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Serum and Glucocorticoid-Regulated Protein Kinases: Variations on a Theme

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Abstract The phosphatidylinositol 3′ kinase (PI3K)-signaling pathway plays a critical role in a variety of cellular responses such as modulation of cell survival, glucose homeostasis, cell division, and cell growth. PI3K generates important lipid second messengers—phosphatidylinositides that are phosphorylated at the 3′ position of their inositol ring head-group. These membrane restricted lipids act by binding with high affinity to specific protein domains such as the pleckstrin homology (PH) domain. Effectors of PI3K include molecules that harbor such domains such as phosphoinositide-dependent kinase (PDK1) and protein kinase B (PKB), also termed Akt. The mammalian genome encodes three different PKB genes (α, β, and γ; Akt1, 2, and 3, respectively) and each is an attractive target for therapeutic intervention in diseases such as glioblastoma and breast cancer. A second family of three protein kinases, termed serum and glucocorticoid-regulated protein kinases (SGKs), is structurally related to the PKB family including regulation by PI3K but lack a PH domain. However, in addition to PH domains, a second class of 3′ phosphorylated inositol phospholipid-binding domains exists that is termed Phox homology (PX) domain: this domain is found in one of the SGKs (SGK3). Here, we summarize knowledge of the three SGK isoforms and compare and contrast them to PKB with respect to their possible importance in cellular regulation and potential as therapeutic targets. J. Cell. Biochem. 98: 1391–1407, 2006.

Key words: SGK; PKB; PI3K; cell survival; oncogenesis

PHOSPHOINOSITIDE SIGNALING

In eukaryotes, the phosphatidylinositol 3′ kinase (PI3K)-signaling pathway triggers a wide range of cellular responses, from cytoskeletal rearrangements to cellular survival. This pathway has been the subject of intensive research since its deregulation is associated with several diseases, including diabetes, and cancer [Cantley, 2002]. There are several classes of PI3K that share the property of selective phosphorylation of the 3′ position of the inositol ring of phosphatidylinositides. Phosphatidylinositol (PI) is not a particularly abundant phospholipid in cell membranes and derivatives that are phosphorylated at its inositol head-group are even rarer. These latter enzymes form a family of molecules that comprise a lipid kinase domain and a regulatory domain. The "Class 1A" subfamily of PI3Ks is comprised of one of three 110 kDa catalytic domains (α, β, or δ) and one of five regulatory domains (p85α, p85β, p55α, p55β, p55γ). Each regulatory domain contains two Src homology-2 (SH2) domains that exhibit tight affinity for phosphotyrosine within specific sequence contexts. The "Class 1B" PI3K consists of a single catalytic subunit (p110γ) and a 101 kDa regulatory subunit.

Stimulation of cells by hormones such as insulin or polypeptide growth factors typically results in the phosphorylation of specific protein-tyrosine kinase receptors at multiple tyrosine residues or in activation of serpentine receptors-induced GTP binding to the Gα subunit of G proteins. The former signal typically couples to members of the Class 1A PI3Ks through phosphotyrosine recruitment of the regulatory subunits via their SH2 domains to

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the transmembrane receptors, whereas GTP-induced dissociation of Gβγ from Gα recruits the Class 1B PI3K to the plasma membrane. The PI3Ks are thus brought into proximity to their membrane-localized substrate leading to the generation of 3′ phosphorylated phosphoinositides in the locality of the activated receptors.

Generation of 3′ phosphorylated phosphoinotides is transient and is reversed by the action of a specific 3′-phosphoinositide 3′-phosphatase termed PTEN. This gene was first identified as a tumor suppressor that is frequently mutated in human cancers such as glioblastoma (in which over 75% of tumors show loss of PTEN), endometrial, prostate, and breast cancers [Yamada and Araki, 2001]. Germline mutations of PTEN cause Cowdens disease, a syndrome resulting in increased susceptibility to breast, brain, thyroid, and endometrial tumors [Scheid and Woodgett, 2001]. The result of those genetic abnormalities is the upregulation of the PI3K pathway signal. Inactivation of the lipid phosphatase removes the natural mechanism for restricting the levels of the PI3K product and, as a consequence, 3′ phosphorylated PI levels are constitutively elevated, leading to chronic activation of downstream processes (see below). But inactivation of PTEN is not the only mechanism by which these important phospholipids are deregulated. Amplification and mutational activation of the receptors that induce the pathway (notably Her2/neu) and the catalytic subunits of PI3K itself are also found in human cancer.

PI3K-Induced Signaling

The membrane localized pockets of newly generated 3′ phosphorylated PI serve to recruit proteins that harbor domains with high affinity for these modified lipids, including pleckstrin homology (PH) domains [Lemmon and Ferguson, 2000]. Over 100 proteins have been identified which these motifs including a series of protein-serine kinases. One of these, termed phosphoinositide-dependent protein kinase-1 (PDK1) plays a critical role in the activation of a many other protein kinases of the "AGC" subfamily [Mora et al., 2004]. PDK1 binds to a phosphorylated hydrophobic motif (HM) in 30+ protein-serine kinases including p70 S6 kinase and RSK, and then phosphorylates these proteins within their so-called "activation loop" (T-loop) leading to their catalytic activation. PDK1 was first discovered by its capacity to phosphorylate protein kinase B (PKBα/Akt1) at Threonine 308. Both PDK1 and PKB contain PH domains and, following growth factor activation of PI3K, both molecules are recruited to the newly synthesized patches of 3′ phosphorylated PI allowing the two enzymes to interact and for PDK1 to phosphorylate PKB. Phosphorylation of PKB at its hydrophobic motif (Serine 473 in PKBα) is also required for full enzyme activation and this is catalyzed by a distinct protein kinase, likely mTor/Rictor (and possibly other members of the PI3K-related kinase family such as ATM and DNA-PK, see below).

The advent of phospho-specific antibodies that recognize only the phosphorylated form of Serine 473 of PKBα permitted a relatively facile means of detecting the activation status of the PI3K pathway in tissue sections. Such analysis revealed that the PI3K pathway is commonly activated in a variety of tumors. Although PKB plays important roles in tumorigenesis (by promoting survival and allowing cells to tolerate significant genetic damage without triggering apoptosis), its activation is unlikely to be sufficient to drive neoplastic transformation. For example, transgenic mice expressing activated alleles of PKBα in the mammary gland do not form mammary tumors—although it does promote tumors initiated by other mutations such as activated Her2/neu [Hutchinson et al., 2004]. This indicates that PKB is a useful marker for, but not necessarily a driver of, tumorigenesis.

