Subcellular location of serum- and glucocorticoid-induced kinase-1 in renal and mammary epithelial cells

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Cordas E, Náray-Fejes-Tóth A, Fejes-Tóth G. Subcellular location of serum- and glucocorticoid-induced kinase-1 in renal and mammary epithelial cells. Am J Physiol Cell Physiol 292: C1971–C1981, 2007. First published January 3, 2007; doi:10.1152/ajpcell.00399.2006.—Serum- and glucocorticoid-induced kinase-1 (SGK1) belongs to a subfamily of Ser/Thr kinases, which, unlike other protein kinases, are predominantly regulated at the transcriptional level. SGK1 was first identified as a glucocorticoid-induced transcript in rat mammary epithelial cells (49). Further studies revealed that SGK1 mRNA is also induced by aldosterone (12, 36) as well as a broad spectrum of stimuli including growth factors, p53, hypertonicity, and ischemic brain injury (25, 32, 35, 47). The multitude of factors that regulate its transcription suggests that SGK1 is involved in the regulation of many physiological events. Work by our group and others (23, 37) showed that SGK1 is one of the mediators of aldosterone-induced reabsorption of Na+ in the renal cortical collecting duct (CCD) of the kidney. When expressed heterologously, SGK1 increases the activity of the epithelial Na+ channel (ENaC) in Xenopus oocytes (2, 12, 36), in the amphibian renal cell line A6 (19), and in cultured mammalian CCD cells (23). In addition to ENaC, SGK1 increases the activity of several other transporters in Xenopus oocytes, including the Na+-K+-2Cl- cotransporter (NKCC2), Na+-K+-ATPase, Kir1.1 (also known as the renal outer medullary K+ channel, ROMK), slowly activating K+ channel subunit (KNC1), and human Na+-dependent neutral amino acid transporter type 2 (hASCT2) (17, 28, 39, 41, 51). In addition to these effects on ion transport, SGK1 has been shown to protect breast cells against apoptosis (34), to regulate cardiomyocyte survival and hypertrophy (3), and to play a role in neuronal excitability (48) and memory formation (29) and in the regulation of cell volume (47).

The subcellular location of SGK1 has not yet been conclusively established. In the majority of the studies, SGK1 was localized to the cytoplasm but not the nucleus (1, 9, 31). However, studies by Firestone and colleagues (11, 33, 40) found SGK1 to be present in the nucleus of rat mammary epithelial tumor cells, but only after serum stimulation. In addition, SGK1 was found to be in the vicinity of the basolateral membranes in rat kidney sections (1). One likely reason for these controversial results is that the half-life of SGK1 protein is very short (8, 9), and thus its endogenous levels may be below the detection limit afforded by available SGK1 antibodies.

Therefore, the goal of this study was to determine the subcellular location of SGK1 in epithelial cells by using SGK1 fused to autofluorescent proteins (SGK1-AFP). We presently report that SGK1-AFP is enriched in the mitochondria of both renal CCD cells (RCCT-28A) and human mammary epithelial cells (MCF10A). In addition, we also have determined that the first 60-amino acid region of SGK1 is necessary and sufficient for its mitochondrial localization. Parts of our findings have been published previously in abstract form (14).

METHODS

cDNA constructs. The various SGK1-AFP constructs used in this study are summarized in Fig. 1. Retroviral full-length human SGK1-green fluorescent protein (SGK1-GFP) and Δ60-SGK1-GFP constructs (generous gifts of Dr. Suzanne Conzen, University of Chicago) were described previously (9). SGK1-enhanced cyan fluorescent protein (SGK1-ECFP) and SGK1-enhanced yellow fluorescent protein (SGK1-EYFP) constructs were generated by subcloning a 1,320-bp Xhol/BamHI fragment of SGK1-GFP into pEYFP-N and pECFP-N expression vectors (Clontech, Palo Alto, CA) between the Saff and BamHI sites, resulting in fusion proteins in which the fluorescent protein is at the COOH terminus of SGK1. A construct encoding only the NH2-terminal 60 amino acids of SGK1 fused to the NH2 terminus of EYFP (called SGK1-N60-EYFP) was generated by inserting the Xhol/BgIII fragment of SGK1 into pEYFP-N. To generate a retroviral construct, we subcloned the Xhol/BsaBI fragment of SGK1-N60-EYFP into the pLNCX vector (Clontech). In addition, we generated a

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perfused with phosphate-buffered saline (PBS) that contained 0.9 mM coverslip into a heated chamber maintained at 37°C. The chamber was temperature for 10 min and mounted with Vectashield (Vector Laboratories). Either the cells were fixed with 4% paraformaldehyde at room temperature for 10 min and mounted with Vectashield (Vector Laboratories, Carlsbad, CA), following the manufacturer's protocol. After 24–36 h, amphotropic Phoenix cells (American Type Culture Collection, Rockville, MD) were grown in DMEM containing 4.5 mg/ml glucose (Mediatech) supplemented with 10% FBS and antibiotics as listed above.

