HAL
open science

The N-terminus of the serum- and glucocorticoid-inducible kinase Sgk1 specifies mitochondrial localization and rapid turnover

Arne Engelsberg, Franziska Kobelt, Dietmar Kuhl

To cite this version:

Arne Engelsberg, Franziska Kobelt, Dietmar Kuhl. The N-terminus of the serum- and glucocorticoid-inducible kinase Sgk1 specifies mitochondrial localization and rapid turnover. Biochemical Journal, 2006, 399 (1), pp.69-76. 10.1042/BJ20060386 . hal-00478553

HAL Id: hal-00478553

https://hal.science/hal-00478553v1

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The N-terminus of the serum and glucocorticoid inducible kinase Sgk1 specifies mitochondrial localization and rapid turnover.

Arne Engelsberg, Franziska Kobelt, and Dietmar Kuhl

Molecular Neurobiology, Department of Biology-Chemistry-Pharmacy, Freie Universität Berlin, 14195 Berlin, Germany

Author for correspondence: Arne Engelsberg, Molecular Neurobiology, Department of Biology-Chemistry-Pharmacy, Freie Universität Berlin, Takustrasse 6, 14195 Berlin, Germany,
Tel: +49 30 838 56940, Fax: +49 30 838 56943 (engelsba@neuroscience.fu-berlin.de)

short title Mitochondrial localization and turnover of Sgk1

Key words Sgk1, mitochondria, half-life, localization, topology, orientation

abbreviations used GFP – green fluorescent protein, DAPI – 4',6-diamidino-2-phenylindole, OMM – outer mitochondrial membrane

Summary

The serine/threonine protein kinase Sgk1 is characterized by a short half-life and has been implicated in the control of a large variety of functions in different subcellular compartments and tissues. Here, we analyzed the influence of the N-terminus of Sgk1 on protein turnover and subcellular localization. Using green fluorescent protein-tagged Sgk1 deletion variants, we identified amino acids 17-32 to function as an anchor for the outer mitochondrial membrane. Subcellular fractionation of mouse tissue revealed a predominant localization of Sgk1 to the mitochondrial fraction. A cytosolic orientation of the kinase at the outer mitochondrial membrane was determined by *in vitro* import of Sgk1 and protease protection assays. Pulse-chase experiments showed that half-life and subcellular localization of Sgk1 are inseparable and determined by identical amino acids. Our data provide evidence that Sgk1 is primarily localized to the outer mitochondrial membrane and shed new light on the role of Sgk1 in the control of cellular function.

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Introduction

The serum and glucocorticoid inducible serine/threonine-kinase Sgk1 consists of 431 amino acids and belongs to a family of structurally related enzymes termed AGC kinases. Among these, Sgk1 shares about 50% sequence similarity on the amino acid level with protein kinase B/Akt, protein kinase C, ribosomal protein S6 kinase and cAMP-dependent protein kinase [1]. Sgk1 has been described to regulate a variety of cellular processes. *In vitro* assays suggested that Sgk1 might control signalling pathways and cell survival by phosphorylation of glycogen synthase kinase 3, B-raf kinase and forkhead family member FOXO3a [2,3,4]. In addition, the action of Sgk1 has been implicated in the control of a variety of plasma membrane transport proteins including sodium channels ENaC and SCN5A, chloride channel ClC2, the epithelial calcium channel TRPV5, glucose transporter SGLT1, the potassium channels Kv1.3, ROMK, KCNE1, and glutamate transporter EAAT1 [5-14]. In the kidney, Sgk1 contributes to sodium homeostasis by mediating the aldosterone induced upregulation of ENaC in the plasma membrane of distal tubule cells. Accordingly, Sgk1 knockout mice display an impaired sodium retention during dietary sodium restriction [15].

Sgk1 was first identified as an immediate early gene expressed in a rat mammary tumor cell line following stimulation with serum or glucocorticoids [1,16]. Depending on the specific tissues or cell lines examined, Sgk1 was shown to be induced by diverse factors, such as changes in osmolarity [17], aldosterone [18], and various growth factors, including fibroblast growth factor, platelet derived growth factor [19], follicle stimulating hormone [20] and transforming growth factor β [21]. Activation of the kinase requires at least two phosphorylation events in the activation loop and the C-terminal hydrophobic motif [22,23] and is further limited by a short half-life, resulting in low levels of protein expression in many tissues and cell lines. In line with these obervations Sgk1 is rapidly ubiquitinated and degraded by the proteasome [24]. Interestingly, although lysine residues at the N-terminus are not subject to ubiquitination, a variant of Sgk1 lacking the N-terminal 60 amino acids has a much longer half-life [24]. Another level of regulation was attributed to the subcellular localization of Sgk1. Various studies suggested that Sgk1 is translocated from the cytoplasm to the nucleus in a stimulus and cell cycle dependent manner via binding to importin-α [25,26,27]. In the nucleus, Sgk1 is thought to phosphorylate specific targets such as FOXO3a [4]. In contrast, two recent reports showed an association of Sgk1 with the plasma membrane in the rat kidney and in different cell lines, and that the N-terminus of the protein plays a major role in membrane targeting [28,24].

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

In order to gain further insight into the regulation and the range of cellular functions of Sgk1 the goals of the present study were to examine the intracellular localization of Sgk1 and to determine the region responsible for localization and degradation.

Experimental

Molecular Cloning

Sgk1-GFP fusion protein variants and variants with a C-terminal FLAG-tag were generated by PCR using a Pwo polymerase (Roche Biochemicals). Primers were extended with restriction sites and fragments were cloned in frame into pEGFP-N3 (BD Clontech), or in case of the FLAG-tagged variants into pcDNA3.1(+) (Invitrogen). The 5' primers contained a Kozak sequence surrounding the ATG start codon. For variants starting with amino acids 17, 33 and 60, we made use of the internal ATGs coding for the respective methionines. In variants starting at position 48, tyrosine 47 was replaced with methionine to obtain a start codon. All constructs were confirmed by DNA sequencing.

Cell Culture and Transfections

Human embryonic kidney HEK293 cells were maintained in DMEM medium (Biochrom, Berlin, Germany), supplemented with 5% fetal calf serum (Biochrom) and 2 mM L-glutamine. For pulse-chase experiments and immunoblot analysis, 2 x 10^6 cells were resuspended in 500 µl of serum-free medium and, after addition of 20 µg of the respective plasmid-DNA, electroporated at 170 V and 960 µF in 4 mm gap electroporation cuvettes (Bio-Rad, Munich, Germany) using a Bio-Rad Gene Pulser II. Transfected cells were plated on a 10 cm diameter dish. For confocal microscopy, 50,000 cells were grown on 12 mm coverslips in 24-wells and transfected with 100 ng Mito-DsRedII (BD Clontech, Palo Alto, CA.) and 200 ng of GFP or the indicated Sgk1-GFP fusion protein variants using the FUGENE transfection reagent (Roche Biochemicals, Mannheim, Germany) according to the manufacturers instructions.