The Serum and Glucocorticoid Regulated Kinase Family

Like PKB, the SGK family is encoded by three genes in mammalian genomes: SGK1, 2, and 3. SGK1 was first identified through a differential screen for glucocorticoid-inducible genes in a rat mammary tumor cell line [Webster et al., 1993]. SGK1 mRNA is also upregulated by additional stimuli such as serum, aldosterone, FSH, transfection with p53, extracellular osmolarity, TGFβ, and hyperosmotic stress [Waldegger et al., 1997; Bell et al., 2000; Gonzalez-Robayna et al., 2000]. The SGK1 promoter region contains a glucocorticoid response element, a TATA box, Sp-1 elements, and putative-binding sites for p53, AP-1, CCAAT/enhancer-binding protein, NF-κB, GATA, and Ets-2 (M.Tessier, unpublished). SGK2 and 3 were cloned by virtue of their similarity to the SGK1 sequence. Their catalytic domains share 80% sequence identity. Despite being structurally related to SGK1,

SGK2, and SGK3 are not transcriptionally regulated [Kobayashi et al., 1999]. All three isoforms of SGK were shown to be post-translationally modified by phosphorylation and to be novel downstream components of the PI3K pathway [Kobayashi et al., 1999; Park et al., 1999]. SGK1 and 3 are ubiquitously expressed, while SGK2 RNA is only present in liver, kidney, and pancreas, and at lower levels in the brain.

The SGK family exhibits structural similarity to PKB and this has contributed to interest in the functions of the family, given the important roles for PKB in various human disorders. The kinase domain of the SGKs share 55% identity with the PKB kinase domain, have a similar carboxy terminal HM as well as conserved residues to those required for PKB activation [Kobayashi and Cohen, 1999]. All three SGK isoforms require PI3K activation for function and are also direct substrates of PDK1. Unlike the PKB family, however, the SGKs do not possess a PH domain—raising the question of how they might be regulated by PI3K. A report by Liu et al. [2000] identifying the mouse homologue of SGK3 as cytokine independent survival kinase (CISK) revealed the presence of a distinct type of phospholipid-binding domain called Phox homology (PX) domain. This domain was initially identified in the NADPH phagocyte oxidase enzyme complex [Kanai et al., 2001]. Most PX domains to date have been found to bind the monophosphorylated lipid, phosphatidylinositol 3′phosphate (PI(3)P), and to direct PX domain proteins to endosomal membranes [Ellson et al., 2002].

These data indicate a disconnection between the similarity in the regulatory residues and the upstream, activating kinases of PKB and SGK, and the dissimilarity in the presence/absence and type of their lipid-binding domains and subcellular localization. In this review, we summarize recent findings regarding the mechanisms of activation and the physiological functions of the SGKs, emphasizing the similarities and differences between PKB and SGK.

MECHANISMS OF REGULATION

PKB Activation

Mechanisms of regulation of PKB have been extensively covered in recent review articles [Woodgett, 2005]. In the interest of space, the current model of PKB activation is only summarized (Fig. 1). Under basal conditions, PKB is primarily localized in the cytoplasm. PI3K stimulants lead to the production of $PI(3,4,5)P_3$. PDK1 and PKB are recruited to the membrane via binding of their PH domains to $PI(3,4,5)P_3$. Colocalization of PDK1 and PKB promotes association of the two proteins although this is likely promoted by phosphorylation of the HM site (S473) of PKB, leading to phosphorylation of PKB at its T-loop site (T308). The identity of the kinase responsible for phosphorylating the HM of PKB is far more controversial. Several third party protein kinases have been shown to be capable of promoting HM site phosphorylation including integrin-linked kinase (ILK), PKCβII, DNA-PK, and ATM [Woodgett, 2005]. Direct evidence for each of these candidates is somewhat lacking. More recently, a rapamycin-insensitive form of mTOR in a complex with Rictor:GβL was shown to induce phosphorylation of PKB at S473 and this is blocked by suppression of expression of mTOR or Rictor by RNAi [Sarbassov et al., 2005]. Since S473 phosphorylation is dependent upon PI3K activation, it is as yet unclear whether or how the mTOR/Rictor complex is regulated by PI3K. It is possible that a S473 phosphatase is inhibited upon PI3K activation. Moreover, a discrete complex of mTOR with a protein termed Raptor phosphorylates and activates p70 S6 kinase. The mTOR/Raptor complex is regulated by the small GTPase, Rheb, which is controlled by the TSC1/2 GTPase activating complex [Nobukini and Thomas, 2004]. Of note, the function of TSC1/2 is dependent upon PKB such that PKB phosphorylation of TSC2 interferes with its GTPase activating function, leading to GTP loading and activation of Rheb and hence, mTor/Raptor. It is unclear whether the two populations of mTOR complexes, one acting upstream of PKB and the other acting downstream, interact in some manner. Finally, dually phosphorylated and activated PKB is released from the plasma membrane, diffuses through the cytosol and the nucleus where it phosphorylates a wide array of substrates involved in cell division, cell growth, survival, and cytoskeletal rearrangements.

SGK Activation

In contrast to the relative maturity of the PKB field, the mechanisms of SGK regulation