For transient transfection, RCCT-28A cells were grown on glass coverslips until they were 70–90% confluent and then transfected with 10% FBS and antibiotics as listed above.

**Cell culture and transient transfections.** RCCT-28A cells, a rabbit CCD cell line (4), and MCF10A cells, a human mammary epithelial cell line (42), were grown in Dulbecco’s modified Eagle’s medium (DMEM-F-12; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 75 μg/ml penicillin, 100 μg/ml streptomycin, 12.5 μg/ml tulosin, and 2 mM t-glutamine. Amphotropic Phoenix cells (American Type Culture Collection, Rockville, MD) were grown in DMEM containing 4.5 mg/ml glucose (Mediatech) supplemented with 10% FBS and antibiotics as listed above.

For transient transfection, RCCT-28A cells were grown on glass coverslips until they were 70–90% confluent and then transfected with different SGK1 constructs and various subcellular compartmental markers by using Lipofectamine Plus reagent. After 24–36 h, either the cells were transfected with 4% paraformaldehyde at room temperature for 10 min and mounted with Vectashield (Vector Laboratories, Burlingame, CA) or live cells were observed by placing the coverslip into a heated chamber maintained at 37°C. The chamber was perfused with phosphate-buffered saline (PBS) that contained 0.9 mM Ca²⁺, 0.5 mM Mg²⁺, and 2 mM glucose.

**Generation of cell lines stably expressing SGK1 constructs.** AmphotropicPhoenix cells were transiently transfected with retroviral expression vectors by using Lipofectamine Plus reagent. After 48 h, the medium was collected and filtered. Subconfluent RCCT-28A or MCF10A cells were incubated in a 1:1 mixture of the retrovirus-containing medium and fresh DMEM-F-12 to which polybrene (4 ng/ml; Sigma, Palo Alto, CA) was added for 24 h. The medium was then replaced with a fresh aliquot of virus and medium and incubated for another 24 h. Cells stably expressing the desired proteins were selected using G418 (500 μg/ml; Sigma), and pools of clones (~500–1,000) were expanded and used as polyclonal cell lines to avoid artifacts due to clonal selection.

**Fluorescence microscopy.** The subcellular location of the various SGK1 fusion proteins was visualized by fluorescence microscopy using an Olympus IMT2 microscope with a ×60 PlanApo oil-immersion objective (numerical aperture 1.4; Nikon). The images were captured with a PXL-cooled charge-coupled device camera (Photometrics, Tucson, AZ) and processed using IPLab (Scanalytics, Fairfax, VA). Confocal images were taken using a Zeiss LSM 510 meta confocal microscope. A Biotech FCS2 chamber (Biotech, Butler, PA) maintained at 37°C was used to examine live cells grown on glass coverslips. To visualize different subcellular compartments, we used Rab 4, 5, and 11 fused to CFP as endosomal markers, and we used pECFP-ER and pECFP-Golgi vectors as markers for the endoplasmic reticulum (ER) and the Golgi apparatus, respectively (Clontech). LysoTracker red (50 nM; Molecular Probes, Eugene, OR) was used as a marker for lysosomes. To visualize mitochondria in live cells, we stained cells with the vital dye rhodamine 123 (500 ng/ml; Eastman Kodak, New York, NY) or with MitoTracker red CMXRos (0.25 nM; Molecular Probes) for 15 min at 37°C. To visualize nuclei in live cells, we stained cells with the cell-permeable DNA dye Hoechst 33342 (10 μM; Polysciences, Warrington, PA) for 15 min at 37°C.

