (HEK) 293 cells treated for 72 h with (+dex) or without (-dex) 1 μ M dexamethasone to enhance SGK1 activity. The results of two independent experiments are shown. B. Arithmetic means $\pm$ SEM ($n = 3$) of relative densities of β -catenin protein expression in HEK 293 cells treated for 72 h with (+dex) or without (-dex) 1 μ M dexamethasone to upregulate SGK1 expression. * indicates significant difference from the absence of dexamethasone ($p < 0.05$).

expressed significantly less β -catenin than colonic tissue from $sgk^{+/+}$ mice. For comparison, β -catenin abundance was also determined in spontaneously tumor-developing $apc^{Min/+}/sgk^{+/+}$ and $apc^{Min/+}/sgk^{-/-}$ mice. As indicated in Fig. 2, SGK1 deficiency also results in reduced β -catenin in tumors from $apc^{Min/+}$ mice.

The results from Western blotting were confirmed by confocal microscopy using the same tumor tissue (Fig. 3). Again, β -catenin protein abundance was significantly reduced in chemically-induced colon tumor tissue from $sgk^{-/-}$ compared to $sgk^{+/+}$ mice.

Next, we investigated whether an *in vitro* activation of SGK1 by exposure of human embryonic kidney (HEK) 293 cells to dexamethasone is similarly capable of

Fig. 5. Regulation of β -catenin by GSK3 β dependent phosphorylation. The multifunctional protein β -catenin is bound to a complex consisting of axin, of adenomatous polyposis coli (APC) and of glycogen synthase kinase 3 β (GSK3 β). The latter phosphorylates β -catenin thus preparing it for degradation. Partial APC deficiency decreases phosphorylation and degradation of β -catenin leading to enhanced β -catenin protein abundance. Dishevelled (which is activated through Wnt and Frizzled) as well as the PKB/Akt and SGK isoforms phosphorylate and by this means inhibit GSK3 β , again increasing β -catenin protein abundance. Conversely, lack of SGK1 disinhibits GSK3 β and blunts the effect of partial APC deficiency.

increasing β -catenin expression. As shown in Fig. 4, the treatment of HEK 293 cells with dexamethasone for 72 h to upregulate SGK1 expression indeed significantly increased the β -catenin protein abundance.

Discussion

The present study reveals that SGK1 plays a critical role in the development of tumors in animals with defective APC. Accordingly, the number of tumors in animals with defective APC is significantly decreased in animals lacking functional SGK1.

In parallel experiments, partial resistance of SGK1 deficient mice to chemical cancerogenesis was observed

[34]. SGK1 has previously been shown to phosphorylate and thus to inhibit FKRL1 (FOXO3) [10, 16, 22, 23], which counteracts cell survival and cell growth [35], at least partially by stimulating the expression of the proapoptotic BIM [36, 37].

The present study further reveals that SGK1 impacts on the abundance of β -catenin. This *in vivo* experimental finding was supported by the analysis of β -catenin in HEK 293 cells showing enhanced protein expression in cells stimulated with dexamethasone. As shown earlier, SGK1 phosphorylates glycogen synthase kinase (GSK) 3 [29] leading to its inhibition and thus to impairment of β -catenin degradation [30]. GSK3 β and thus β -catenin degradation is further regulated by dishevelled (Fig. 5), which is activated by Wnt through the receptor Frizzled [5, 38].

SGK1 may influence tumor growth by further mechanisms. For instance, SGK1 upregulates a wide variety of nutrient transporters [14, 39, 40] and may thus contribute to the enhanced nutrient delivery required by fast proliferating cells. Moreover SGK1 stimulates channels [14, 41, 42], which may in turn be involved in the regulation of cell proliferation [43]. SGK1 further inhibits the expression of FAS and FASL, thus affecting the doxorubicin-dependent caspase 8 activation in kidney cancer cells [44].

SGK1-dependent signaling may in addition participate in the stimulation of angiogenesis and thus foster the vascularization of tumors. SGK1 has been shown to be upregulated by ischemia [45–47] and may well participate in the mechanisms increasing blood supply.

SGK1 participates in the glucocorticoid- or colony stimulating factor 1-induced stimulation of invasiveness, motility and adhesiveness [48] and may thus participate in the signaling required for tumor metastasis.

SGK1 may further confer resistance of tumor cells to therapy. SGK1 expression is stimulated by ultraviolet (UV) radiation, oxidative stress, and heat shock. Inhibition of SGK1 expression by silencing RNA increases the toxicity of chemotherapeutic drugs [18]. Along those lines overexpression of SGK1 by glucocorticoids inhibits the proapoptotic effect of chemotherapy on breast cancer cells [49]. With the same token SGK1 transcription was stimulated following radiation of tumor cells resistant to radiation but not in tumor cells sensitive to radiation [50].

Decreased SGK1 transcript levels have been shown in some tumors, including colon carcinoma [51], prostate cancer [19], ovarian tumors [20] and hepatocellular carcinoma [21]. These observations are in seeming contrast to the present observation that tumor abundance is decreased in SGK1 deficient mice. It should be kept in

mind that the SGK1-dependent functions may be accomplished by the other SGK isoforms and the protein kinase B/Akt isoforms [14]. In tumors with excessive activation of the PI3K/PKB/Akt isoforms, SGK1 expression may thus not be required for sufficient stimulation of SGK1-dependent functions [52]. As a matter of fact, the enhanced expression of SGK1 dependent functions due to excessive activation of PKB/Akt may lead to a negative feedback leading to the downregulation of SGK1 expression. The survival of tumor cells without enhanced PKB/Akt activity, but not the survival of tumor cells with excessive PKB/Akt activity may critically depend on the genomic upregulation of SGK1. Excessive activation of PKB is, for example, observed following loss-of-function mutations of the phosphatase and tensin homolog PTEN [53], which usually disrupts PI3-kinase-dependent signaling by degradation of PIP3 [54]. Moreover, transcript levels may not always reflect SGK1 activity. In normal intestine, for instance, SGK1 transcript levels are highest in enterocytes [55]. Despite those high transcript levels, the intestinal

glucose transport is similar in SGK1 knockout mice (*sgk1^{-/-}*) and their wild type littermates (*sgk1^{+/+}*). Only challenging the mice with glucocorticoid treatment reveals a difference between *sgk1^{-/-}* and *sgk1^{+/+}* mice [56] indicating that only under this condition the epithelium expresses functional SGK1.

In conclusion, SGK1 is critically important for the appearance of intestinal tumors in mice with defective APC. In APC-deficient tissues, SGK1 may play a role in the regulation of β -catenin.

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