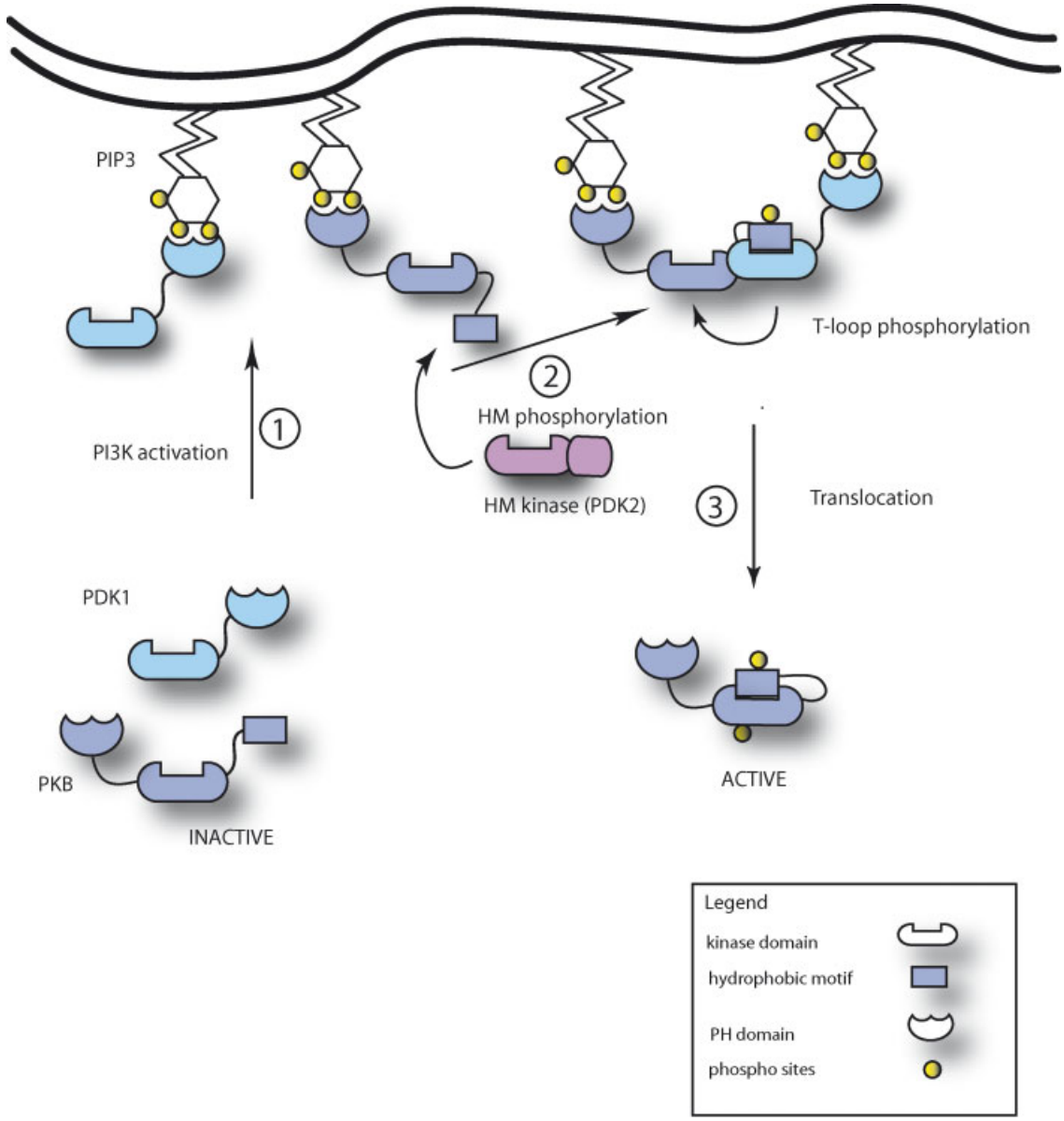

Fig. 1. Mechanism of PKB/Akt activation. Inactive PKB resides in the cytoplasm in an unphosphorylated state. **1**: Activation of the PI3K pathway results in generation of the 3′ phosphorylated lipid, phosphatidylinositol 3,4,5′ phosphate (PIP3) in the plasma membrane that then attracts binding of PH domain containing proteins, such as PKB. **2**: Once at the membrane, the hydrophobic motif of PKB is phosphorylated by an HM kinase (PDK2) at Serine 473. **3**: The HM motif phosphorylated PKB is then phosphorylated at its T-loop (Threonine 308) by PDK1 which is co-localized to PKB via its own PIP3 binding PH domain. The doubly phosphorylated PKB adopts a fully active conformation and diffuses throughout the cell to target its substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

have only recently been probed. In addition to its transcriptional regulation, SGK1 is also regulated by ubiquitination. Several groups reported difficulty in expressing full length SGK1. Removal of the first 60 amino acids of the protein led to a more stable protein, suggesting that this region may be important in the turnover rate of SGK1. Indeed, the amino terminal domain of SGK1 contains a region responsible for its ubiquitination. Under basal conditions, SGK1 levels are kept low by poly-ubiquitination and subsequent degradation by the 26S proteosome.

Being the first identified member of the SGK family, SGK1 has benefited from the most attention. In 1999, SGK1 was demonstrated to be regulated at a third level, by post-translational modification [Kobayashi and Cohen,

1999; Park et al., 1999]. Induction of the PI3K pathway increased SGK1 catalytic activity by 3–12-fold. This activation was abolished by pretreatment with LY294002, a specific PI3K inhibitor, indicating that SGK1 is a downstream component of the PI3K pathway. Mutation to alanine of the residues in the HM and T-loop of SGK1, equivalent to the activating sites in PKB, inhibited activation of SGK1. Thus, like PKB, two phosphorylation events are needed for full activation of SGK1 (Fig. 2).

It is widely accepted that PDK1 phosphorylates SGK1 on its T-loop residue (Fig. 2). PDK1 can activate SGK1 both in vitro and in vivo [Kobayashi and Cohen, 1999]. A pocket in the kinase domain of PDK1 was termed PDK1-interacting fragment (PIF)-binding domain, due to the binding of a fragment of protein

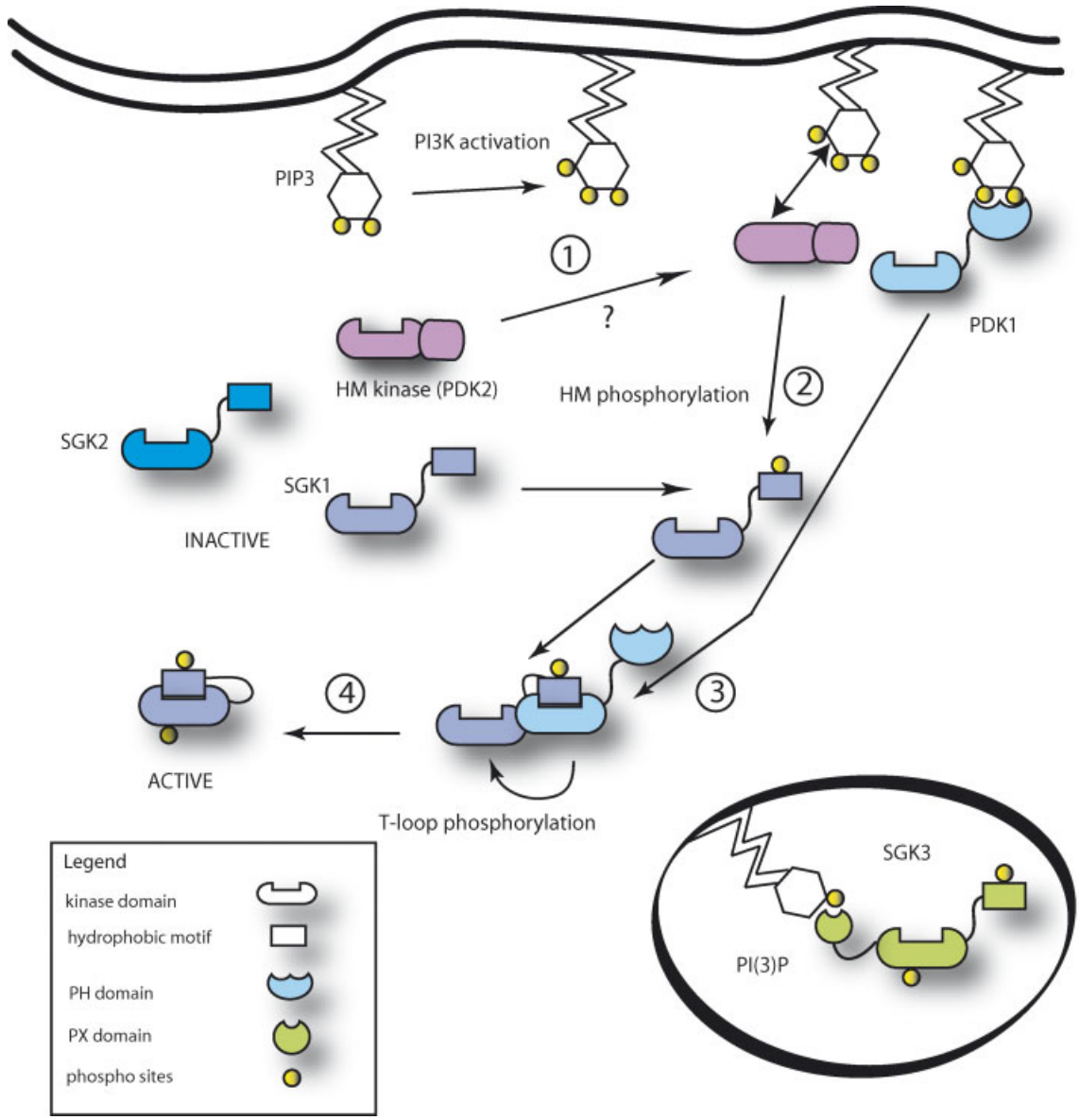

Fig. 2. Mechanisms of SGK activation. Inactive SGKs are located in the cytoplasm (SGK1 and 2) or are associated with the endosomes (SGK3). **1**: Upon PI3K activation, an HM kinase becomes activated. **2**: This kinase phosphorylates the SGKs at their hydrophobic motifs. **3**: This forms a binding site for PDK1 that associates with the HM and phosphorylates the T-loop site. **4**: The doubly phosphorylated protein adopts an active conformation and targets its substrates. SGK3 activation is restricted to the endosomes due to its PX domain that has high affinity for 3′ phosphorylated phosphatidylinositol (PI(3)P). This likely restricts the substrates accessible to this isoform. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