For immunocytochemistry, RCCT-28A cells were fixed using periodate-lysine-paraformaldehyde for 10 min and then permeabilized with 0.5% Nonidet P-40 for 15 min followed by a 5-min incubation in PBG (0.5% BSA and 0.1% gelatin in PBS). The permeabilized cells were then incubated with an anti-GFP antibody (Molecular Probes) diluted 1:200 at 1 h at room temperature and then with Alexa 568 red-conjugated anti-rabbit antibody (Molecular Probes) diluted 1:3,000 for 1 h at room temperature. The cells were rinsed again with PBS, refixed with 4% paraformaldehyde and mounted onto slides using the anti-fade medium Vectashield (Vector Laboratories). Non-specific binding was determined by incubating cells with the secondary antibody only.

Photobleaching experiments were performed on RCCT-28A cells stably expressing SGK1-AFP chimeras with a Zeiss LSM 510 meta confocal microscope. Cells were grown on glass coverslips and maintained at 37°C during the experiments in a Biotech FCS2 chamber (Biotech).

**Subcellular fractionation.** The mouse kidney and liver were removed and rinsed in chilled PBS, weighed, minced, and homogenized using a Potter-Elvehjem homogenizer in an isolation buffer (0.2 mM EDTA, 10 mM Tris-HCl, pH 7.8, and 0.25 M sucrose) supplemented with protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 1,000 g for 10 min at 4°C to remove unbroken cells and nuclei. The supernatant was then centrifuged at 12,000 g for 15 min at 4°C to pellet the mitochondria. The pellet was washed twice by being resuspended in the isolation buffer, centrifuged at 12,000 g for 15 min, and resuspended again in the same buffer (mitochondrial fraction). The supernatant of the 12,000-g spin was further centrifuged at 100,000 g for 30 min to separate “cytosol” (supernatant) and “microsome” (pellet, resuspended in the isolation buffer) fractions. Protein concentration of the lysate and subcellular fractions was determined with BCA protein assay reagent (Pierce Biotechnology, Rockford, IL).

To assess the purity of the mitochondrial fraction, we performed immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a cytosolic marker) using an antibody from American Research Products (Belmont, MA). The mitochondrial fraction contained no detectable GAPDH signal. The enrichment of the mitochondrial

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**Fig. 1.** Graphic representation of the various serum- and glucocorticoid-induced kinase-1 (SGK1)-autofluorescent fusion proteins (SGK1-AFP) that were transiently or stably expressed in RCCT-28A and MCF10A cell lines. Fusions of the various SGK1 mutants were made to enhanced cyan fluorescent protein (ECFP), enhanced green fluorescent protein (EGFP), and enhanced yellow fluorescent protein (EYFP) as described in METHODS.
fractions was evaluated by determining citrate synthase activity, as previously described (43), as well as by Western blot analysis using antibodies against two other mitochondrial marker proteins, mitochondrial heat shock protein 70 (mtHsp70); Affinity BioReagents, Golden, CO) and cyclooxygenase IV Cox IV; Cell Signaling Technology, Beverly, MA). Citrate synthase activity was approximately fourfold higher in the mitochondrial fraction compared with the supernatant after broken cells were pelleted out (“total” fraction). Similar enrichment was found by immunoblot analysis with mtHsp70 and Cox IV antibodies.

Western blot analysis. Protein samples (6–20 μg) were electrophoresed in 10% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Ponceau S dye (Sigma) was used to confirm equal loading of the protein. The membranes were blocked in SuperBlock blocking buffer (Pierce Chemical) containing 0.5% Tween 20 or 5% dry milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween, and 124 μM thimerosal) for 1 h at room temperature and incubated with the primary antibodies diluted in blocking reagent under the following conditions. Rabbit SGK1 antibodies (Epitomics, Beverly, MA; Cell Signaling Technology; Upstate Biotechnology, Lake Placid, NY; and Sigma) were applied at a 1:500 dilution (except the Sigma SGK antibody, which was used at 1:5,000 dilution) and incubated overnight at 4°C. A rabbit antibody against Cox IV (Cell Signaling Technology; 1:2,000) and mouse monoclonal antibodies against GAPDH (American Research Products; 1:10,000) and mtHsp70 (Affinity BioReagents; 1:2,000) were incubated at room temperature for 1–3 h. After a series of washes with TBST, the membranes were incubated for 1–2 h at room temperature with either an anti-rabbit horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology) diluted 1:5,000 for SGK1 and 1:2,000 for Cox IV or with an anti-mouse HRP-linked antibody (Zymed Laboratories, San Francisco, CA) diluted to 1:5,000. Finally, after a series of washes with TBST, blots were visualized using an enhanced chemiluminescent substrate (SuperSignal West Dura extended duration substrate; Pierce Biotechnology). The membranes were imaged with a charge-coupled device camera (ChemilImager 5500; Alpha Innotech, San Leandro, CA).