Confocal Laser Scanning Microscopy

48 hours after transfection, HEK293 cells were washed with PBS, fixed with 4% paraformaldehyde and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) before mounting. Microscopy was performed with a Zeiss LSM510 inverted laser scanning microscope (Carl Zeiss, Jena, Germany) using the following configuration: DAPI: $\lambda_{exc.}$ = 364 nm, BP $\lambda_{em.}$ =

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

385-470 nm, GFP: $\lambda_{exc.}$ = 488 nm, BP $\lambda_{em.}$ = 505-550 nm, DsRed2: $\lambda_{exc.}$ = 543 nm, LP $\lambda_{em.}$ = 560 nm. Images were processed with the Zeiss LSM image browser software.

Pulse Chase Experiments and Immunoprecipitation

Transfected HEK293 cells were incubated for 45 minutes in methionine- and cysteine-free DMEM medium, and then pulse-labelled for 10 minutes with 200 µCi [^{35}S]methionine/cysteine labelling mix (Hartmann, Braunschweig, Germany). Incorporation of label was stopped by addition of complete medium supplemented with 1.5 mM nonradioactive methionine and 0.5 mM nonradioactive cysteine to a final volume of 5 ml. After 0 minutes, 15 minutes, 60 minutes, and 4 hours, cells from 1 ml suspension were pelleted and lysed in 50 mM Tris pH7.5, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF and 1.5 µg/ml aprotinin for 30 minutes at 4°C. Lysates were depleted of nuclei and cell debris by centrifugation for 10 minutes at 14,000 rpm in a microcentrifuge and precleared with normal rabbit serum. GFP fusion proteins were recovered by immunoprecipitation with anti-GFP rabbit serum (gift by Ralf Schülein) and Protein A-sepharose (Sigma, Steinheim, Germany), followed by five washes in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40. Sepharose-beads were boiled in reducing sample buffer and subjected to PAGE. Gels were dried and signals were obtained with 2,5-diphenyloxazol fluorography.

Subcellular Fractionation

Fractions were prepared from livers and kidneys of Sgk1 knockout mice and their wild-type littermates [15]. Organs were homogenized in TES-buffer (10 mM Tris, 250 mM sucrose, 1 mM EDTA, pH 7.4) in a Dounce homogenizer by 12 strokes with a tight fitting pestle. After centrifugation at 500 g for 10 minutes, crude mitochondria were pelleted from postnuclear supernatant at 10,000 g for 20 minutes. The pellet was resuspended in TES, layered on a discontinuous sucrose gradient consisting of 1 M and 1.5 M sucrose and centrifuged at 80,000 g for one hour. Highly purified mitochondria were harvested at the 1 M/1.5 M interphase and washed twice in TES. Cytosolic and microsomal fractions were prepared by ultracentrifugation of the postmitochondrial supernatant for 1 hour at 100,000 g in a TLA 110 rotor (Beckman). The pellet served as microsomal fraction. For the purification of nuclei and plasma membranes from the 500 g-pellet, the pellet was resuspended in 120 mM Tricine, 150 mM KCl, 30 mM $MgCl_2$, pH 7.5 supplemented with 1 mM DTT and 0.1 % Triton X-100, and nuclei and plasma membrane sheets were pelleted by centrifugation through a 1.75 M sucrose cushion in a Beckman SW-

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

32Ti rotor for 2 hours at 38,000 g. In some experiments, mice were injected with 100 µg/kg body weigth dexamethasone (Sigma) 4 hours before the preparation.

In vitro protein import into mitochondria

Coupled *in vitro* transcriptions and translations were performed in the presence of [^{35}S]methionine according to the manufacturer's instructions using the Promega TnT kit (Promega, Mannheim, Germany). Reticulocyte lysates containing the radiolabled Sgk1 variants were cleared of low molecular weight components by passage through Sephadex G-50 and added for the indicated periods of time at room temperature to purified C57BL/6 kidney mitochondria in import buffer consisting of 250 mM sucrose, 80 mM KCl, 10 mM $Mg(OAc)_2$, 2,5 mM EDTA, 2 mM KH_2PO_4, 2.5 mM ATP, 5 mM NADH, 2.5 mM disodiumsuccinate, 50 mM Hepes pH 7.3, 0.2 mg/ml BSA. Mitochondria were washed twice in ice-cold import buffer and subjected to PAGE. For the analysis of membrane association and orientation, *in vitro* translated proteins were added to 1 ml mouse kidney postnuclear supernatant from C57BL/6 mice supplemented with 2.5 mM ATP, 5 mM NADH, and 2.5 mM disodiumsuccinate, where import in mitochondria occurred for 20 minutes at 30°C. Mitochondria were pelleted at 10,000 g for 15 minutes, and were washed once with TES.

Membrane association, sodium carbonate extraction and protease protection assays

For protease protection assays, mitochondria were incubated with 200 µg/ml proteinase K (Roth, Karlsruhe, Germany) for 15 minutes at room temperature with or without 0.5 % Triton X-100. The reaction was stopped by addition of 5 mM PMSF.

Mitochondria were sonicated and then separated in soluble and membrane fractions ultracentrifugation for one hour at 300,000 g in a Beckman TLA 100.3 rotor. For sodium carbonate extractions, a membrane pellet was resuspended in 100 mM Na_2CO_3, incubated for one hour at 4°C under agitation and again pelleted for one hour at 300,000 g. Samples were separated by PAGE and radiolabeled proteins were visualized by fluorography.

Immunoblots

Transfer of proteins onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) or onto PVDF membranes for anti-Sgk1 and anti-FLAG M2 blots (Immobilon P, Millipore) was conducted using a tank blot chamber (Biorad, Munich, Germany). After blocking in 5% non-fat dry milk in PBS containing 0.05% Tween20 (PBS-T), antibodies were applied in PBS-T as

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

follows: Anti-Sgk1 (Sigma) 1:50,000, anti-Sgk1 (Cell Signaling Technology, Danvers, MA.) 1:2,000, anti-Sgk1 [28] 1:5000, anti-Sgk1 [36] 1:2000, anti-Sgk1 (this publication, see below) 1:10,000, anti-FLAG M2 (Sigma) 1:10,000, anti-Hsp60 (Stressgen, San Diego, CA.) 1:4,000, anti-Cytochrome C (Santa Cruz, Heidelberg, Germany) 1:5,000, anti-Smac/Diablo (ProSci Inc., Poway, CA.) 1:1,000, anti-Bip/Grp78 (Stressgen) 1:500, anti-GAPDH (Chemicon, Temecula, CA.) 1:2,500,000, anti-Na/K-ATPase α-subunit (Upstate) 1:10,000, and anti-Histone H1 (Santa Cruz) 1:1,000. For detection of Sgk1 with a sheep anti-Sgk1 antibody [36], membranes were blocked with Rotiblock reagent (Carl Roth, Karlsruhe, Germany) instead of dry milk. Membranes were washed, incubated with peroxidase-conjugated secondary antibodies (Vector labs), washed again and subjected to chemiluminescence detection.