kinase C-related kinase 2 to that region of PDK1. Introduction of the PIF peptide blocks the activation of SGK1 by PDK1, thus associating the PIF-binding pocket to a substrate recognition pocket [Biondi et al., 2001]. It mediates the interaction and phosphorylation of SGK1 by PDK1. Prior phosphorylation of SGK1 at its carboxy terminal HM promotes the binding of the PIF-binding pocket of PDK1 to SGK1 leading to PDK1-dependent phosphorylation of its T-loop. SGK1 is not activated in embryonic stem cells in which a PDK1 PIF pocket mutant was knocked in, replacing the wild-type PDK1, while activation of PKB was normal in these cells [Collins et al., 2003]. Further knock-in studies have revealed that the phosphate and hydrophobic grooves of PDK1, another region that interacts with its substrates, are required for maximal activation of SGK [Collins et al., 2005]. It is likely that SGK1, in lacking a PH domain, relies more heavily than PKB on structural interactions with PDK1 to achieve full activation. While to date there have been no data published regarding the identity of the HM kinase acting on the SGKs, it is reasonable to assume that this enzyme(s) will be similar to that acting on PKB.

A few reports have described activation of SGK1 without PI3K activation. In a yeast two-hybrid screen using ERK5/BMK1 as bait, SGK1 was identified as a potential binding partner [Hayashi et al., 2001]. BMK1 can phosphorylate SGK1 at S78, independently of PDK1. Adhesion of MDCK cells to immobilized fibronectin leads to activation of SGK1 that is insensitive to inhibition of PI3K [Shelly and Herrera, 2002; Imai et al., 2003]. One study also found that SGK1 is activated in a PI3K-independent manner by a Ca^{2+}/calmodulin-dependent protein kinase pathway in CHO cells [Imai et al., 2003]. The possibility that SGK2 and 3 are activated via PI3K-independent mechanisms has not been addressed.

Unlike PKB, where the three human isoforms are regulated in a very similar manner, the mechanisms of activation of the SGK isoforms appear to differ considerably. First, unlike SGK1, the genes encoding SGK2 and SGK3 are not transcriptionally modulated. SGK2 and SGK3 appear to be regulated entirely by phosphorylation at T193 and S356, and T253 and S419, respectively [Kobayashi et al., 1999].

Another major difference between the SGK isoforms is the presence of a PX domain only in SGK3. SGK1 and 2 contain a truncated and non-functional PX domain and their localization is mainly cytoplasmic. The presence of a PX domain in SGK3 requires rethinking of the means by which SGK is regulated (Fig. 2). Studies on CISK showed that the PX domain targets the protein to endosomes and that this localization is PI3K-dependent [Xu et al., 2001]. However, immunofluorescence experiments to determine the effects of PI3K inhibition on CISK localization did not include a marker of the endocytic compartment. Since PI3K is itself involved in endosome dynamics, the possibility remained that endosomal structure is disrupted upon treatment of cells with PI3K inhibitors. Thus, redistribution of SGK3 from the endosomal compartment to the cytosol upon PI3K inhibition cannot necessarily be attributed to a change of localization. There is also some controversy regarding the nature of the phospholipids with highest affinity for the PX domain of CISK. One group reported that the CISK PX domain binds strongly and selectively to PI(3)P [Virbasius et al., 2001], whereas a second group reported that it interacts with PI(3,4)P2, PI (3,4,5)P3 and to a lesser extent to PI(4,5)P2 [Xu et al., 2001]. A protein-lipid overlay assay carried out in our laboratory confirmed selective binding of the PX domain of human SGK3 to the monophosphorylated PI, PI(3)P, the vast majority of which is found in endosomes. A crystal structure of the isolated PX domain of CISK identified the presence of a phosphoinositide-binding pocket that determines the lipid specificity [Xing and Xu, 2003]. Coupling of a hydrophobic loop region with an hydrophilic β-turn promotes the localization of CISK by non-specific interactions with the membrane. When the linker region between the PX and the kinase domain was included in the protein fragment used for crystallization, dimerization was observed. This dimerization may be important in determining the efficiency of CISK localization and regulation. To date, no crystal structures of the kinase domain of SGKs have been reported.

In addition to being important for targeting CISK to endosomes, the PX domain also modulates protein kinase activity. A mutation in the phospholipid-binding pocket of the PX domain abrogates phospholipid binding and abolishes most of CISK kinase activity towards exogenous substrates [Virbasius et al., 2001; Xu et al., 2001]. This finding could be explained by

one of several possibilities. Binding of SGK3/CISK to a phospholipid bilayer could be important for its activation or that the proper localization of the protein may be a determining factor to achieve activation.

Other important facets in understanding signaling pathways are cell-type specific effects, the nature of the agonists that trigger activation, the duration of the stimulation of the pathways and the temporal kinetics of activation of upstream and downstream protein kinases. Similar PI3K agonists, like insulin and IGF-1, lead to activation of PKB and SGKs. However, these agonists typically cause only a twofold increase in SGK activity as measured by an in vitro kinase assay—which may indicate that PI3K signaling is required but not sufficient for efficient activation of SGKs. In addition, it is possible that relative timing is a factor in the action of PKB and SGKs. In our hands, SGK3 is activated more slowly than PKB, suggesting that the endosomal location of SGK3 causes a delay in the activation process compared with the plasma membrane. Given that PKB and SGK3 share at least one upstream activator, namely PDK1 and possibly mTOR/Rictor, and given that recruitment of PDK1 to SGK3 requires phosphorylation of the HM of SGK3 which is located on the endosomal membranes, the question is, how does PI3K activation lead to SGK3 phosphorylation at the HM? Studies carried out in our laboratory indicate that the targeting of SGK3 to endosomes, mediated by its PX domain, is essential for proper SGK3 activation, likely due to co-localization of SGK3 with an endosomal, PI3K-dependent, and staurosporine-sensitive HM kinase (M.Tessier, unpublished).

