review

The sphingosine and diacylglycerol kinase superfamily of signaling kinases: localization as a key to signaling function

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Abstract The sphingosine and diacylglycerol kinases form a superfamily of structurally related lipid signaling kinases. One of the striking features of these kinases is that although they are clearly involved in agonist-mediated signaling, this signaling is accomplished with only a moderate (and sometimes no) increase in the enzymatic activity of the enzymes. Here, we summarize findings that indicate that signaling by these kinases is strongly dependent on their localization to specific intracellular sites rather than on increases in enzyme activity. Both the substrates and products of these enzymes are bioactive lipids. Moreover, many of the metabolic enzymes that act on these lipids are found in specific organelles. Therefore, changes in the membrane localization of these signaling kinases have profound effects not only on the production of signaling lipid phosphates but also on the metabolism of the upstream signaling lipids.—Wattenberg, B. W., S. M. Pitson, and D. M. Raben. **The sphingosine and diacylglycerol kinase superfamily of signaling kinases: localization as a key to signaling function. *J. Lipid Res.* 2006. 47: 1128–1139.**

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Agonist-dependent activation of signaling enzymes usually involves an enormous increase in the catalytic activity of those enzymes: an on or off switch. This is not true, however, for sphingosine kinases (SKs) and diacylglycerol kinases (DGKs), which exhibit activity that is only moderately increased by agonist activation and sometimes not increased at all. Yet, the signaling function of these related lipid kinases is now recognized to be critical in a wide variety of processes. In this review, we explore how translocation to distinct sites is a key feature of activation that promotes the signaling function of the SKs and DGKs.

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Phosphorylation of a range of lipids is now recognized to be a major mode of production of signaling second messengers. Much attention has been paid, for example, to the phosphatidylinositol (PI) phosphates. However, recently, there has been intense interest in other phosphorylated lipids, such as sphingosine-1-phosphate, phosphatidic and lysophosphatidic acids, and ceramide-1-phosphate. These lipids have potent roles in a variety of cellular responses, including cell proliferation and survival, cell migration, and calcium fluxes. The substrates and products of the lipid kinases have special characteristics as signaling second messengers because their hydrophobic nature constrains their localization and movement within the cell. The localization of the lipid kinases, therefore, can have a marked impact on signaling. This can be envisioned to occur in a variety of ways. First, access of these enzymes to their hydrophobic substrates will depend on whether the enzymes and substrates are localized to the same sites. Second, the nature of the membrane surface with which they interact will have a strong impact on the activity of the enzymes. Third, these signaling enzymes will produce their downstream products at specific sites and therefore control access of those signaling lipids to their downstream effectors. Finally, localized production of the signaling lipids will determine their proximity to enzymes that terminate signaling by metabolizing the lipid phosphates.

Here, we focus primarily on the mechanisms and consequences of localization of the related lipid signaling enzymes SK and DGK. Ceramide kinase is also in this superfamily (1); however, study of this enzyme is in its infancy and

Abbreviations: CRD, cysteine-rich domain; DAG, diacylglycerol; DGK, diacylglycerol kinase; ERK2, extracellular signal-regulated kinase-2; K_{cat}, (V_{max}/enzyme concentration); PA, phosphatidic acid; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PS, phosphatidylserine; SK, sphingosine kinase; TNF-α, tumor necrosis factor-α.

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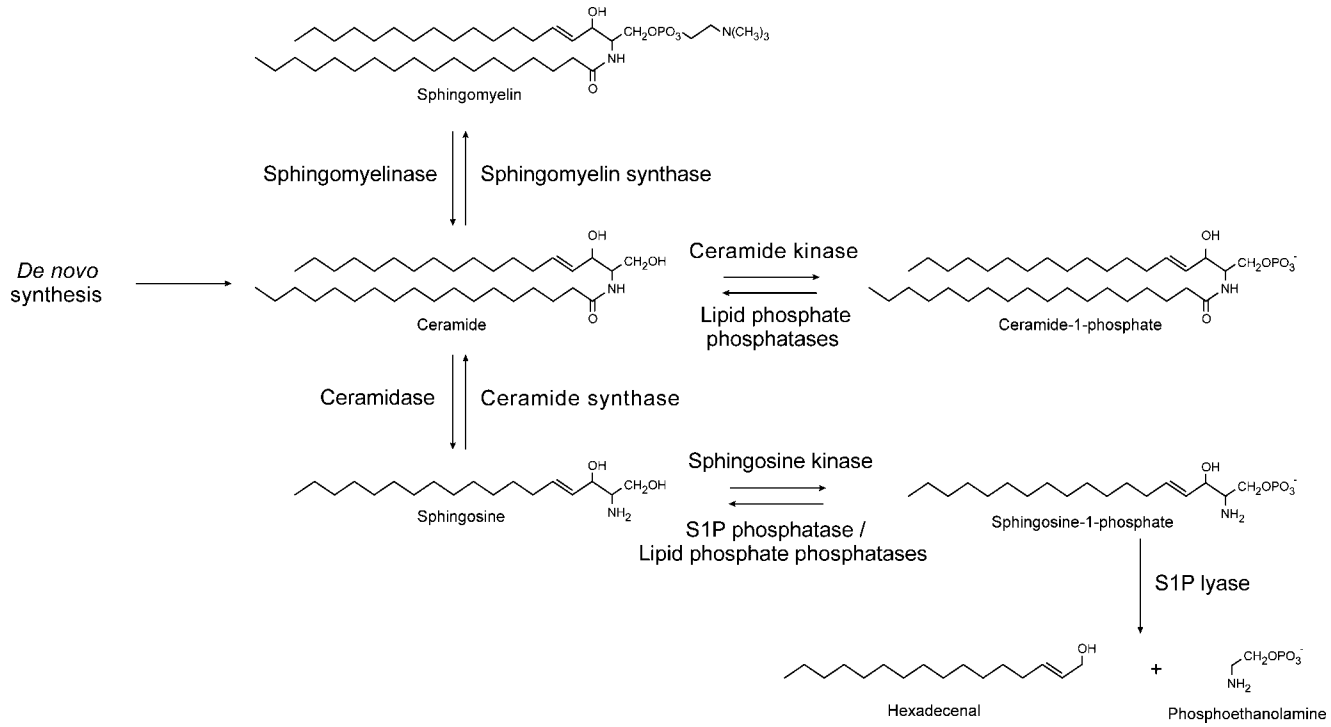