We gave rise to an anti-Sgk1 antibody by immunization of a rabbit five times with 500 µg of Sgk1 amino acids 290-431 fused to an N-terminal His-tag. The protein was expressed in *E.coli* and purified using Ni-NTA spin columns (Qiagen, Hilden, Germany). IgGs were purified from the serum using Protein A-sepharose.

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Results

A signal peptide between amino acids 17 and 32 targets Sgk1 to mitochondria

Previous studies indicated that Sgk1 is localized to the cytosol, the nucleus and the plasma membrane in response to different external stimuli. We used the PSORTII prediction algorithm [29] to calculate the probability of Sgk1 localization to different cellular compartments. In this analysis, Sgk1 has a 43,5% probability to be a cytosolic protein and a 34,8% probability to localize to mitochondria. According to TargetP V1.0 [30], and MitoProt II 1.0a4 [31], two programs that predict mitochondrial localization, Sgk1 is likely to be imported into mitochondria and a predicted mitochondrial signal peptide might be cleaved at amino acid position 33 (Figure 1A). Analysis using the Kyte-Doolittle plot suggested that the N-terminus of Sgk1 might contain a transmembrane segment (Figure 1B).

To experimentally examine the subcellular localization of Sgk1, we transfected HEK293 cells with a series of Sgk1 variants that harboured a C-terminally fused green fluorescent protein (GFP) for analysis with confocal laser scanning microscopy. We focussed on the N-terminus of the kinase, because the first 60 amino acids were previously proposed to be responsible for plasma membrane association [24] and the mitochondrial targeting signal was predicted to reside in this region. When we transiently transfected HEK293 cells with full length Sgk1-GFP, we obtained a reticulo-tubular staining pattern that is characteristic for mitochondria in this cell line. To corroborate mitochondrial localization of Sgk1-GFP, we cotransfected mito-DsRedII as mitochondrial marker and stained nuclei with 4',6-diamidino-2-phenylindole (DAPI)**.** Our data demonstrate that GFP- and DsRedII fluorescence signals were overlapping (Figure 2A). To narrow down the region responsible for organelle targeting, we next generated and analyzed N-terminal deletion variants starting with amino acids 17, 33, and 48 (Figures 2C, E, G). Our analysis shows that amino acids 1-16 are dispensable for protein targeting as a Sgk1 variant containing amino acids 17-431 is still localized to mitochondria (Figure 2C). In contrast, Sgk1 33-431 (Figure 2E) and Sgk1 48-431 (Figure 2G) show a fluorescence pattern indistinguishable from that of GFP alone (data not shown). To investigate if the C-terminal regions of Sgk1 contribute to protein targeting, we fused the same deletion variants but ending at amino acid position 60 of Sgk1 to GFP. Confocal analyses of HEK293 cells transfected with the variants Sgk1 1-60-GFP (Figure 2B), Sgk1 17-60-GFP (Figure 2D), Sgk1 33-60-GFP (Figure 2F) and Sgk1 48-60-GFP (Figure 2H) revealed no difference between these and the long variants arguing against an influence of the C-terminal parts of Sgk1 on protein targeting. Additionally, we analyzed the variants Sgk1 1-16-GFP and Sgk1 1-32-GFP. Sgk1 1-16-GFP was distributed

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

throughout the cell (Figure 2I), whereas Sgk1 1-32-GFP localized to mitochondria (Figure 2J). Taken together, our data demonstrate that the region between amino acids 17 and 32 is necessary and sufficient for mitochondrial localization of Sgk1. These findings are in good accordance with the amino acid composition of Sgk1 in this region (Figure 1). Here, negatively charged residues are rare and arginine is overrepresented as is the case in most mitochondrial targeting sequences [30]. In a few cells, Sgk1-GFP signals were not restricted to mitochondria. However, these cells showed a strong overexpression of the transgene. In no instance were any Sgk1-GFP variants enriched in the nucleus even when cells were serum-stimulated or exposed to heat shock (data not shown). Moreover, none of the fusion protein variants were detectable at the plasma membrane.

Sgk1 is localized to mitochondria *in vivo*

To confirm a mitochondrial localization of Sgk1 *in vivo*, we performed subcellular fractionation experiments from mouse tissue. We first tested five commercial and non commercial antibodies for specificity of Sgk1 binding in Western blot analyses. Although all antibodies readily detected overexpressed Sgk1 in cell lines, we were not able to detect Sgk1 expression in any mouse tissue including kidney and liver (Supplementary Figure 1). We therefore increased levels of Sgk1 protein expression by administration of dexamethason. Specificity of the Sgk1 signal was verified by direct comparison with corresponding tissue from knockout animals. Four hours after intraperitoneal application of dexamethasone we fractionated mouse livers from Sgk1 knockouts and their wild-type littermates into cytosol, nuclei and plasma membranes, microsomes, and mitochondria by differential centrifugation and processed them for immunoblot analyses. The purity of the fractions was examined with antibodies directed against GAPDH, Histone H1, Na/K-ATPase, BiP/Grp78, cytochrome C and the α-subunit of the Na/K-ATPase (Figure 3). We obtained robust Sgk1 signals from the mitochondrial fraction and weak signals from the cytosolic and microsomal lane (Figure 3). The signals originated from Sgk1 as they were absent in the knockout tissue fractions.