PHYSIOLOGICAL FUNCTIONS

PKB and SGK Substrates

Numerous potential and confirmed substrates of PKB have been identified (Table I) (reviewed in [Bellacosa et al., 2005]) which have yielded clues to the main cellular functions of this protein kinase family, namely insulin action and metabolism, cell survival, cell proliferation and cell growth. By contrast, most of the physiological substrates of the SGKs, and, by extension, their functions within cells, remain elusive (Table I) [Kobayashi and Cohen, 1999; Liu et al., 2000; Brunet et al., 2001; Zhang et al., 2001; Palmada et al., 2003; Boehmer et al., 2003c; Chun et al., 2004; Dieter et al., 2004; Murray et al., 2004; Rangone et al., 2004; Snyder et al., 2004; David and Kalb, 2005]. One of the reasons is that several AGC family members other than SGKs, like PKB, RSK, and S6K, share similar substrate specificities. Peptide library screening has shown that each of these kinases prefers to phosphorylate an R-X-R-X-X-S/T motif, with a few subtle differences. It is also probably that these protein kinases have substrates in common. However, based on the fact that the mouse models of these protein kinases exhibit distinct phenotypes and given their regulatory distinctions, it is reasonable to presume that they also have specific functions and targets. Early studies of PKB and SGK substrate specificity demonstrated that PKB exhibits a preference for a serine residue as the phosphoacceptor and requires the presence of a bulky hydrophobic amino acid at the +1 position. In contrast, SGKs prefer a threonine at the phosphorylation site and have no requirement for a hydrophobic residue at the +1 position [Kobayashi and Cohen, 1999]. These results raised the possibility that SGK may phosphorylate the same substrates as PKB at the same sites or at different sites, and that SGK may also have targets that are not PKB substrates.

The Cohen group confirmed that SGK is more flexible in its preference for the amino acids surrounding the phosphorylation site motif [Murray et al., 2004]. This group developed a method for the identification of protein kinase substrates termed KESTREL and has utilized the technique to discover NDRG1 as substrate of SGK1 and not of PKB. This protein is phosphorylated by SGK1 at three sites in vitro and the phosphorylation of NDRG1 is abrogated when expression of SGK1 is knocked down and in SGK1 null cells. Using the sequence surrounding the SGK1 phosphorylation sites on NDRG1, these investigators showed that the inability of PKB (or RSK1) in phosphorylating it resides in the presence of three amino acids within the motif: a serine at n−4, a serine at n + 1 and a glutamate at n + 2. Although this finding may help to identify further specific substrates of SGK1, sequences outside of the recognition motif also affect phosphorylation, one example being the importance of the HM interactions. Also, the three-dimensional structure of proteins in vivo determines if the substrate motif is accessible to be recognized and phosphorylated.

TABLE I. Direct Substrates of PKB and SGK Families of Kinases

	PKB		SGK1		SGK2	SGK3	
Functions	Substrates	References	Substrates	References	Substrates	Substrates	References
Metabolism	GSK3	Reviewed in Bellacosa et al. [2005]	GSK3	Kobayashi et al. [1999]	N/A	Nedd4-2	Dieter et al. [2004]
	PDE-3B						
	6-PF2-K		Nedd4-2	Snyder et al. [2004]		GSK3	Liu et al. [2000]
	mTOR		SCN5A	Boehmer et al. [2003c]			
	TSC2						
	Wnk1						
	PIKfyve		ROMK1	Palmada et al. [2003]			
	ArgBP2						
	Synip						
	AS160						
	Nedd4-2						
	PTP1B						
Apoptosis	BAD		Raf	Zhang et al. [2001]			
	PEA-15						
	Caspase 9						
	ASK1						
	MLK3						
	SEK1						
	Raf						
	Gab2						
	EDG-1						
Transcription factors	FKHR		FKHRL1	Brunet et al. [2001]			
	FoxO2						
	FKHRL1		CREB	David et al. [2005]			
	AFX						
	IKK						
	ALPHA						
	Tpl2/Cot						
	CREB						
	AR						
	YAP						
	YB-1						
	Trachealess						
Cell cycle	p21/cip1/waf1						
	p27/kip1						
	MDM2						
	ARK5						
Immune system	Nurr77						
	HO-1						
	p47						
	phox						
Neurochemistry	GABA-R		NDRG1	Murray et al. [2004]			
	Huntingtin		NDRG2	Rangone et al. [2004]			
	NDRG2		Huntingtin	Chun et al. [2004]			
	Ataxin-1		Tau				
Angiogenesis	eNOS						
Cytoskeleton	Filamin C						

Cell Survival, Proliferation, and DNA Damage

Due to its resemblance with PKB, the first potential function of SGK1 to be tested was cell survival and proliferation. The first hint that SGK1 may play a role in cell-cycle progression derived from the observation that SGK1 translocates from the cytoplasm to the nucleus with the help of importin-α in a manner dependent on growth factors/hormonal stimulation and on the phase of the cell cycle [Buse et al., 1999; Maiyar et al., 2003]. In the presence of serum and

during S phase, SGK1 is predominantly nuclear; in the absence of growth factors and during other cell-cycle phases, SGK1 is cytoplasmic. BMK1-mediated phosphorylation of SGK1 is required for entry of cells into S phase [Hayashi et al., 2001]. The localization of SGK2 is believed to be primarily cytoplasmic [Kobayashi et al., 1999]. SGK3 is endosomally localized, but contains an atypical NLS within the kinase domain that only functions in the context of a mutated PX domain (M.Tessier, unpublished).

Given this nuclear translocation, it is likely that at least some SGK1 targets (and maybe certain SGK3 targets) will be nuclear. One candidate is the Forkhead family of transcription factors, which induce apoptosis and/or cell-cycle arrest. SGK1 phosphorylates T32 and S315 on the forkhead transcription factor FKHRL1/FOXO3a in neuronal cells, whereas PKB phosphorylates T32 and S253 in vitro and in vivo [Brunet et al., 2001]. By phosphorylating FOXO3a, SGK1 promotes its exit from the nucleus and binding to 14-3-3, thereby inhibiting function. Phosphorylation of all three sites is needed for efficient suppression of FKHRL1-mediated transcription, suggesting that, at least for this function, SGK1 may act in concert with PKB. In addition, SGK1 has an indirect effect on the cell cycle through inhibition of the forkhead transcription factors that have, among others, the p27 CDK inhibitor protein as a target. Another important modulator of survival signaling in mammalian cells is attachment to the extracellular matrix. SGK1 is activated by HGF and integrins in MDCK cells. Detachment induced dephosphorylation of FKHRL1 in MDCK cells is prevented by activated SGK1, another indication that SGK1 regulates cell survival [Shelly and Herrera, 2002].

Suppression of forkhead activity by SGK1 was also observed in *C. elegans*. DAF-16 is responsible for regulating diapause, longevity, stress response, metabolism, and reproductive growth in the nematode [Hertweck et al., 2004]. Previous work indicated that RNAi of Akt-1 and Akt-2 in worms failed to fully shutdown insulin signaling and that mutation of the consensus Akt-1 and Akt-2 phosphorylation sites in DAF-16 did not yield dauer formation or increased longevity, suggesting the existence of additional components of this pathway. Through genetic and biochemical analyses, Hertweck et al. showed that *C. elegans* SGK1 (sgk-1) is strictly dependent on PDK1 for its activation and that this activation leads to phosphorylation, nuclear exclusion, and inhibition of DAF-16 activity. These data suggest that sgk-1 is a critical factor in the control of development, stress response, and longevity. The nematode studies support the concept that PKB and SGK1 have distinct but overlapping functions.