Fig. 1. The sphingolipid metabolic pathway. S1P, sphingosine-1-phosphate.

will not be treated in depth here. One important signaling feature of these enzymes is that both the substrates and the lipid products are bioactive, so activation of these enzymes has a 2-fold effect on signaling by abrogating signals produced by the upstream metabolites and initiating signals produced by the phosphorylated lipid products. SK is poised at a critical junction of the sphingolipid pathway (**Fig. 1**). Upstream are the potent sphingolipids sphingosine and ceramide. Sphingosine-1-phosphate is not only a second messenger but also is the substrate for the only essentially irreversible reaction in the sphingolipid pathway, sphingosine-1-phosphate lyase. Activation of SK, therefore, not only produces sphingosine-1-phosphate but also reduces the levels of sphingosine and ceramide. It has been proposed that SK is the fulcrum of the "sphingolipid rheostat" (2). Similarly, diacylglycerol (DAG) levels must be tightly regulated given their well-documented role in modulating protein kinase C (PKC) activity and other DAG binding proteins. One of the proposed roles of DGKs is to terminate the DAG signal to these proteins. Additionally, termination of the phospholipid-derived DAG signals by DGKs generates another signaling lipid, phosphatidic acid (PA), suggesting that DGKs may function as a "phospholipid rheostat" (3) (**Fig. 2**). The downstream functions of the products of these enzymes, sphingosine-1-phosphate (4, 5) and PA (6, 7), have been the subject of several excellent reviews and so will not be addressed in detail here.

The catalytic activity of SK is acutely activated by a number of agonists, but this activation is moderate. When measured in cell extracts, agonist stimulation results in an

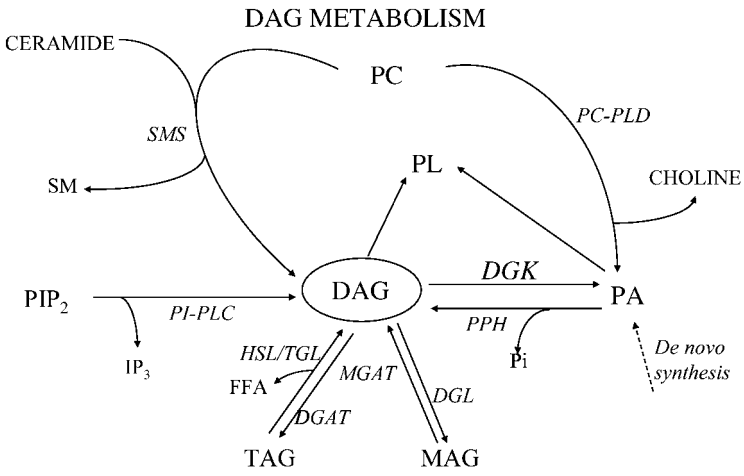

Fig. 2. Relationship between diacylglycerol (DAG) and phosphatidic acid (PA) metabolism. DAG and PA are interconverted through the actions of diacylglycerol kinases (DGKs; converting DAG to PA) and phosphatidic acid phosphohydrolases (PPHs; converting PA to DAG). In addition to their signaling roles, DAG and PA are intermediates in a large number of other lipid metabolic pathways. DGAT, diacylglycerol acyltransferase; DGL, diacylglycerol lipase; HSL, hormone-sensitive lipase; IP_3, inositol trisphosphate; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; PC, phosphatidylcholine; PC-PLD, phosphatidylcholine-specific phospholipase D; Pi, inorganic phosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PL, phospholipid; SM, sphingomyelin; SMS, sphingomyelin synthase; TAG, triacylglycerol; TGL, triacylglycerol lipase.

increase of activity of only 2- to 3-fold. The maximal activation of the enzyme from the basal to the maximally activated state is calculated to be 14-fold (8). The catalytic activity of the DGKs may not be activated at all by agonists. This strongly suggests that signaling function is not principally dependent on an increase in enzyme activity. For both sets of enzymes, it is becoming clear that localization of the proteins is a key to their signaling capacity and that agonist-mediated activation involves translocation to key sites. SK activation, therefore, involves both control of overall enzyme activity and movement of the enzyme, whereas DAG modulation by agonists may in most cases be completely a function of enzyme translocation.