Sgk1 is localized to the cytosolic surface of the outer mitochondrial membrane

Mitochondrial proteins can be localized to the inner or outer mitochondrial membrane (OMM), the intermembrane space or the matrix. To overcome the low endogenous Sgk1 protein expression levels, we investigated the localization and orientation of *in vitro* translated radiolabeled Sgk1 after import into intact mouse kidney mitochondria. Figure 4 shows that full

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

length Sgk1 is imported by purified mitochondria in a time-dependent manner. In constrast, the Δ1-59 variant, lacking the mitochondrial targeting sequence, completely remained in the supernatant after pelleting the organelles. When mitochondria were disrupted and separated into a soluble and a membrane fraction after protein import, Sgk1 was detectable only in the membrane fraction even after treatment with 100 mM sodium carbonate (Figure 5A, upper panel). Under these conditions, peripheral membrane proteins are released to the supernatant as is the case for cytochrome C, which is attached to the inner mitochondrial membrane mainly by electrostatical force (Figure 5A, lower panel). These experiments indicate that Sgk1 is an integral mitochondrial membrane protein. Based on the observation that Sgk1 can be phosphorylated by cytosolic kinases, we speculated that Sgk1 might be localized to the OMM with the kinase domain and the C-terminus facing the cytosol. To test this assumption, imported Sgk1 was subjected to protease protection assays. As control for correct protein import, we used the *in vitro* translated and radiolabeled mitochondrial matrix enzyme fumarate hydratase (Figure 5D). Proteinase K treatment of mitochondria led to complete digestion of Sgk1. To demonstrate that mitochondria remained intact during protease treatment, we performed Western blot analyses with antibodies directed against the intermembrane space protein Smac/Diablo and the matrix protein Hsp60. Figure 5C shows that both proteins were protected unless Triton X-100 was added. Cytochrome C is extremly resistent to digestion by proteinase K [37] and served as loading control (Figure 5C, lowest panel). The degradation of Sgk1 suggests a largely cytoplasmic orientation at the OMM. From these studies, we conclude that Sgk1 is targeted and anchored via amino acids 16-33 to the OMM and faces the cytosol with the C-terminus containing the catalytic domain.

Half-life of Sgk1 is determined by amino acids 17-47

It has been documented previously [23,24] that Sgk1 protein levels are very low even after overexpression in cell lines because of rapid ubiquitin mediated degradation by the proteasome [23,24]. The short life span is conferred by the first 60 amino acids of Sgk1, but is not due to ubiquitination of lysine-residues within this region [24]. To more precisely determine the region responsible for proteasomal degradation, we performed pulse-chase experiments using Sgk1-GFP deletion variants. After a 10 minute pulse with [^{35}S]methionine/cysteine, transfected HEK293 cells were immediately lysed or chased for 15 minutes, 60 minutes, and 4 hours. Sgk1 fusion proteins were recovered by immunoprecipitation with an anti-GFP serum and subjected to autoradiography (Figure 6). GFP alone does not show any apparent degradation within a 4

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

hour chase, nor does Sgk1 60-431, as previously described [24]. In contrast, full length Sgk1 and Sgk1 1-60 are rapidly degraded. Fine mapping of the regions responsible for the fast turnover showed that amino acids 17-32 and 33-47 are sufficient for targeting Sgk1 for proteasomal degradation (Figure 6). Therefore, the short half-life of Sgk1 and mitochondrial targeting are conveyed by the identical peptide sequence.

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Discussion

Here we demonstrate that Sgk1 is anchored via the N-terminus to the OMM, that mitochondrial targeting is conferred by amino acids 17-32, that the C-terminus containing the kinase domain faces the cytosol, and that mitochondrial targeting is inseparably linked to the rapid degradation of Sgk1 by the proteasome. Using an *in vitro* import assay, confocal microscopy of Sgk1-GFP deletion variants in HEK293 cells, and biochemical fractionation of murine liver tissue, we provide three independent experimental lines of evidence that Sgk1 is predominantly localized to mitochondria and resides at the OMM. Mitochondrial localization is mediated by amino acids 17-32 which resemble a bona fide mitochondrial targeting signal. However, this region is not a classical mitochondrial presequence, since Sgk1 is not imported into the mitochondrial matrix and is not cleaved by the mitochondrial processing peptidase. Several OMM proteins with cytoplasmic orientation are targeted by a non-cleavable N-terminal signal anchor consisting of a hydrophobic transmembrane domain and a net positive charge at the corresponding C-terminal flanking segment [32,33]. Similarly, the N-terminal region of Sgk1 is hydrophobic and is followed by three positively charged residues (Figure 1). Although the carbonate extraction data support that Sgk1 is an integral component of the OMM, a stretch of ten hydrophobic amino acids as indicated by the Kyte-Doolittle plot is generally considered to be too short for a membrane spanning segment. However, even if not integral, the experiments indicate that this segment at least confers an unusually strong peripheral membrane association. The cytoplasmic orientation of Sgk1 meets the requirement to be accessible for activation by cytosolic kinases [22,23].

Our data provide strong evidence that Sgk1 is primarily localized to the outer mitochondrial membrane, but do not exclude other sites of localization. Previous reports describe that Sgk1 is found in association with membranes [24, 28]. Our data indicated that these membranes might have been of mitochondrial origin. Moreover, our data support the observation of Brickley and coworkers that a variant of Sgk1 lacking the first 60 residues does not exhibit membrane association, is not ubiquitinated, and consequently, has a prolonged half-life [24]. Finally, the N-terminus of Sgk1 does not contain a classical targeting signal, and targeting of the kinase to different membrane systems and compartments might depend on the specific cell lines or culture conditions used. For example, the Bcl-2 protein can associate with both, the OMM and microsomal membranes via a C-terminal hydrophobic domain [38].

At the plasma membrane, Sgk1 was proposed to interact and phosphorylate the E3 ubiquitin ligase Nedd4-2 and thereby controlling plasma membrane insertion of ENaC sodium transporters [5]. However, using surface plasmon resonance analysis, a direct interaction of

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

Sgk1 with Nedd4-2 could not be confirmed [34]. Moreover, consistent with our results, a recent report described activation of ENaC by Sgk1 independent of Nedd4-2 [6].

Using confocal fluorescence microscopy, we also never observed significant localization of Sgk1 to the nucleus, not even after serum stimulation or heat shock, two stimuli that were reported to lead to nuclear translocation of Sgk1 [25,26,27]. Consistent with this, we demonstrate that endogenous Sgk1 is not present at detectable levels in the nuclear fraction of mouse liver tissue. However, we would like to point out that in these experiments pretreatement of mice with steroid hormones was necessary to induce detectable levels of Sgk1 protein expression. In mammary epithelial and tumor cells, steroid hormone treatment was shown to lead to a translocation of Sgk1 from the nucleus to a perinuclear compartment which contains mitochondria [25, 27]. Moreover, different cell lines and tissues are likely to differ in their capacity to import Sgk1 into the nucleus.

We here show that amino acids 17-32 are essential for organelle targeting and determine the half-life of Sgk1 protein. This makes it likely that degradation of Sgk1 is facilitated rapidly upon arrival at its mitochondrial destination. A corollary of this finding is that a large number of studies using an active variant of Sgk1, lacking the N-terminal 60 residues, need to be critically revisited. This long-lived variant, which has often been used in kinase assays for the detection or validation of kinase targets, is not directed to its physiological subcellular localization and is therefore prone to interact with and phosphorylate nonphysiological protein targets. Moreover, our data indicate that the use of Sgk1 variants with N-terminal tags should be avoided, because these are likely to alter the subcellular localization. In this context it is interesting to note that Helms and colleagues [35] reported that a Δ1-60 variant showed less biological activity than full length Sgk1 on ENaC function.