RESULTS

SGK1-EGFP localizes to mitochondria in epithelial cells. First, we determined the subcellular location of transiently expressed SGK1-EGFP in live RCCT-28A cells. As shown in Fig. 2, SGK1 was primarily located in the cytoplasm and was enriched in tube-shaped compartments. We did not observe SGK1 in the nucleus, as demonstrated by a lack of colocalization with the cell-permeant DNA stain Hoechst 33342. To determine the subcellular compartment in which SGK1 is enriched, we cotransfected cells with SGK1-AFP and known subcellular compartment markers. SGK1 did not colocalize with markers for the ER, endosomes associated with Rab 4, 5, and 11, or lysosomes (Fig. 3).

Since the tubular structures with which SGK1-AFP was associated were reminiscent of mitochondria, we tested whether it colocalized with a mitochondrial marker, rhodamine 123, in live RCCT-28A cells. As shown in Fig. 4, we observed a high degree of colocalization of SGK1-EGFP with rhodamine 123, indicating that transiently expressed SGK1 is enriched in the mitochondria RCCT-28A cells.

To exclude the possibility that the observed subcellular location of SGK1 is an artifact due to the high expression levels usually associated with transient transfections, we generated RCCT-28A cells stably expressing SGK1-EGFP (EGFP was fused to the COOH terminus of SGK1). Our results, shown in Fig. 5, demonstrate that in these cells, just as in transiently transfected cells, SGK1-EGFP colocalized with the mitochondrial marker (in this case, MitoTracker). This dominant pattern of mitochondrial localization of SGK1-EGFP was observed in 98% of cells in three separate experiments. In the remaining 2% of the cells, the fluorescence intensity of the EGFP was too low to assess localization.

To address the possibility that the unexpected mitochondrial localization of SGK1-EGFP is a unique feature of CCD cells,
we also expressed SGK1-EGFP stably in the human mammary epithelial cell line MCF10A. We observed that similarly as in RCCT-28A cells, SGK1-EGFP was localized to mitochondria in MCF10A cells as well (Fig. 6). These results demonstrate that the mitochondrial location of SGK1-EGFP is not an artifact associated with the transient transfection and is not restricted to renal epithelial cells but is a more general phenomenon.

Since AFPs become fully fluorescent only after a maturation period of hours, which is much slower than the reported half-life of SGK1 (6, 8), in theory mitochondrial localization of SGK1-AFP in our study may be accentuated, because short-lived molecules in other compartments may not have been detected. To test this possibility experimentally, we determined the localization of SGK1 in RCCT-28A cells stably expressing SGK1-YFP or SGK1-N60-YFP by using an anti-GFP antibody that reacts with YFP. These experiments also verified that the majority of the SGK1-AFP fusion protein is enriched in mitochondria, because the fluorescence patterns of the fusion protein and the immunostaining were nearly identical (Fig. 7).

The NH2-terminal region is necessary and sufficient for localization of SGK1 to mitochondria. To determine the region of SGK1 that is responsible for its mitochondrial localization, we first analyzed SGK1 sequences using Mitoprot II 1.0a4 program. Interestingly, this analysis revealed a potential mitochondrial targeting sequence within the NH2-terminal region of SGK1 with a probability of export to mitochondria of 97.7 and 95% for mouse and human SGK1, respectively. Therefore, we first examined the role of the NH2-terminal 60-amino acid region of SGK1 from two approaches: examining the subcellular localization of 1) the NH2-terminally truncated SGK1 and 2) a construct encoding the first 60 amino acids of SGK1.

Data shown in Fig. 8 demonstrate that elimination of the first 60 amino acids resulted in a dramatic change in subcellular localization of SGK1 (compare Figs. 4 and 5 with Fig. 8A). In contrast with the full-length SGK1, Δ60-SGK1-EGFP did
not colocalize with MitoTracker, but rather it was distributed throughout the cell, in both the cytoplasm and the nucleus (Fig. 8A). The localization of Δ60-SGK1-EGFP is similar to that previously observed by Brickley et al. (9) in transiently transfected breast cancer cells. These results suggest that the NH2-terminal 60-amino acid region is necessary for mitochondrial targeting of SGK1. Interestingly, this NH2-terminally truncated SGK1, although maintaining its kinase activity, fails to increase ENaC currents when stably expressed in M1 cells, whereas the full-length SGK1 significantly increases Na+ transport (23).