The role of SGK1 as a stress response protein can be extended to mammalian cells. Indeed, a variety of environmental stresses, like UV irradiation, heat shock, oxidative stress, and hyperosmotic stress, induce SGK1 protein levels through the p38/MAPK pathway and SGK1 phosphorylation through the PI3K pathway in non-tumorigenic mammary epithelial cells [Leong et al., 2003]. Each stress has different kinetics and durations of induction. Interestingly, under conditions where SGK1 is induced and activated, PKB was shown in an unphosphorylated, inactive state. Wild-type and constitutively active forms of SGK1 confer protection of cells from sorbitol, heat, UV, and H_2O_2-induced cell death. This is due at least in part by decreasing Forkhead-dependent transcription.

As noted above, the identification of CISK, as a gene that allows IL-3-independent survival in murine lymphoblast cells suggested that it could act as a pro-survival gene. In support of this, there are data showing that CISK inhibits FKHRL1-dependent transcription and might affect the phosphorylation status of a Bcl2 family member, Bad, on a different site than that targeted by PKB [Liu et al., 2000]. Another evidence for a potential role of SGK in survival of immune system cells is the upregulation of SGK1 in neutrophils treated with granulocyte macrophage-colony stimulating factor, a cytokine that prolongs neutrophil survival, and its downregulation during neutrophil apoptosis [You et al., 2004; Kobayashi et al., 2005].

In contrast to its function in cell survival, the involvement of FKHRL1 in response to DNA damage is less clear. In mouse embryonic fibroblasts, p53 induced by DNA damage leads to FKHRL1 phosphorylation, nuclear exclusion, and transcriptional inhibition [You et al., 2004]. There is some evidence to suggest that SGK1 is induced by p53 via an ERK1/2-dependent post-translational modification after DNA damage. However, the transactivation domain of p53 is required for induction of SGK1. The effect of p53 on SGK1 is cell-type specific: several p53-

binding sites were mapped within the promoter of SGK1 in non-tumorigenic and tumorigenic mammary epithelial cells and shown to play a role in increasing SGK1 promoter activity [Maiyar et al., 1996]. However, in Rat-2 fibroblasts, transcriptional activation of SGK1 by p53 was repressed. PKB was found to be dispensable for p53-dependent suppression of FKHRL1 [You et al., 2004].

Another connection between SGK and growth control is that SGK1 acts in the Raf-MEK-ERK kinase cascade. SGK1 phosphorylates B-Raf on S364 and inhibits its activity in response to serum or Ras [Zhang et al., 2001]. The PKB sites on B-Raf are different and SGK1 seems to be a more potent inhibitor of B-Raf. SGK1 has also been shown to phosphorylate S166 and S337 of MEKK3, thereby inhibiting its function and promoting cell-cycle progression and cell proliferation [Chun et al., 2003]. SGK1 was also identified as an ERK-inducible gene using subtractive screening in an inducible B-Raf cell system [Mizuno and Nishida, 2001]. The protein levels of SGK1 are increased with growth factors that activate the ERK/MAP kinase pathway, and this induction is blocked in the presence of the MEK inhibitor, U0126. It is possible that SGK1 expression and activation are controlled by the ERK pathway and the PI3K pathway, respectively.

Transgenic and Knockout Mouse Models

Cell and transgenic mouse models expressing activated alleles of the three PKB isoforms have proven valuable in deciphering their role in whole organisms. The phenotypes of the knockouts and transgenic animals for PKB and SGK are summarized in Table II (for PKB—reviewed in [Yang et al., 2004]) [Wulff et al., 2002; McCormick et al., 2004]. With no SGK2 knockout and no transgenic mouse models of any SGK isoforms, there is a clear need for better in vivo models of the SGKs.

A potential connection between SGKs and the Wnt pathway emerged from the generation of SGK3 knockout mice. SGK3 null mice do not exhibit any of the phenotypes associated with the various reported PKB knockout mice [McCormick et al., 2004; Alonso et al., 2005]. Mice lacking SGK3 are viable and fertile, and display a defect in post-natal hair follicle development. Starting at post-partum day 4 (PP4), a clear difference in hair follicle progression was observed which became more pronounced at PP19 where the wild-type hair follicles were in the telogen phase (the last phase of hair growth) and the null hair follicles were in anagen (the first phase). In addition, the hair follicle of the knockout animals was disorganized, suggesting that SGK3 may also be involved in cell differentiation and migration. Results of PCNA nuclear staining, in vitro proliferation assays, and TUNEL staining indicated that the hair follicle defect is likely due to reduced proliferation. The defect correlated with lower levels of β-catenin nuclear localization in knockout mice. Furthermore, SGK3 was able to mediate EGF/TGF-α activation of β-catenin/LEF-1-dependent transcription in cultured keratinocytes. The combined effect of activating LEF1 and inhibiting forkhead transcription may contribute to the proliferative status of the hair follicle. The SGK3 knockout phenotype is consistent with the phenotypes of

TABLE II. Animal Models of PKB and SGK Families

Mouse models	Phenotypes	References
Whole body knockouts		
Akt1/PKBα	Growth and apoptosis defect	Reviewed in Yang et al. [2004]
Akt2/PKBβ	Glucose metabolism defect	
Akt3/PKBγ	Reduced post-natal brain growth	
SGK1	Induced sodium balance intolerance	Wulff et al. [2002]
SGK2	N/A	
SGK3	Transient defect in hair follicle development	McCormick et al. [2004]
Transgenics		
Heart PKB	Hypertrophy and increased contractility	Reviewed in Yang et al. [2004]
Thymus PKB	Early onset of thymic lymphoma	
Mammary gland PKB	Fatty milk synthesis and involution delay	
Pancreas PKB	Hypertrophy, hyperplasia and hyperinsulinaemia	
Prostate PKB	Neoplasia	
SGK	N/A	

the EGF receptor null mice, Wa-2 mice, TGF-α null mice, keratinocyte-specific PTEN null mice, and the keratinocyte-specific β-catenin overexpression mice. The similarity in phenotypes between the PTEN null and SGK3 null mice is unexpected since PTEN negatively regulates SGK3. Thus, it is possible that the phenotype is caused by distinct mechanisms in at least these two models, and perhaps the others. In addition to the follicle phenotype, the SGK3 null mice also show a transient growth defect until 7 weeks of age. This delayed growth may be due to a decrease in intestinal glucose transport through the sodium-dependent glucose transporter SGLT1 (see Salt Homeostasis/Channels and Transporter Proteins section).

Oncogenic Potential

Given the participation of SGK1 (and SGK3 to some extent) in cell survival and proliferation through the PI3K pathway, study of the involvement of these kinases in cancer initiation and progression is warranted. Asano and colleagues compared the roles of PKB and SGK1 in glucose metabolism, DNA synthesis and oncogenic activity [Sakoda et al., 2003]. They found that myristoylated PKB, which is constitutively active, could increase glucose transport, increase phosphorylation of GSK3, promote DNA synthesis and oncogenic transformation in soft agar. A constitutively active mutant of SGK1 failed to enhance glucose transport, DNA synthesis, or oncogenic transformation, but phosphorylated GSK3 to the same extent as PKB.