The influence of Sgk1 on a broad spectrum of cellular functions and its activation by several different stimuli indicates that Sgk1 plays a central role in cell signalling. Our data provide evidence that Sgk1 acts at the OMM. This finding may lay the basis for a further analysis of the function of Sgk1. The low protein expression levels of Sgk1 however renders a thorough biochemical examination of this molecule difficult. Detailed analysis of the molecules involved in the degradation of Sgk1 will clearly provide more insight into the biology of this kinase.

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Acknowledgements

We thank Burkhard Wiesner from the confocal imaging unit of the Institute for Molecular Pharmacology, Berlin, for help with laser scanning microscopy, Ralf Schülein for providing anti-GFP rabbit serum, and Cecilia Canessa and James Murray for providing anti-Sgk1 antibodies. We also thank Volker Haucke for discussions.

References

1 Webster, M.K., Goya, L., Ge, Y., Maiyar, A.C., and Firestone, G.L. (1993) Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Mol. Cell. Biol. **13**, 2031-2040

2 Sakoda, H., Gotoh, Y., Katagiri, H., Kurokawa, M., Ono, H., Onishi, Y., Anai, M., Ogihara, T., Fujishiro, M., Fukushima, Y., et al. (2003) Differing roles of Akt and serum- and glucocorticoid-regulated kinase in glucose metabolism, DNA synthesis, and oncogenic activity. J. Biol. Chem. **278**, 25802-25807

3 Zhang, B.H., Tang, E.D., Zhu, T., Greenberg, M.E., Vojtek, A.B., and Guan, K.L. (2001) Serum- and glucocorticoid-inducible kinase SGK phosphorylates and negatively regulates B-Raf. J. Biol. Chem. **276**, 31620-31626

4 Brunet, A., Park, J., Tran, H., Hu, L.S., Hemmings, B.A., and Greenberg, M.E. (2001) Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol. Cell. Biol. **21**, 952-965

5 Debonneville, C., Flores, S.Y., Kamynina, E., Plant, P.J., Tauxe, C., Thomas, M.A., Munster, C., Chraibi, A., Pratt, J.H., Horisberger, et al. (2001) Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. EMBO J. **20**, 7052-7059

6 Diakov, A., and Korbmacher, C. (2004) A novel pathway of epithelial sodium channel activation involves a serum- and glucocorticoid-inducible kinase consensus motif in the C terminus of the channel's alpha-subunit. J. Biol. Chem. **279**, 38134-38142

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

7 Boehmer, C., Wilhelm, V., Palmada, M., Wallisch, S., Henke, G., Brinkmeier, H., Cohen, P., Pieske, B., and Lang, F. (2003) Serum and glucocorticoid inducible kinases in the regulation of the cardiac sodium channel SCN5A. Cardiovasc. Res. **57**, 1079-1084

8 Palmada, M., Dieter, M., Boehmer, C., Waldegger, S., and Lang, F. Serum and glucocorticoid inducible kinases functionally regulate ClC-2 channels. (2004) Biochem. Biophys. Res. Commun. **321**, 1001-1006.

9 Embark, H., Setiawan, I., Poppendieck, S., Van De Graaf, S., Boehmer, C., Palmada, M., Wieder, T., Gerstberger, R., Cohen, P., Yun, C., et al. (2004) Regulation of the epithelial Ca2+ channel TRPV5 by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase isoforms SGK1 and SGK3 expressed in Xenopus oocytes. Cell. Physiol. Biochem. **14**, 203-212

10 Dieter, M., Palmada, M., Rajamanickam, J., Aydin, A., Busjahn, A., Boehmer, C., Luft, F.C., and Lang, F. (2004). Regulation of glucose transporter SGLT1 by ubiquitin ligase Nedd4-2 and kinases SGK1, SGK3, and PKB. Obes. Res. **12**, 862-870.

11 Henke, G., Maier, G., Wallisch, S., Boehmer, C., and Lang, F. (2004) Regulation of the voltage gated K+ channel Kv1.3 by the ubiquitin ligase Nedd4-2 and the serum and glucocorticoid inducible kinase SGK1. J. Cell. Physiol. **199**, 194-199

12 Yun, C.C., Palmada, M., Embark, H.M., Fedorenko, O., Feng, Y., Henke, G., Setiawan, I., Boehmer, C., Weinman, E.J., Sandrasagra, S., et al. (2002) The serum and glucocorticoid-inducible kinase SGK1 and the Na+/H+ exchange regulating factor NHERF2 synergize to stimulate the renal outer medullary K+ channel ROMK1. J. Am. Soc. Nephrol. **13**, 2823-2830.

13 Embark, H.M., Bohmer, C., Vallon, V., Luft, F., and Lang, F. (2003) Regulation of KCNE1-dependent K(+) current by the serum and glucocorticoid-inducible kinase (SGK) isoforms. Pflugers Arch. **445**, 601-606.

14 Boehmer, C., Henke, G., Schniepp, R., Palmada, M., Rothstein, J.D., Broer, S., and Lang, F. (2003) Regulation of the glutamate transporter EAAT1 by the ubiquitin ligase Nedd4-2 and the serum and glucocorticoid-inducible kinase isoforms SGK1/3 and protein kinase B. J. Neurochem. **86**, 1181-1188.

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

15 Wulff, P., Vallon, V., Huang, D.Y., Volkl, H., Yu, F., Richter, K., Jansen, M., Schlunz, M., Klingel, K., Loffing, J., et al. (2002) Impaired renal Na(+) retention in the sgk1-knockout mouse. J. Clin Invest. **110**, 1263-1268.

16 Webster, M.K., Goya, L., and Firestone, G.L. (1993) Immediate-early transcriptional regulation and rapid mRNA turnover of a putative serine/threonine protein kinase. J. Biol. Chem. **268**, 11482-11485

17 Waldegger, S., Barth, P., Raber, G., and Lang, F. (1997) Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. Proc. Natl. Acad. Sci. USA **94**, 4440-4445

18 Naray-Fejes-Toth, A., Canessa, C., Cleaveland, E.S., Aldrich, G., and Fejes-Toth, G. (1999) Sgk is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial na+ channels. J. Biol. Chem. **274**, 16973-16978

19 Mizuno, H., and Nishida, E. (2001) The ERK MAP kinase pathway mediates induction of SGK (serum- and glucocorticoid-inducible kinase) by growth factors. Genes Cells **6**, 261-268

20 Alliston, T.N., Maiyar, A.C., Buse, P., Firestone, G.L., and Richards, J.S. (1997) Follicle stimulating hormone-regulated expression of serum/glucocorticoid-inducible kinase in rat ovarian granulosa cells: a functional role for the Sp1 family in promoter activity. Mol. Endocrinol. **11**, 1934-1949

21 Waldegger, S., Klingel, K., Barth, P., Sauter, M., Rfer, M.L., Kandolf R., and Lang, F. (1999) h-sgk serine-threonine protein kinase gene as transcriptional target of transforming growth factor beta in human intestine. Gastroenterology **116**, 1081-1088

22 Park, J., Leong, M.L., Buse, P., Maiyar, A.C., Firestone, G.L., and Hemmings, B.A. (1999) Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. EMBO J. **18**, 3024-3033.