Next, we asked whether this NH2-terminal region is sufficient for the observed mitochondrial localization of SGK1 or whether other targeting motifs also are required. To answer this question, we first cotransfected RCCT-28A cells with a construct encoding the NH2-terminal 60-amino acid region of SGK1 fused to EFYP (SGK1-N60-EFYP) and the full-length SGK1-ECFP. Interestingly, we observed a perfect colocalization between the two fusion proteins (Fig. 8, J and K). In addition, stably expressed SGK1-N60-EFYP also colocalized with the mitochondrial marker MitoTracker in both RCCT-28A cells (Fig. 8, D–F) and MCF10A cells (data not shown). These results indicate that the NH2-terminal 60-amino acid region of SGK1 is necessary and sufficient for the observed localization of SGK1 to the mitochondria.

Finally, we examined the subcellular location of a mutant in which we inserted EFYP in the middle of SGK1 (SGK1-EFYP-middle). Our rationale for testing this chimera was that the subcellular location of SGK1 varies depending on whether the fluorescent protein is fused to its COOH or NH2 terminus (Ref. 38 and unpublished data), probably due to interference with possible targeting domains such as the COOH-terminal PDZ binding domain (38) or the NH2-terminal region of SGK1. However, this fusion protein also was enriched in mitochondria (Fig. 8, G–I), and its localization was indistinguishable from that of the full-length SGK1 or the NH2-terminal 60-amino acid region of SGK1. These data suggest that the COOH-terminal fusion of the fluorescent protein does not cause artificial localization to the mitochondria due to disruption of the PDZ binding domain.

To assess the dynamics of SGK1’s mobility in mitochondria, we performed fluorescence recovery after photobleaching (FRAP) experiments in RCCT-28A cells stably expressing SGK1-EGFP. Initial FRAP experiments with a bleach spot diameter of 2 μm resulted in only partial fluorescence recovery. Approximately 30% of the lost fluorescence in the bleached area was regained within 15 s with a half-time of recovery of 3–5 s. However, no further recovery occurred during the subsequent observation period, indicating that roughly 70% of the fluorescence is present in a practically immobile form (Fig. 9). To identify the mechanism responsible for the initial recovery phase, we performed photobleachings with various spot sizes. These experiments revealed that the size of the mobile fraction was dependent on the bleach area...
and that with bleach diameters >5 μm, no significant fluorescence recovery occurred within 6–8 min. These studies also showed that recovery occurred primarily at the boundary of the bleached and unbleached areas, suggesting that it most likely originates from either mitochondrial movement and/or recovery within partially bleached mitochondria and/or fusion of bleached with unbleached mitochondria. Time-lapse experiments indeed demonstrated that movement as well as fusion/fission of mitochondria is quite rapid. In addition, FRAP with a diffraction-limited spot size showed that the intramitochondrial lateral diffusion of SGK1-EGFP was quite rapid, as demonstrated in Supplemental Movie 1 and Supplemental Fig. 1. (The online version of this article contains supplemental data.) These data indicate that the lateral mobility of SGK1 in mitochondria is quite high; however, the rate of exchange between a cytoplasmic and mitochondrial compartment is rather slow.

**SGK1 immunoblot analysis in subcellular fractions from mouse kidney.** To confirm that endogenous SGK1 is also localized to the mitochondria, we performed Western blot analysis in cellular fractions obtained by differential centrifugation from the mouse kidney and liver. We used four different commercially available anti-SGK antibodies and monoclonal antibodies generated in our laboratory, but we were unable to detect endogenous SGK1 by using any of the antibodies. These antibodies recognized multiple bands, some of them with molecular weight close to the expected size of SGK1. However, the patterns were very similar in tissues from wild-type and SGK−/− mice (see Supplementary Fig. 2), indicating nonspecific reaction of the antibodies. We observed similar results using cultured cells instead of tissues.