In this review, we discuss the enzymology of these enzymes to gain some focus on how they might function in different cell compartments. We then summarize what is known about activation of these enzymes, both in terms of increases in enzyme activity and in terms of translocation to key signaling sites.

SK

Enzymology of SKs

SKs have been purified or cloned from a variety of sources, including *Saccharomyces cerevisiae* (9), *Arabidopsis thaliana* (10), *Drosophila* (11), rat (12), mouse (13), and human (14–17). Where analyzed, these enzymes share similar biochemical properties and display similar kinetic parameters. In general, the K_m values for ATP are ~25–100 μM, whereas those for D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine are in the range of 2–12 μM. The surface dilution kinetics (i.e., the measurement of SK activity at a membrane interface) has not been exhaustively studied. However, data from an early study examining surface kinetics in octylglucoside mixed micelles demonstrated that SK principally uses sphingosine in the context of a membrane interface and not as a soluble substrate (18). This supports the concept that SK is a membrane-active enzyme and that localization to membranes will be key to its function.

Two mammalian isoforms of SK have been identified through molecular cloning. Although these two enzymes, designated SK1 and SK2, are quite different in size (43 and 65 kDa, respectively) and originate from different genes (chromosomes 17 and 19, respectively), they possess a high degree of sequence similarity. In fact, almost all of the SK1 polypeptide sequence aligns with regions of the larger SK2 sequence, with an overall identity of ~45% and 80% similarity (14). SK2, however, also possesses two additional polypeptide regions at its N terminus and in the middle of its sequence that are quite distinct from SK1 and, indeed, from any other protein. Although the molecular basis for their existence is not currently known, a number of apparent alternatively spliced isoforms of both human SK1 and SK2 have also been identified that generate N-terminal extensions of 14 and 86 amino acids for SK1 and 36 amino acids for SK2 (19). All of the variant forms of human SK1 and SK2 appear to display similar activity and substrate specificity. The mouse variants of SK1, however, do have distinct activities, stability, and subcellular localization (20). Further analysis will be needed to determine the implications of these differences.

SK possesses five regions in its polypeptide sequence, designated C1–C5, that show very high sequence identity across all of the known SKs. The combination of these five regions is unique to the SKs (and ceramide kinases) and thus can be used to define this family of proteins. The complete SK sequences do not show any obvious similarity to known domains of other proteins, with the exception of their putative catalytic domain, which shows some similarity to DGK and ceramide kinase. Similarly, the SK sequences show no clear homology to the well-established ATP binding motifs of other kinases. The identification of an inactivating mutation in a *Drosophila* DGK in a region of homology to SK gave a hint to where the ATP binding site might be. Using site-directed mutagenesis and covalent modification with an ATP analog, glycine 82 and lysine 103 were determined to be essential in nucleotide binding in SK1. A motif of SGDGx$_{17–21}$K seems to represent the nucleotide binding region of SKs (21). Although this nucleotide binding region is unique to this family of proteins, it does show some weak sequence similarity to the well-characterized glycine-rich loop motifs of protein kinases (22, 23) and PI phosphate kinases (23–25). Subsequent sequence analysis has also suggested that SK, DGK, and ceramide kinase may share some structural similarity in their nucleotide binding domains with NAD kinases and 6-phosphofructokinases (26).

Recently, Yokota et al. (27) suggested that the SK C4 region is involved in sphingosine binding, because mutation of aspartate 177 within this region in murine SK1a markedly increased the K_m of this enzyme for sphingosine. As expected, this region of SK shows no sequence similarity to DGK, although it does show some similarity to the comparable region in ceramide kinases.

The activity of mammalian SKs is enhanced, at least in vitro, by the presence of acidic phospholipids such as phosphatidylserine (PS), PI, and PA (3, 14, 17). Because these lipids are enriched on the inner aspect of the plasma membrane, it is tempting to speculate that translocation to that site is accompanied by activation of SK. There is also speculation that translocation is accomplished in part by enhanced binding to one or more of these lipids (see below).

One of the major distinctions between SK1 and SK2, apart from an ~10-fold difference in specific activity (28), is a variation in their substrate specificities. Although both enzymes efficiently use D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine, SK2 has much greater activity than SK1 against phytosphingosine (14, 17) and the artificial substrate, ω-biotinyl D-*erythro*-sphingosine (28). SK2 also demonstrates the surprising ability to phosphorylate D, L-*threo*-dihydrosphingosine (14), an inhibitor of SK1 (13, 17). Furthermore, recent studies have indicated that SK2 is the main enzyme responsible for phosphorylation and activation of the immunosuppressive agent FTY720 and related analogs, novel substrates that SK1 demonstrates

only very low activity toward (19, 29, 30). Thus, SK2 is considerably more promiscuous than SK1 in the substrates it can use. The physiological significance of this difference in substrate specificity remains unknown.