23 Kobayashi, T., and Cohen, P. (1999) Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. Biochem. J. **339**, 319-328.

24 Brickley, D.R., Mikosz, C.A., Hagan, C.R., and Conzen, S.D. (2002). Ubiquitin modification of serum and glucocorticoid-induced protein kinase-1 (SGK-1). J. Biol. Chem. **277**, 43064-43070

25 Buse, P., Tran, S.H., Luther, E., Phu, P.T., Aponte, G.W., and Firestone, G.L. (1999) Cell cycle and hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative and proliferative cell signaling pathways. J. Biol. Chem. **274**, 7253-7263.

26 Maiyar, A.C., Leong, M.L., and Firestone, G.L. (2003) Importin-alpha mediates the regulated nuclear targeting of serum- and glucocorticoid-inducible protein kinase (Sgk) by recognition of a nuclear localization signal in the kinase central domain. Mol. Biol. Cell. **14**, 1221-1239

27 Leong, M.L.L., Maiyar, A.C., Kim, B., O'Keeffe, B.A., and Firestone, G.L. (2003) Expression of the serum- and glucocorticoid-inducible protein kinase, Sgk, is a cell survival response to multiple types of environmental stress stimuli in mammary epithelial cells. *J. Biol. Chem.* **278**, 5871-5882

28 Alvarez de la Rosa, D., Coric, T., Todorovic, N., Shao, D., Wang, T., and Canessa, C.M. (2003) Distribution and regulation of expression of serum- and glucocorticoid-induced kinase-1 in the rat kidney. J. Physiol. **551**, 455-466.

29 Nakai, K. and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci., **24**, 34-35

30 Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. **300**, 1005-1016

31 Claros, M.G., and Vincens, P. (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur. J. Biochem. **241**, 779-786

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

32 Suzuki, H., Maeda, M., and Mihara, K. (2002) Characterization of rat TOM70 as a receptor of the preprotein translocase of the mitochondrial outer membrane. J. Cell Sci. **115**, 1895-1905

33 Kanaji, S., Iwahashi, J., Kida, Y., Sakaguchi, M., and Mihara, K. (2000) Characterization of the signal that directs Tom20 to the mitochondrial outer membrane. J. Cell Biol. **151**, 277-288

34 Henry, P.C., Kanelis, V., O'Brien, M.C., Kim, B., Gautschi, I., Forman-Kay, J., Schild, L., and Rotin, D. (2003) Affinity and specificity of interactions between Nedd4 isoforms and the epithelial Na+ channel. J. Biol. Chem. **278**, 20019-20028

35 Helms, M.N., Fejes-Toth, G., and Naray-Fejes-Toth, A. (2002) Hormone-regulated transepithelial Na+ transport in mammalian CCD cells requires SGK1 expression. Am. J. Physiol. Renal Physiol. **284**, 480-487

36 Murray, J.T., Campbell, D.G., Morrice, N., Auld, G.C., Shpiro, N., Marquez, R., Peggie, M., Bain, J., Bloomberg, G.B., Grahammer, F., Lang, F., Wulff, P., Kuhl, D., Cohen, P. (2004) Exploitation of KESTREL to identifiy NDRG family members as physiological substrates for SGK1 and GSK3. Biochem. J. **384**, 477-488

37 Uren, R.T., Dewson, G., Bonzon, C., Lithgow, T., Newmeyer, D.D., Kluck,R.M. (2005) Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. J. Biol. Chem **280**, 2266-2274

38 Janiak, F., Leber, B., Andrews, D.W. (1994) Assembly of Bcl-2 into Microsomal and Outer Mitochondrial Membranes. J. Biol. Chem. **269**, 9842-9849

Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Figure Legends

Figure 1. Amino acid sequence and hydropathy of the Sgk1 N-terminus.

A. The N-terminal 60 amino acids of murine Sgk1. Residues are shown in the one-letter code. Starting amino acids for the constructs used in this study are in bold, in some constructs tyrosine 47 (Y47) was replaced by methionine. Grey box highlights a predicted mitochondrial targeting sequence, and arrowhead indicates a potential cleavage site for the mitochondrial processing peptidase at amino acid position 33. Black bar indicates a potential transmembrane segment as predicted by the Kite-Doolittle analysis.

B. Kite-Doolittle hydropathy plot of Sgk1. The N-terminus of Sgk1 contains a potential transmembrane segment.

Figure 2. Amino acids 17-33 target Sgk1 to mitochondria in HEK293 cells.

HEK293 cells were transfected with the mitochondrial marker Cytochrome C oxidase subunit VIII coupled to DsRedII and either full length Sgk1-GFP (A) or Sgk1-GFP deletion variants (B-J). Cells were grown on coverslips, formaldehyde-fixed and nuclei were stained with DAPI. Left panels show GFP fluorescence signals (green), middle panels show DAPI (blue) and mitochondrial staining (red) and right panels show merged images. Yellow color indicates colocalization.

Figure 3. Sgk1 localizes to mitochondria *in vivo*.

The distribution of the Sgk1 protein in subcellular fractions from liver tissue was analyzed four hours after intraperitoneal administration of 100 µg/kg body weight dexamethasone. To be able to identify Sgk1 signals, Sgk1 knockouts (-/-) and their wild-type littermates were analyzed in parallel. Signals were obtained predominantly from the mitochondrial fraction and only weakly from the cytosolic and microsomal fraction. Lower panels show immunoblots with antibodies against the indicated organelle markers to demonstrate purity of the fractions. 20 µg protein were loaded per lane for detection of Sgk1 and 10 µg were loaded for detection of organelle markers. For the detection of Sgk1 we employed the antibody obtained from Sigma. PM: Plasma membrane.

Figure 4. Sgk1 associates with purified mitochondria in a time dependent manner.

Radiolabeled *in vitro* translated full length Sgk1 or Sgk1 Δ1-59 were exposed to highly purified mitochondria from mouse kidneys for the indicated times. Mitochondria were washed, lysed,

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

and separated by PAGE. Full length Sgk1 is imported in a time-dependent manner whereas the truncated variant is not. Lower panels show aliquots from the supernatants of the import reaction. Signals were visualized by fluorography.