**DISCUSSION**

The inherent substrate specificity of PKB/Akt and SGK family members is very similar (40). Nevertheless, their biological effects are significantly different from each other. For instance, SGK1 but not Akt increases the activity of the ENaC (5, 12). One way that the different biological actions in face of similar substrate specificity can be brought about is to target these kinases to different subcellular compartments, thereby restricting their substrate availability. Indeed, Akt and SGK family members have different subcellular targeting domains. All three Akt isoforms contain a pleckstrin homology (PH) domain at the NH2 terminus that targets them to phosphatidylinositol 3,4,5-trisphosphate-rich domains of the
plasma membrane (22). In contrast, none of the three SGK isoforms contains a PH domain, but SGK3 possesses an NH2-terminal complete phox homology (PX) domain that targets the protein to endosomes (45). The sgk1 gene also encodes an NH2-terminal PX domain; however, in the most common splice variant, this region is incomplete and thus most likely not functional.

SGK1 has several additional domains that could determine its intracellular localization. The main finding of this study is that SGK1 localizes to mitochondria and that the NH2-terminal

Fig. 8. Subcellular localization of SGK1 mutants in live RCCT-28A cells. Live RCCT-28A cells stably expressing Δ60-SGK1-EGFP (A), SGK1-N60-EYFP (D), or SGK1-EYFP-middle, a construct in which EGFP is inserted between amino acids 60 and 61 of SGK1 (G) were costained with MitoTracker (B, E, and H, respectively). C, F, and I: overlayed images of corresponding images at left and middle. Δ60-SGK1-EGFP is found throughout the cytoplasm and the nucleus and does not resemble the mitochondrial pattern observed with full-length SGK1. J and K: fluorescent images of a cell that was cotransfected with full-length SGK1-ECFP (J) and SGK1-N60-EYFP (K). The NH2-terminal 60-amino acid region of SGK1 is sufficient for mitochondrial localization of SGK1, as shown by the perfect colocalization of SGK1-N60-EYFP and the mitochondrial stain MitoTracker (D–F) as well as SGK1-ECFP (J) and SGK1-N60-EYFP (K). The fusion protein SGK1-EYFP-middle is also localized to mitochondria (G–I), indicating that this localization is not due to the COOH-terminal fusion of AFP.
region of SGK1 is necessary and sufficient for this localization. In addition, SGK1 contains a COOH-terminal conserved PDZ binding domain (38) and an unconventional nuclear localization signal in its catalytic domain (33). Recent work also identified an NH2-terminal hydrophobic motif that targets SGK1 for proteosomal degradation (6, 8). The presence of multiple targeting signals is not unusual, since many proteins express more than one targeting motif that may compete with each other. This process may be involved in the regulation of the localization and function of proteins.

The presence of competing targeting motifs and the possibility that these might be utilized depending on cell type and stimulus may be one reason why thus far no consensus has emerged regarding the subcellular localization of SGK1. Although our study showed mitochondrial localization, previous reports have localized SGK1 to the cytoplasm (1, 9), nucleus (11, 33, 40), or the ER (6, 8).1

Based on our data, it seems that the NH2-terminal region is the most important for subcellular targeting of SGK1. Our previous work showed that when AFP was fused to the NH2 terminus of SGK1, its localization was cytoplasmic and endosomal (38). This is in strong contrast with the mitochondrial location of the COOH-terminally fused AFP (present data). This phenomenon is most likely due to interference of the NH2-terminal tag with the mitochondrial localization signal. In support of this interpretation, the probability of mitochondrial localization using Mitoprot II 1.0a4 program is 85% for SGK-1 with AFP fused to its COOH terminus, but this probability drops to only 8% if the tag is fused to the NH2 terminus of SGK1 and to 0.3% if the NH2-terminal 60 amino acids are removed. If the PDZ binding domain were important for subcellular localization of SGK1, a COOH-terminal fusion

would also interfere with localization. Therefore, to assess the relative contribution of the NH2-terminal mitochondrial targeting signal vs. the COOH-terminal PDZ binding domain, we determined the subcellular distribution of a fusion protein in which the AFP was inserted into the middle of SGK1. The localization of this fusion protein was indistinguishable from that of the COOH-terminal GFP fusion, indicating that the dominant subcellular localization signal in SGK1 is the NH2-terminal mitochondrial signal.

In the present study, we never observed nuclear localization of SGK1 in renal or mammary epithelia, independent of stimulation with serum or other agents. In contrast, work from Firestone and colleagues (11) using a polyclonal antibody found SGK1 in the nucleus of ~30% of mammary tumor cells and equally localized between the cytoplasm and nucleus of ~50% of the cells after serum stimulation. However, SGK1 was found to be excluded from the nucleus in several studies by other laboratories using different antibodies or GFP-tagged-SGK1 (2, 8, 9, 15). In addition, using the same antibody as Firestone and colleagues (11), Loffing et al. (31) failed to observe nuclear localization of SGK1 in rat kidney sections. The resolution in this study was insufficient to determine whether the staining over the cytoplasm was associated with mitochondria (31).