Unlike many signaling enzymes, in the absence of activation SK1 exhibits substantial basal activity. It has been demonstrated conclusively that this is truly intrinsic catalytic activity rather than a low level of activation. First, recombinant enzyme produced in *Escherichia coli*, and thus not subject to mammalian modifications, exhibits this basal activity (17). This unstimulated activity has a Vmax/enzyme concentration (k_{cat}) of $\sim$85 s^{-1}, which compares favorably with, for example, the intrinsic activity of bacterial DGK, which has a k_{cat} of $\sim$12 s^{-1} (31), and therefore constitutes a considerable level of enzyme activity. Second, overexpression of a dominant-negative form of SK1 blocks endogenous SK1 activation by agonists while leaving the intrinsic activity unaltered (32). Finally, a mutated SK1 lacking the activating phosphorylation site (see below) retains full activity (8). Below, we outline a model that proposes a housekeeping function for the unstimulated activity of SK and a signaling function for the activated enzyme.

Agonist activation of SKs

SK activity is upregulated by both posttranslational and transcriptional processes. Here, we focus on the acute, posttranslational control of SK signaling. The catalytic activity of SK1 is transiently activated over a time course of minutes by a range of agonists, including tumor necrosis factor-α (TNF-α) (33), interleukin-1β (34), platelet-derived growth factor (35), vascular endothelial growth factor (36), epithelial growth factor (37), formylmethionine peptides (38), platelet-activating factor (39), substance P (39), bradykinin (40), nerve growth factor (41), basic fibroblast growth factor (41), lysophosphatidic acid (42), and, interestingly, sphingosine-1-phosphate itself (43). The activation of SK2 has not been as well studied, but it has been reported to be activated by epithelial growth factor (44) and in mast cells by Fc-receptor engagement (45). SK1 activation is the result of phosphorylation of serine 225 (in the human SK1 sequence) (8). Mutation of this serine to alanine completely blocks the ability of TNF-α or PKC activation to stimulate activity. This mutation, however, does not affect the constitutive basal activity of the enzyme. The protein kinase responsible for SK1 activation is the mitogen-activated protein kinase extracellular signal-regulated kinase-2 (ERK2) or a closely related kinase. In vitro experiments combining purified recombinant SK1 and ERK2 have demonstrated that phosphorylation alone, in the absence of any other modifications or accessory factors, is directly responsible for the catalytic activation of SK1. This activation results in an increased k_{cat} for the enzyme, with minimal changes in the K_m for either substrate (8, 41). The enzymatic activation of SK1 is relatively moderate. As measured in cell extracts from either untreated or agonist-treated cells, activity is typically increased 1.5- to 3-fold by agonists (35). The maximal activation of SK1, comparing unphosphorylated enzyme with quantitatively phosphorylated enzyme, is 14-fold, as determined using the in vitro phosphorylation system cited above.

Agonist-induced translocation of SK 1

Localization of SK before stimulation. SK1 does not have obvious membrane-anchoring or -docking sequences and therefore appears to be a soluble enzyme. The predominant view is that before activation, the bulk of SK1 is freely diffusing within the cytosol. There are some hints, however, that there may be a small pool of SK1 that is membrane-bound or associated with the cytoskeleton. When membrane association is measured by fractionation in cells in culture, SK1 is found to be mostly, but not exclusively, cytosolic (13, 46). An early report, before cloning of the enzyme, measured an endoplasmic reticulum-associated SK activity (47) from cells in culture. In tissue samples, the amount of SK activity associated with a pelletable fraction varied from 21% to 68%, depending on the tissue (48). This fraction may represent either membrane- or cytoskeleton-associated enzyme.

Examination of the sites of unstimulated SK1 by microscopy also indicates a principally cytosolic, but partially immobilized, distribution. Precisely localizing endogenous SK1 is difficult because levels of the enzyme are low. Interpreting the imaging of ectopically expressed enzyme must be done cautiously, because of overexpression artifacts and also because even soluble, cytosolic proteins can appear to have nonhomogeneous distributions that may be mistaken for specific intracellular structures. Various groups have reported distributions of nonstimulated SK1, often using a fusion between SK1 and green fluorescent protein. These distributions have primarily been described as cytosolic, with some undefined perinuclear densities (8, 46, 49, 50). However examination of the staining of endogenous SK1 suggests a filamentous distribution, which could be reflective of an association with the endoplasmic reticulum or, as the authors interpret it, the cytoskeleton (see Fig. 4 in 49).

The recent cloning of SK2 has added a new wrinkle to the localization of SK. SK2 is found in the nucleus and cytoplasm of some cells in culture (51), although localization to the nucleus is cell type-dependent. This localization appears to be regulated, as the proportion in the nucleus increases with the increased confluence of cells. Indeed, SK activity has been detected in nuclear fractions in a growth factor-dependent manner (52). Remarkably, Igarashi and colleagues (53) discovered a putative nuclear export signal in the SK1 sequence. Mutation of this nuclear export signal leads to the partial localization of SK1 in the nucleus. This implies that the steady-state localization of SK1 in the cytosol is the result of a constant cycling into the nucleus and back to the cytosol. The regulation of such a cycle could result in the nuclear localization of SK. Another view of SK2 is provided by the work of Spiegel and colleagues (54), who found an endoplasmic reticulum localization of the overexpressed enzyme under conditions of serum starvation. In their system, overexpressed SK2 is proapoptotic and SK1 is antiapoptotic. They attribute the proapoptotic effects of SK2 to its endoplasmic reticulum

localization. To what extent the results with the overexpressed enzyme reflect that of the endogenous enzyme remains to be determined.