Figure 5. Sgk1 associates with the outer mitochondrial membrane.

A. Membrane association of imported radiolabeled Sgk1 was assessed by preparation of mitochondrial (mitos) soluble (s/n) and membrane (mem) fractions. Radiolabeled proteins were visualized by autoradiography (AR). The peripheral membrane protein cytochrome C (CytC) was released by Na_2CO_3-treatment and visualized by Western blot (WB, lower panel). Asterisk: this signal sometimes occurred after *in vitro* translation and is most likely a degradation product (also in B).

B. After import of *in vitro* translated radiolabled Sgk1, mitochondria were treated with 200 µg/ml proteinase K (PK) in the presence or absence of Triton X-100 or left untreated. Sgk1 was subject to complete digestion indicating a localization to the cytoplasmic face of the OMM. ivt: an aliquot of the *in vitro* translation product.

C. Aliquots of the protease protection assay were subjected to Western blot analyses with antibodies directed against the matrix protein Hsp60, and the intermembrane space proteins Smac/Diablo and cytochrome C (CytC). CytC is resistent to proteinase K treatment under the conditions used, even when the membranes are permeabalized by Triton X-100 [37], and therefore served as loading control. Hsp60 and Smac/Diablo remain intact in the absence of Triton X-100 demonstrating the integrity of the organelles.

D. The capability of the preparation to correctly import proteins is shown by the import of *in vitro* translated fumarate hydratase (FH) in parallel. Imported FH migrates slightly faster than the *in vitro* translation product (ivt) because of the removal of the signal peptide by the processing peptidase after import into the matrix.

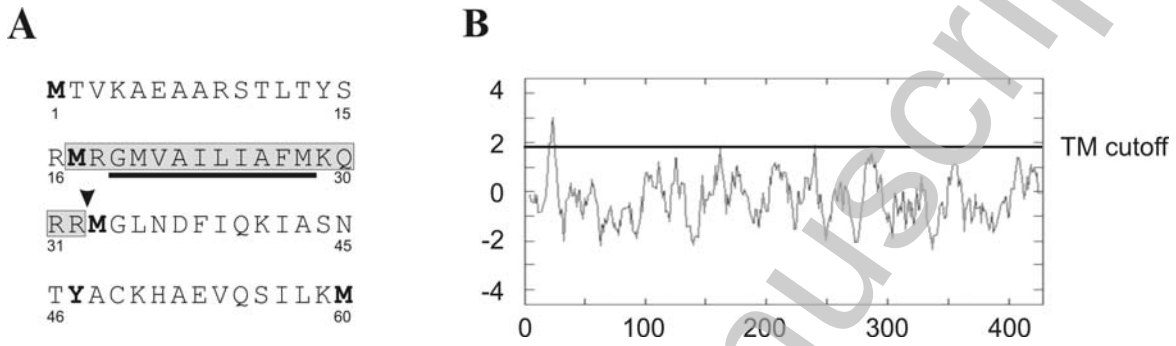
Figure 6. Rapid turnover of Sgk1 is determined by amino acids 17-47.

Sgk1-GFP fusion protein variants or GFP alone were transfected into HEK293 cells and subjected to pulse-chase analysis. After a pulse of 10 minutes, proteins were chased for 0 minutes, 15 minutes, 1 hour, or 4 hours, and the respective fusion proteins were recovered by immunoprecipitation with an anti-GFP antiserum. All variants containing amino acids 17-47 were rapidly degraded.