Nevertheless, other indirect lines of evidence suggest that SGK may contribute to tumorigenesis. Initial cloning of SGK1 was carried out in a rat mammary tumor cell line [Webster et al., 1993] and we have isolated human SGK3 from a mammary carcinoma. SGK1 expression is increased in some hepatocellular carcinomas [Chung et al., 2002] and in ductal breast carcinoma in situ as shown by cDNA microarray analysis [Adeyinka et al., 2002]. Moreover, SGK1 is overexpressed in 29 out of 38 breast cancer tissue samples on the National Cancer Institute "TARP-2" breast cancer tissue array [Zhang et al., 2005], and in 48% of tumor samples in a group of 40 independent breast cancer specimens from the University of Chicago [Sahoo et al., 2005]. Interestingly, there is a statistically significant association between SGK1 and phospho-PKB in the samples tested, suggesting they play complementary roles. SGK1 may also have a function during IL-6 survival signaling in choliangiocarcinomas [Meng et al., 2005]. It will be interesting to determine if SGK1 overexpression is a trait of other cancers.

A recent report outlines a possible mechanism for the role of SGK1 in cancer formation. Loss of RhoB, an important cell proliferation checkpoint in normal cells, correlates with elevated SGK1 phosphorylation, predominant SGK1 localization to the cytosol, exclusion of GSK3 from the nucleus, and inactivation of GSK3 by SGK1, not PKB [Huang et al., 2005]. This results in c-myc stabilization and a strong push towards tumorigenesis.

SGK1 may play a role in promoting the survival of breast cancer cells. SGK1 is overexpressed in breast cancer cells in response to glucocorticoids and protects these cells from growth factor starvation-induced apoptosis [Mikosz et al., 2001]. SGK1 may be a potential regulator of c-fms, a proto-oncogene involved in adhesiveness of breast cancer cells, in response to glucocorticoids or to colony stimulating factor-1 [Tangir et al., 2004]. In mammary epithelial tumor cells, an IPTG-inducible SGK1 approach indicated that SGK1 could protect against growth factor-starvation cell death [Leong et al., 2003]. Dexamethasone pretreatment of breast cancer cells inhibits chemotherapy-induced apoptosis in a glucocorticoid receptor-dependent manner and is associated with the transcriptional induction of SGK1 [Wu et al., 2004]. This finding has clinical relevance since dexamethasone is often administered prior to chemotherapy in the treatment of breast cancer. Herceptin, a widely used monoclonal antibody therapy against Her2/Neu, impacts the PI3K pathway. Treatment of cells with Herceptin reduces PDK1 activity and PKB phosphorylation in breast cancer cells and has similar effects on SGK3 (M. Tessier, unpublished). Given the established role of PI3K activation in tumorigenesis, the above evidence suggests that SGKs may be potential therapeutic targets for cancer treatment including breast cancer.

Salt Homeostasis/Channels and Transporter Proteins

The most compelling physiological function for SGK1 is in modulation of salt balance. SGK1 knockout animals generated in the Pearce

laboratory demonstrate a defect in sodium homeostasis [Wulff et al., 2002]. Sodium reabsorption occurs in the aldosterone-sensitive distal nephron of the kidney through the epithelial sodium channel (ENaC). When fed a normal salt diet, the SGK1 knockout animals are undistinguishable from their wild-type littermates. However, when SGK1 knockout mice are placed on a low-salt diet, they show signs of aldosterone resistance, such as low sodium retention, weight loss, and low blood pressure. The phenotype of other knockouts of components of the sodium transport chain is early death due to severely impaired renal salt wasting. The mild phenotype of the SGK1 knockout suggests the presence of compensatory mechanisms—possibly via the actions of SGK2 and SGK3. Indeed, these SGK isoforms are expressed in the kidney and are both able to stimulate ENaC. However, there is no impairment in renal salt transport in the SGK3 null mice.

The molecular mechanisms by which SGK1 regulates sodium reabsorption are unclear. A current model is that SGK1 integrates PI3K, cAMP and mineralocorticoid regulation of epithelium sodium transport [Snyder et al., 2004]. SGK1 is transcriptionally induced by aldosterone and activated by PI3K. SGK1 then increases ENaC activity partly by increasing the abundance of this receptor at the apical membrane. There is mounting evidence that the link between ENaC and SGK1 is the E3 ubiquitin ligase Nedd4-2, which serves to promote the ubiquitination and thus degradation of ENaC. Several groups have shown that Nedd4-2 is phosphorylated by SGK1 in vitro and in vivo and that this event disrupts the interaction of Nedd4-2 with ENaC, probably because phosphorylated Nedd4-2 binds 14-3-3, thereby causing an accumulation of ENaC and increased sodium transport [Asher et al., 2003; Bhalla et al., 2005]. However, it remains possible that SGK1 acts on sodium transport in a Nedd4-2-independent manner. For example, it has recently been reported that ENaC harbors an SGK1 phosphorylation site [Vallon and Lang, 2005]. SGK1 and Nedd4-2 may have a reciprocal relationship. Zhou and Snyder [2005] proposed that phosphorylation of Nedd4-2 stimulates the degradation of SGK1 by the 26S proteosome. This feedback mechanism would allow for sensitive control of sodium balance in vivo. In addition, Brickley et al. [2002] reported that the membrane is associated but not the cytosolic fraction of SGK1 is polyubiquitinated. The fact that Nedd4-2 regulates SGK1 degradation is consistent with this finding.

Transporter regulatory roles for SGK1 are not restricted to ENaC. Through coexpression studies in *Xenopus* oocytes and in vitro assays, SGK1 has been found to affect a variety of channels: calcium, chlorine, potassium, sodium channels, like TRPV5 [Vallon and Lang, 2005], ClC-2 [Palmada et al., 2004], ROMK1 [Palmada et al., 2003], SCN5A [Boehmer et al., 2003c], KCNE1 [Vallon and Lang, 2005], Kv1.3 [Henke et al., 2004], and the cystic fibrosis transmembrane conductance regulator [Wagner et al., 2001]. SGK1 also leads to upregulation of several transporters such as NHE3 [Wang et al., 2005], NKCC2 [Fillon et al., 2001], SGLT1 [Dieter et al., 2004], SN1 [Boehmer et al., 2003b], EAAT1 [Boehmer et al., 2003a], LAT1 [Wagner et al., 2000], SLC6A8 [Shojaiefard et al., 2005], and hASCT2 [Palmada et al., 2005]. The mechanisms involved in regulation of transport by SGK1 include, as with ENaC, phosphorylation of Nedd4-2, as well as the presence of cofactors, like NHERF2, which is needed for the stimulation of TRPV5, ROMK1, and NHE3 by SGK1, and direct phosphorylation of the transporters/channels, such as in the case of ROMK1 and NHE3. Most channel studies were carried out with both SGK1 and 3; only in the case of Na^+/K^+-ATPase, Kv1 and ClC-2, were the studies extended to SGK2 [Gamper et al., 2002; Palmada et al., 2004].

Despite the relative wealth of information about the effects of SGK on channels and transporters, most of the papers dealing with ion transport present results that only establish a relationship between SGK and channel activity—not direct phosphorylation of their targets. For that reason, the mechanisms by which the channel/transporters are regulated remain unclear.