Another interesting aspect of the basal localization of SK1 in cells concerns the observations of Hla and coworkers (55), who reported that a small proportion of the cellular content of this enzyme is constitutively exported into the medium by human umbilical vein endothelial cells and HEK293 cells. This finding has been supported by studies with airway smooth muscle cells (56). SK1 lacks a classical signal peptide sequence, so this secretion would be the result of the so-called nonclassical secretory pathway (57). Because the levels of extracellular SK1 are extremely low relative to those found in cells in culture, it is difficult to rule out the possibility that the measured extracellular enzyme is not the result of cell breakage. In both studies, the exported SK1 was implicated in the localized extracellular generation of sphingosine-1-phosphate and the subsequent triggering of cell surface sphingosine-1-phosphate receptors.

Agonist-dependent translocation of SK. Activation of SK1 by TNF-α (57a), platelet-derived growth factor (58), muscarinic agonists (50), lysophosphatidic acid (50), anaphylatoxin C5a (59), insulin-like growth factor binding protein-3 (60), PKC activation (8, 49), and calcium mobilization (50) results in translocation of the enzyme from internal sites to the plasma membrane. Obeid and colleagues (49) observed that treatment of cells with phorbol esters to activate PKC results in a decrease in cytosolic SK and an increase in membrane SK, as measured both biochemically by fractionation of lysates and by quantitative fluorescent imaging. The translocation is significant, but it clearly does not result in the quantitative redistribution of the enzyme. Pitson and colleagues (8) demonstrated that either PKC or TNF-α activation of SK1 results in translocation to the plasma membrane. Translocation is dependent on the same ERK-mediated phosphorylation that results in increased enzyme activity. Spiegel and colleagues (58) found that platelet-derived growth factor stimulates SK1 translocation to the leading edge of plasma membrane lamellae of migrating cells. This observation indicates that translocation may involve not just global localization to the plasma membrane but targeting to specific membrane domains.

At present, the molecular mechanisms that mediate SK1 translocation to the plasma membrane remain unclear. Recently, the involvement of anionic lipids as membrane binding sites has received attention. Because the catalytic activity of SK1 is known to be stimulated by anionic lipids, it is plausible that the interaction between anionic lipids and SK1 directs membrane localization. Delon and coworkers (61) have shown that the generation of PA by phospholipase D can drive SK1 to membranes both in cell extracts and in intact cells, primarily to the Golgi apparatus. As the Golgi does not seem to be a major site of SK1 agonist-stimulated translocation, this localization may be a result of the overexpression systems they used. Nonetheless, their data strongly suggest that the generation of high localized membrane concentrations of PA could drive SK1 translocation. The involvement of PA, however, has been challenged by the recent observation that PS has a major role in the localization of SK1 to membranes (62). These investigators, using physical techniques, demonstrated a preferential interaction of SK1 with PS-containing model membranes. They then generated mutations in SK1 that block this interaction and found that translocation of SK1 to membranes was ablated in these mutants. Based on experiments with mutations in the SK1 phosphorylation site, these investigators believe that agonist-dependent phosphorylation exposes a PS binding site in SK1. In this model, the opened binding structure then drives SK1 to the plasma membrane. There are some ambiguities in the models suggested by these experiments, one important one being that in addition to phosphorylation of SK1 there appears to be a second signal that is also required for translocation. This could be the generation or rearrangement of plasma membrane lipids. In addition to these lipid effects, Young and coworkers (50) suggest a direct role of calmodulin in the translocation process. The calmodulin inhibitor W-7 blocks SK1 translocation to the plasma membrane in response to calcium-mobilizing stimuli. SK1 binds calmodulin, and this association may be important for the phosphorylation-dependent change in SK1 phosphorylation.

In addition to calmodulin, several other SK1-interacting proteins have been identified. These include platelet endothelial cell adhesion molecule-1 (63), δ-catenin (64), TNF-α receptor-associated factor 2 (65), a protein kinase A-anchoring protein-like protein SKIP1 (66), aminoacylase 1 (67), and RPK118 (68). Overexpression of some of these proteins appears to have modest effects on the activity and activation of SK1. Some of these proteins, including RPK118, platelet endothelial cell adhesion molecule-1, and aminocyclase 1, also appear to alter SK1 localization when overexpressed, suggesting their possible involvement in the restriction of this enzyme to various cellular locations before stimulation. Further analysis, however, is required to establish the exact roles of the interaction of these proteins with SK1.