Besides the differential utilization of multiple targeting motifs, another possible reason for these apparently contradictory results is the different methodology used in the different studies, i.e., immunohistochemistry with different antibodies vs. subcellular localization of SGK1-AFP fusion proteins. The advantage of using immunohistochemistry is that one detects the endogenous protein, which is biologically more relevant. However, if the half-life of the protein is very short, as is the case with SGK1 (~30 min), the antibodies will recognize all SGK1 proteins pools, including the pool of ubiquitinized SGK1 that is targeted for rapid proteosomal degradation (8, 9, 52). If this pool is much larger than the fraction of SGK1 that is not ubiquitinated and consequently has a longer half-life, the latter protein pool may be difficult to detect.

A further consequence of the short half-life is that endogenous SGK1 levels are very low, and this fact combined with the poor quality of available SGK1 antibodies may be another reason for the controversial localization data. For instance, using two different SGK1 antibodies, immunohistochemical studies gave diverging results on the intrarenal distribution of SGK1 in the same species. Whereas Loffing et al. (31) showed that SGK1 is highly expressed in the connecting and collecting tubules, Alvarez de la Rosa et al. (1) showed the highest expression of SGK1 in the thick ascending limb. An additional technical problem may be that the mitochondrial membrane is not permeabilized by the usual techniques applied for immunohistochemistry.

To circumvent these problems, we and others used AFP-tagged SGK1 to determine its subcellular localization. In general, this is a valid approach, since previous work has demonstrated an excellent correlation between the subcellular location of AFPs and the corresponding endogenous proteins (13). This approach can be used in live cells, and there are no membrane permeability problems involved. Since AFPs become fully fluorescent only after a maturation period of hours (16, 30), this approach is especially well suited to studying proteins with longer half-lives. Thus, in theory, the mitochon-

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1 While this work was in progress, an article published online also showed that SGK1 localizes to mitochondria (18).
drial localization of SGK1-AFP in our study may have been accentuated, because short-lived molecules in other compartments may not have been detected. However, our immunocytochemical experiments using an AFP antibody as the AFP-associated fluorescence (Fig. 7) suggest that there are no large pools of shorter-lived SGK1 molecules in other compartments, or we would have detected these compartments with the anti-AFP antibody.

On the other hand, if the mitochondrial localization protects SGK1 from proteosomal degradation, this would lead to an enrichment of fully functional SGK1 in the mitochondria. Previous work has demonstrated that the NH2-terminal region in SGK1 is critical for ubiquitination and subsequent degradation (8, 9). Most interestingly, this is also the region that we identified as being responsible for mitochondrial targeting. We speculate that this overlap between mitochondrial targeting and degradation signal is biologically relevant, because it could ensure that the pool of synthesized SGK1 molecules that is not destined to import into mitochondria is rapidly degraded. This idea is supported by the current FRAP experiments showing that the rate of exchange between cytoplasmic and mitochondrial pools is very slow compared with the overall half-life of SGK1 in the cell, confirming that mitochondrial SGK1 is protected from rapid degradation. This conclusion is in agreement with recent findings that ubiquitination of SGK1 occurs primarily on the cytoplasmic surface of the ER (8).

SGK1’s localization to the mitochondria is congruous with previous work that has found SGK1 not in the cytosol but in a 250,000-g pellet that contained mitochondria and from which nuclei were preisolated, prepared from various rat tissues including the kidney, jejunum, ileum, distal colon, and whole brain (1, 15). Similarly, Brickley et al. (9) found SGK1-FLAG to be enriched in a 100,000-g pellet (from which mitochondria were not preseparated) in breast cancer cells.