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Accepted Manuscript

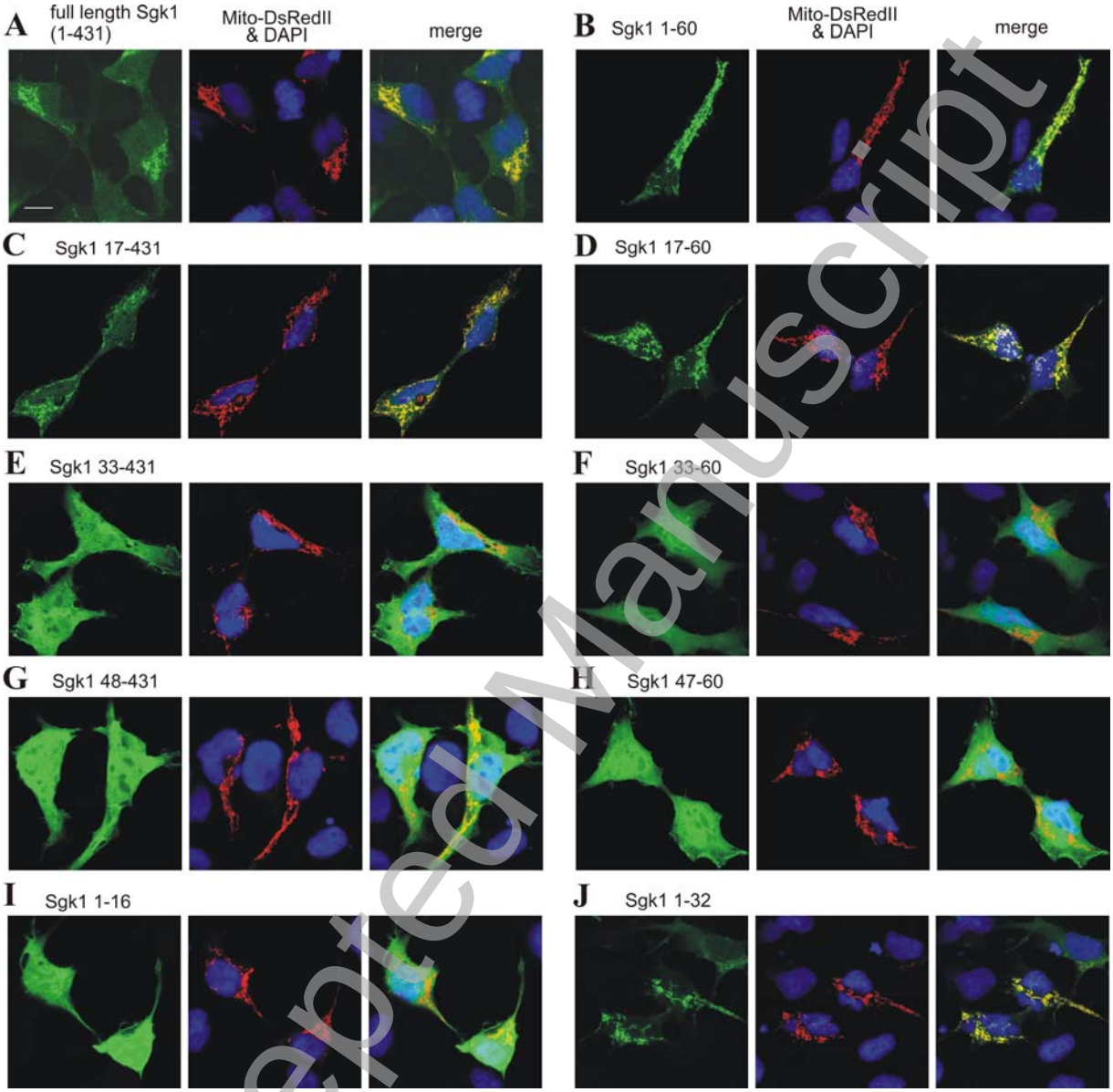
Figure 1



Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

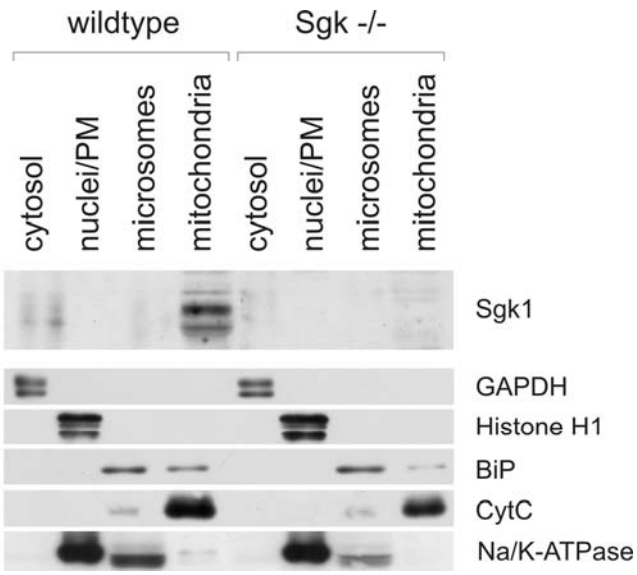
Figure 2



Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

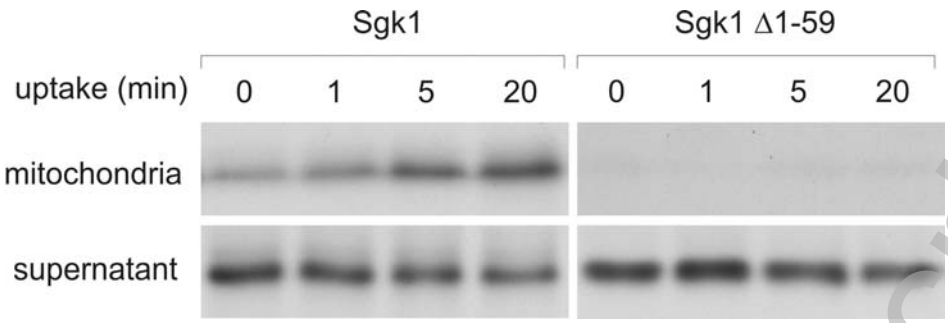
Figure 3



Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

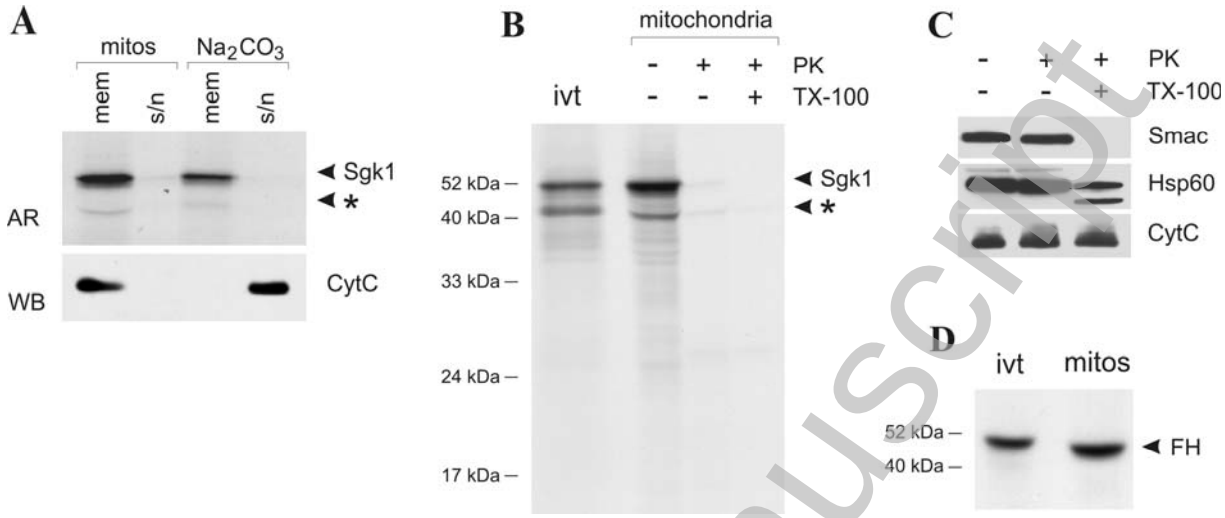
Figure 4



Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

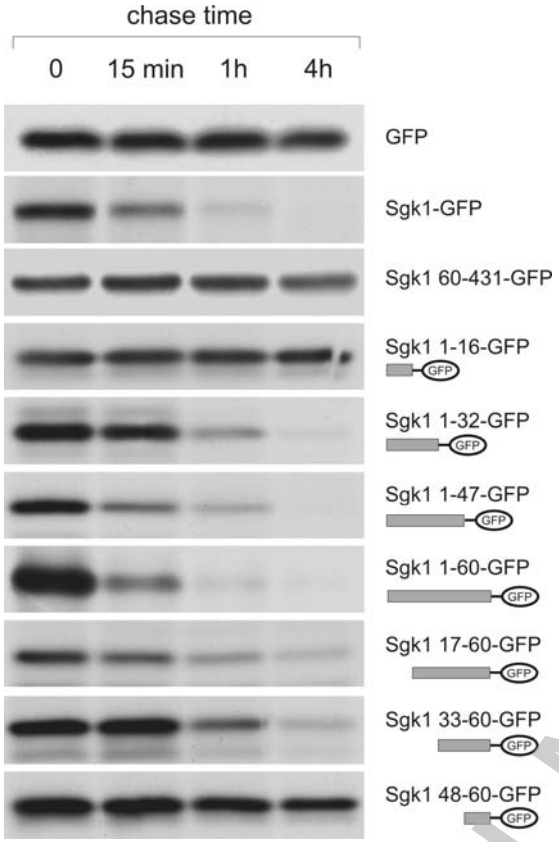
Figure 5



Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society

Figure 6



Accepted Manuscript

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2006 Biochemical Society