In addition to the agonist-induced translocation of SK1 from the cytosol to the plasma membrane, recent studies have established that during macrophage phagocytosis SK1 translocates from the cytosol to the phagosome membrane (69), where it appears to be involved in phagosome maturation (70). Interestingly, this translocation of SK1 to the phagosome is independent of the enzyme's catalytic activity, and unlike the agonist-dependent translocation to the plasma membrane, it is also independent of the phosphorylation of SK1 at serine 225 (69). Thus, these studies suggest that translocation of SK1 to the phagosome occurs via a different mechanism from that operating during translocation of this enzyme to the plasma membrane.

Effects of localization of SK

Downstream signaling. Recent studies have demonstrated that the phosphorylation-induced translocation of SK1 to the plasma membrane is an essential step in mediating pro-

survival, proproliferative signaling by this enzyme (57a). In contrast to wild-type SK1, expression of SK1 with a mutation in the activating phosphorylation site provided no survival or proliferative effects on cells. Constitutive localization of this nonphosphorylatable mutant to the plasma membrane via attachment of the Lck tyrosine kinase myristoylation/dual palmitoylation motif, however, restored its prosurvival, proproliferative signaling. The type of interaction of SK1 with the membrane may be an important determinant of its signaling function. A form of SK1 containing the single myristoylation site of c-Src (71) markedly inhibited cell proliferation while still conferring protection to cells from apoptosis induced by serum withdrawal. The reasons for the apparent different effects of the two plasma membrane-localized SK1 proteins remain to be elucidated. However, it is notable that myristoylation and palmitoylation of proteins via the Lck motif has been shown to drive the localization to Triton X-100-insoluble plasma membrane fractions (72). In contrast, myristoylation alone via the c-Src motif drives localization to Triton X-100-soluble plasma membrane fractions (73). Thus, it is possible that the localization of SK1 to different plasma membrane microdomains elicited by these different acylation motifs may result in divergent effects on cell proliferation.

Access to substrate. Sphingosine and dihydrosphingosine, the lipid substrates of SK, are generated at both the plasma membrane and lysosomes by hydrolysis of ceramides (sphingosine) and biosynthetically in the endoplasmic reticulum (dihydrosphingosine). Directing SK to either of these sites could, theoretically, increase the levels of sphingosine-1-phosphate produced. Indeed, artificially directing SK1 to the plasma membrane does increase the levels of sphingosine-1-phosphate produced (58). Furthermore, differential localization to the plasma membrane versus the endoplasmic reticulum could regulate the source of sphingosine/dihydrosphingosine used. Recent evidence suggests that sphingosine-1-phosphate and dihydrosphingosine-1-phosphate can have different signaling roles (74). Moreover, directing SK to use dihydrosphingosine as a substrate could control the levels of ceramide generated by biosynthesis. Precise studies of the localization of sphingosine remain to be done. However, sphingosine has a relatively high critical micelle concentration (16 μM) (75) and therefore a high soluble monomer concentration. This would facilitate the rapid exchange of sphingosine between membranes. Indeed, externally added sphingosine is rapidly phosphorylated by SK in fibroblasts (76), platelets and megakaryoblastic cells (77), and astrocytes (78), indicating that its transport from outside of the cell to intracellular sites where SK is located is not rate-limiting. However, it is still important to determine whether there are localized pools of sphingosine to which SK might be targeted. Furthermore, sphingosine is a potent mediator in its own right. Localized levels of sphingosine may have distinct signaling effects. In this light, it will be essential to identify to what extent SK affects levels of sphingosine at specific intracellular sites and to what extent the localization of SK relative to sphingosine pools affects the generation of sphingosine-1-phosphate.

Metabolism of sphingosine-1-phosphate. Sphingosine-1-phosphate is metabolized both by lipid phosphatases, of which there are several, and by a specific sphingosine-1-phosphate lyase. All of these enzymes are membrane-bound, so their localization relative to the sites where sphingosine-1-phosphate is generated is likely to be important. There are several phosphatases that control sphingosine-1-phosphate levels: both phosphatases that are relatively specific for sphingosione-1-phosphate (79, 80) and lipid phosphatases that have a broader substrate specificity [reviewed by Pyne and Pyne (81)]. The sphingosine-1-phosphate-specific phosphatases have been localized to the endoplasmic reticulum (80, 82). The less specific phosphohydrolases have both plasma membrane and internal localizations. The less specific phosphatases are generally thought to regulate extracellular surface sphingosine-1-phosphate and its access to cell surface receptors. However, manipulation of the levels of these phosphatases clearly affects intracellular levels of sphingosine-1-phosphate (83), so their function is obviously more complicated than initially thought. Sphingosine-1-phosphate lyase is found in the endoplasmic reticulum (84, 85). Interestingly, the active sites of the phosphatases and the lyase are on opposite sides relative to the cytoplasm. The active site of the lyase is exposed to the cytoplasm and therefore has easy access to the sphingosine-1-phosphate generated there. The phosphatase active sites are oriented toward the lumen of the endoplasmic reticulum or on the extracellular surface of the plasma membrane. Therefore, access of sphingosine-1-phosphate to these enzymes would require a transbilayer movement of the lipid. In addition, ceramide synthase, which can use either sphingosine or dihydrosphingosine as a substrate, is localized in the endoplasmic reticulum, with its active site oriented toward the endoplasmic reticulum lumen.