Previous work also has suggested that the subcellular location of SGK1 may be stimulus dependent: following osmotic stress and dexamethasone, SGK1 was predominantly in the cytoplasm, whereas under heat shock, UV irradiation, or H2O2 treatment, it was heterogeneously expressed (11). The same study suggested that location of SGK1 might be cell cycle dependent, given that in mammary tumor cells, SGK1 was cytoplasmic in the G1 phase and nuclear in the S and G2/M phases (11). In contrast, Coric et al. (15) failed to observe any nuclear localization of SGK1 in either the proliferating or resting cells of the distal colon. Similarly, we did not observe differences in subcellular location of SGK1-EGFP in unsynchronized RCCT-28A cells or after serum stimulation or phosphatidylinositol 3-kinase inhibition. Under all these conditions, SGK1 showed typical mitochondrial localization (data not shown). A possible explanation of this apparent controversy is that mitochondrial localization is a regulated process that may depend on the actual postsynthetic modifications on SGK1. For instance, SGK1 contains a conserved PKC-e phosphorylation site at T11, and if this site is mutated to the phosphominic asparate, the predicted mitochondrial localization probability falls from 94.7% (T11, no phosphorylation) to 13.7% (T11D).

An obvious question is: What is the function of SGK1 in the mitochondria? A logical assumption is that SGK1 is involved in the regulation of energy production and metabolism. SGK1 was found to regulate many ion channels and transporters when overexpressed in Xenopus oocytes (46). In native epithelia some of these transporters are located in the apical membrane, whereas others, such as Na+-K+-ATPase, are located in the basolateral membrane. Since apical and basolateral targeting of membrane occurs via distinct vesicle trafficking events, it seems unlikely that SGK1 regulates a common trafficking mechanism. On the other hand, an increased function of such overexpressed transporters is dependent on the cells’ energy status, and thus ATP generation is likely to be rate limiting. Increased mitochondrial energy production by SGK1 could be the common mechanism involved in the increased activity observed with these ectopically overexpressed transporters.

The mitochondrial targets of SGK1 remain to be identified, but PKB/Akt is known to increase mitochondria-associated hexokinase activity (21) and phosphorylate the β-subunit of ATP synthase and mitochondrial glycosyn synthase kinase (7). Another possibility for such a mechanism could be via the stimulatory effect of SGK1 on potassium channel Kv1.3 (24). Recently, Szabó et al. (44) reported that Kv1.3 is present in the inner mitochondrial membrane of T lymphocytes, in addition to its well-established location in the plasma membrane. K+ is required for optimal oxidative phosphorylation (26), and in energized mitochondria there is a significant K+ flux (44). Studies with Kv1.3-deficient mice (27) suggest that Kv1.3 regulates energy homeostasis and body weight (50). Thus SGK1, via the regulation of the activity of mitochondrial Kv1.3, may regulate basal metabolic rate.

SGK1, like Akt, has been shown to play a role in cell survival. The localization of SGK1 to the mitochondria could also play a role in cell survival. This was demonstrated in breast cancer cell lines, HEK-293 T cells, a CCL39 fibroblast cell line, and a rat mammary epithelial cell line (10, 11, 34, 49). The mitochondrial membrane contains a number of proapoptotic and antiapoptotic proteins (20). Thus a mitochondrially localized SGK1 could be involved in the inhibition of proapoptotic proteins or activation of antiapoptotic proteins.

One of the best-known functions of SGK1 is regulation of ENaC-mediated Na+ transport in kidney cells. Interestingly, in contrast to stably overexpressed SGK1, an NH2-terminally truncated SGK1 mutant, which does not localize to mitochondria (present study), failed to increase transepithelial Na+ current in renal collecting duct cells even though it is expressed at much higher levels than the full-length SGK1 (23), suggesting a link between mitochondrial localization and function. On the other hand, our previous studies showed that SGK1 in which the COOH-terminal PDZ binding domain was removed, when coexpressed with ENaC in Xenopus oocytes, had the same stimulating effect on ENaC function as the full-length protein, indicating that this domain is not essential for SGK1’s increase in ENaC activity (38).

Finally, it must be emphasized that although our data provide strong evidence for the mitochondrial localization of the SGK1-EGFP fusion protein, they do not exclude the localization of SGK1 to other cellular compartments that may have been below the limit of detection in our studies. In fact, the proposed interaction of SGK1 with Nedd4-2 (which thus far has been demonstrated only with overexpressed proteins) is more likely to take place in the cytoplasm. It also should be noted that the observation that the same protein has multiple localizations within the cell would not be unique for SGK1.

In summary, we have shown that transiently and stably expressed SGK1 is enriched in the mitochondria of renal and
mammary epithelial cells. This finding sheds new light on the possible functions and substrates of SGK1. The mitochondrial localization may help to explain the apparent multifunctional nature of SGK1. SGK1 may be involved in a more general cellular process that is necessary for channel activation, preventing apoptosis, neuronal excitability, and regulating cell volume.

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