Neurological Functions

Given its regulation of channels that modulate neuronal excitability, its expression pattern and its induction upon injury to the brain [Nishida et al., 2004], SGK1 may be a key player in neuronal signaling and by consequence, in neurological disorders. To identify molecular processes that contribute to Parkinson's disease (PD), expression studies have been performed in several chemical murine models of PD. SGK1

was found to be strongly upregulated prior to the histological appearance of dopaminergic neuronal death [Stichel et al., 2005]. In addition, SGK1 upregulation correlates with increased FKHRL1 phosphorylation and decreased expression of Kv channels [Schoenebeck et al., 2005]. SGK1 was also dysregulated in the brains of Huntington's (HD) patients where there is abnormal expansion of polyglutamine (Q) repeats in the protein Huntingtin [Rangone et al., 2004]. SGK1 phosphorylates Huntingtin at S241 in vitro and in cells, and this modification reduces the toxicity of poly Q Huntingtin and increases the survival of striatal neurons expressing polyQ Huntingtin. S241 is also phosphorylated by PKB. Given the differing time frames of PKB activation and of SGK1 induction and activation, their role in HD may be complementary. SGK1 was also reported to phosphorylate S214 of tau in vitro and to associate with tau and 14-3-3 in cells [Chun et al., 2004]. Tau is a microtubule-associated protein and when hyperphosphorylated, causes microtubule dysfunction and neuronal degradation due to accumulation of insoluble paired helical filaments. Hyperphosphorylated tau and the resulting neurofibrillary tangles are common hallmarks of neurological disorders such as Alzheimer's disease. However, several other protein kinases in addition to SGK1 are known to phosphorylate Tau at sites that promote its aggregation.

SGK1 could also be important for memory consolidation in murine models, and maybe in humans. Glucocorticoid receptor (GR) deficient animals and knock-in mice of GR mutants display impaired spatial learning and memory. Since SGK1 is a glucocorticoid-regulated gene, its involvement in learning and memory has been investigated. Differential display PCR, Northern blotting and in situ hybridization showed that SGK1 is overexpressed in the hippocampus of fast learning rats versus slow learning rats based on a water maze learning task [Tsai et al., 2002]. Enrichment training also significantly induced SGK1 mRNA and protein levels in the hippocampus, through the AMPA receptor [Lee et al., 2003]. Expression of mutant SGK1 in the hippocampus impaired spatial learning, fear conditioning learning, and recognition learning. In addition, SGK1 was recently identified as a potential plasticity gene [David et al., 2005]. Overexpression of constitutively active SGK1 doubles the number of primary dendrites and dendritic branches in cortical neurons. Reports by Florian Lang's laboratory indicate that SGK3 and SGK1 may play roles in hippocampal long-term potentiation and spatial memory through their ability to specifically regulate the abundance of GluR1 and GluR6, subunit glutamate receptors, and consequently increase GluR1- and GluR6-mediated glutamate-induced currents [Strutz-Seebohm et al., 2005a,b]. SGK2 was less effective in stimulating GluR6. The mechanism by which SGK1 and 3 affect glutamate-induced currents is unknown. Also, SGK3 null mice were assessed for spatial learning and exploratory behavior. SGK3 KO mice show a deficit in precision and goal-directed navigation in space, reduced exploratory activity, and reduced locomotion, probably due to deranged neuronal regulation of transporters and ion channels in the absence of SGK3 [Lang et al., 2005]. Further experiments, such as examination of neuronal cells within the hippocampus of SGK1 and SGK3 null animals, are required to better define the neuronal functions of the SGKs.

Diabetes, Hypertension, Cardiac Dysfunction, and Obesity

In addition to its roles in growth and neurological disorders, SGK1 has also been implicated in diabetes, hypertension, heart conditions, and obesity. Insulin activates SGK1 in cells and SGK1 inhibits phosphomannomutase-2, an enzyme necessary for the synthesis of GDP-mannose but ectopic expression of SGK1 had no effect on glucose transport [Menniti et al., 2005]. Increased extracellular glucose concentrations stimulate transcription of SGK1 and SGK1 RNA levels are induced in diabetic nephropathy [Lang et al., 2000]. Elevated levels of glucocorticoids can be associated with the development of diabetes, partly by impairing insulin secretion. Glucocorticoid analogs increase SGK1 transcription in insulin-secreting β-islet cells, and glucocorticoids are unable to stimulate insulin release in SGK1 null islets [Ullrich et al., 2005]. These data suggest that glucocorticoid-induced expression of SGK1 protein stimulates voltage gated potassium channel activity, thereby reducing calcium entry through voltage-gated Ca^{2+} channels and insulin release.

Since SGK1 acts on the ENaC and Na^+, K^+, $2Cl^-$ cotransporter, this induction could contribute to deranged electrolyte concentrations

and increased blood pressure. SGK1 mRNA and protein are upregulated in rat smooth muscle cells by endothelin-1, an important modulator of salt-sensitive hypertension. In fact, genetic variants in SGK1 (E8CC/CT and I6CC) were found to be significantly associated with increased blood pressure and high body mass indexes [von Wowern et al., 2005]. Another connection between SGK1 and hypertension is its binding and activation, by a PI3K-dependent mechanism, by *WNK1*, a gene encoding a kinase in hypertensive patients [Xu et al., 2005a,b]. The activation of SGK1 by WNK1 results in increased sodium transport through the epithelial sodium channel and contributes to blood pressure regulation. SGK1, SGK3, and PKB were found to enhance glucose transport through SGLT1, a sodium-coupled glucose transporter. Therefore, in addition to leading to hypertension, overactivation of SGK1 could enhance SGLT1 activity and lead to obesity [Dieter et al., 2004].

CONCLUSIONS

Although there remain many unanswered questions about the SGKs, what has been uncovered to date clearly demonstrates important roles in a variety of cellular responses. Perusal of this data indicates that SGK1 demonstrates several similarities to PKB in mechanism of regulation and substrate specificity and it is reasonable to suggest that SGK1 and the PKBs have coordinated rather than redundant roles in cells. By contrast, the second most studied SGK isoform, SGK3, appears quite distinct from both SGK1 and PKB, primarily due to the endosomal targeting of the protein via its PX domain. This difference in localization is important, because it may determine the specific identity or regulation of SGK3 upstream kinases and its mechanism of activation. The differential location also suggests that SGK3 and PKB experience different pools of substrates and may therefore have more discrete functions from one another than for SGK1 and PKB.

Analysis of the individual and compound PKB isoform knockouts was required to discern the various physiological functions of the variants and these studies revealed significant redundancy in some aspects, yet exquisite specificity in others. A similar type of analysis will be required to reveal the distinctions and redundancies among the SGKs. Likewise, as for the PKBs, transgenic expression of activated alleles may provide insight into their specific roles in growth regulation and development. To date, no selective inhibitors have been developed for the SGKs although proof of principle experiments should be possible through the use of siRNA approaches. Given their position downstream of the PI3K pathway and the promising effects of inhibitors of other downstream kinases such as PKB and mTOR in treating various cancers, the time may be right to target these enzymes.

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