The localization of these sphingolipid metabolic enzymes to the endoplasmic reticulum raises the intriguing prospect that this localization serves a regulatory function that depends on SK localization. One possibility is that in the absence of agonist stimulation, SK is freely diffusible or loosely associated with the endoplasmic reticulum and encounters the bulk of its substrates, sphingosine and dihydrosphingosine, in the widely distributed endoplasmic reticulum membrane. Sphingosine-1-phosphate lyase, localized in the endoplasmic reticulum, then rapidly degrades the product. The result is a flux of sphingosine and dihydrosphingosine for degradation through SK and then sphingosine-1-phosphate lyase without generating high, signaling levels of sphingsine-1-phosphate. Alternatively, the sphingosine-1-phosphate generated at the endoplasmic reticulum membrane is imported into the lumen, dephosphorylated, and used for the generation of ceramides. Indeed, Reizman and coworkers (86) have demonstrated that in yeast, exogenous sphingosine must be converted to sphingosine-1-phosphate by SK to be incorporated into ceramide. Moreover, this could explain why overexpression of SK2, with its potential association with the endoplasmic reticulum, results in increased cellular ceramide levels, as suggested previously (54). Together, these observations suggest that controlling the access of

SK to the endoplasmic reticulum could determine the levels of free sphingosine-1-phosphate. Furthermore, regulating the import of sphingosine-1-phosphate into the lumen of the endoplasmic reticulum could determine the relative levels of sphingosine-1-phosphate degradation versus reutilization in the generation of ceramides. This might be considered a housekeeping, metabolic function for SK. This is consistent with the high unstimulated activity of SK. What, then, about the signaling function of SK? Upon agonist stimulation, SK translocation to the plasma could sequester the sphingosine-1-phosphate produced away from the lyase (and phosphatases) to generate higher, signaling levels of sphingosine-1-phosphate.

Localization of sphingosine-1-phosphate to sites of its effectors. One consequence of an agonist-driven translocation of SK to the cell surface would be to stimulate sphingosine-1-phosphate production near the cell surface receptors that bind the lipid. Sphingosine-1-phosphate exerts a number of effects through stimulating cell surface receptors (reviewed in 87, 88). There is strong evidence for intracellular effectors as well (89), but the identity of these, and thus confirmation of their action, has remained elusive. Stimulation of the cell surface sphingosine-1-phosphate receptors in an autocrine manner is well established (90). Secretion of sphingosine-1-phosphate has been measured in a number of systems (8, 78, 91), but the mechanism of this secretion, whether secretion is mediated by a transporter or is attributable to spontaneous diffusion through the bilayer, is still unclear. In platelets, there is an agonist-stimulated secretion of sphingosine-1-phosphate that appears to be mediated by a member of the ABC transporter family. From these studies, it seems likely that sphingosine-1-phosphate secretion will generally require a specific transporter, but future studies will be required to identify this transporter in various cell types. Whatever the mechanism of secretion might be, concentrating sphingosine-1-phosphate at the plasma membrane would enhance secretion and therefore the stimulation of the sphingosine-1-phosphate receptors. Indeed, the agonist-dependent translocation of SK1 appears to result in the selective secretion of sphingosine-1-phosphate produced outside the cell (8, 45, 57a, 92). The access of sphingosine-1-phosphate to intracellular effectors of sphingosine-1-phosphate could also be regulated by the localization of SK.

DGK

Enzymology of DGKs

Ten mammalian isoforms of DGK have been identified and organized into five categories based on the arrangement of similar structural motifs identified in their primary sequences (6, 7, 93, 94). These motifs are thought to be involved in calcium binding (type I DGKs, "EF-hand" motif), protein binding (types II and V, pleckstrin homology domains; type IV, ankyrin repeats), a membrane binding domain (type III), and lipid binding motifs [types I–V, cysteine-rich domains (CRDs)]. Except for DGK-ε (95, 96), none of the DGKs are integral membrane proteins; therefore, like the SKs, they are presumed to have transient associations with the membranes that contain their DAG substrates.

The DAG binding site has not been identified with certainty, although one prevailing hypothesis is that the CRDs bind DAG for catalysis. The notion that these domains are involved in binding DAG results from the observation that these CRDs are homologous to the C1A and C1B domains of PKCs. CRDs have been shown to bind DAG or phorbol ester in PKC, *n*-chimaerin, the *unc-13* gene product in *Caenorhabditis elegans* (97, 98), and protein kinase D (99). It has not been clearly established, however, that in the DGKs these domains are responsible for binding the DAG that serves as the substrate for catalysis. The involvement of CRDs in catalysis is questioned by the observation that the *Drosophila* DGK1 does not contain any CRDs (100). Additionally, Sakane et al. (101) showed that a construct of porcine DGK-α lacking its CRDs retains an apparent K_m for DAG that is similar to that of the wild-type enzyme. On the other hand, these domains appear to be critical for DGK-α and DGK-θ activity in vitro (94, 102), and Topham and Prescott (unpublished observations described in 103) found that removal of either CRD from DGK-ζ results in an inactive enzyme. The significance of inactivation by these deletions will require demonstrating that the effect is directly on DAG binding and not the result of an indirect effect, such as protein misfolding.

The identity of the ATP binding site is somewhat more clear. Based initially on a DGK mutation in *Drosophila*, and then confirmed in SK1 (see above), it is likely that the GXGXXXG motif found within the putative catalytic domain of DGKs is responsible for coordinating ATP in the catalytic pocket. Consistent with this, other studies in which the second glycine in this motif was mutated to an aspartate or alanine rendered the DGK catalytically inactive (104–106). These data clearly indicate that this motif is part of the ATP binding site of DGKs.

The kinetic parameters of some of the eukaryotic DGKs have been determined in a limited set of studies (101, 107–110). In general, these studies indicate that the K_m for ATP is ~100–150 μM, similar to that of SKs. Determining the kinetic parameters for the lipid substrate is more complex. Like other lipid-metabolizing enzymes, DGKs are bisubstrate enzymes that use a soluble substrate (ATP) and a hydrophobic substrate (DAG). It is likely, therefore, that these enzymes exhibit interfacial kinetics that depends not only on the affinity of the enzyme for its substrates but also on the binding of the enzyme to the membrane surface. Clearly, these parameters must be determined for each individual enzyme. For this analysis, it is important to have a system in which both the bulk (expressed in terms of molarity) and surface (expressed in terms of mole fractions) concentrations of substrates (ATP and DAGs) can be varied. Although most studies have not considered the interfacial kinetic aspects when evaluating DGKs, a close approximation of such a system has been used to characterize the apparent V_{max} and K_m for DAG and ATP (MgATP) for two DGKs: DGK-ε (111) and DGK-α (101).

These studies indicate that the K_m for DAG is ~2–3 mol% and that for ATP is ~100–150 μM. The V_{max} for these enzymes is ~1–2 nmol/min/mg.

In one recent study, we examined the kinetic parameters of a *Dictyostelium* DGK, DGKA, that is related to the mammalian DGK-θ (112, 113). Our results suggest that under some conditions, DGKA has only a very transient association with the membrane (or micelle) surface, but sometimes it has an appreciable interaction with the surface that contains its lipid substrate. The enzyme was shown to catalyze the phosphorylation of solubilized medium-chain (DiC8 and DiC6) DAGs in a Michaelis-Menten manner. When physiologically relevant long-chain DAGs were tested, enzyme kinetics were dependent on substrate surface concentration and on the detergent used. DGKA displayed Michaelis-Menten kinetics with respect to bulk substrate concentration when the surface substrate concentration was ≤3.5 mol% in octylglucoside mixed micelles. At higher surface concentrations in this detergent, however, there was a sigmoidal relationship between the initial velocity and the bulk substrate concentration. This may mean that DAG itself activates DGKA in an allosteric manner. The catalytic activity of DGKA was significantly enhanced by PS and PA. It is tempting to speculate, therefore, that the enzyme activity is modulated by either DAG or phospholipids binding to a second allosteric site, such as a CRD or pleckstrin homology domain (PH) domain, on the enzyme. This would allow for further "localized regulation" in that alterations of the local membrane composition could have a profound effect on the enzymatic activity.

To date, there is no information on the three-dimensional structure of either DGK or SK. This is a major impediment to a detailed understanding of these enzymes. Particularly important will be determining where the lipid substrate binds, the points of contact of the enzyme with lipid surfaces, and how the substrates are transferred from lipid bilayers into the catalytic pocket. Both SK and DGKs (α, β, γ, ζ, ι, θ) are strongly activated in vitro by anionic phospholipids (96, 114–117). This results in an increase in V_{max}. How lipid binding increases the catalytic efficiency of these enzymes has important consequences for the subcellular activation at sites where anionic lipids such as PS and phosphatidylinositol phosphates may be concentrated.

Agonist activation and translocation of DGKs

Agonists can acutely affect DGK activity at specific intracellular sites, but current evidence suggests that this is entirely attributable to translocation to those sites rather than to an increase in specific activity of the enzyme preexisting there. This is a clear distinction from the well-documented activation of SK. For the most part, the mechanisms regulating these translocations are not understood.

DGKs are classically considered to be involved in the PI cycle by converting DAG to PA for the resynthesis of PI. Therefore, it is interesting that three isoforms (DGK-α, DGK-β, and DGK-γ) are calcium-sensitive, consistent with the presence of EF-hand domains (103). It is tempting to speculate that DGKs may be regulated by an increase in intracellular calcium that accompanies the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). However, DGK-α translocation to the nucleus does not appear to be dependent on intracellular calcium increases, indicating that calcium may not universally be involved in DGK translocation (118). It has also been suggested that DAG itself, the other product of PIP_2 hydrolysis, regulates the distribution of DGKs. Studies designed to examine this regulation are complicated by the facts that DAG activates PKC and some DGKs are influenced by this enzyme (see below) (94, 106, 119–125). There are data, however, suggesting that DAG levels alone do not lead to an increase in apparent DGK activity (126), although in those studies the effect of DAG on DGK localization was not studied.

There is increasing evidence that phosphorylation may affect the membrane association of certain DGK isoforms. Phosphorylation of DGK-θ by a novel PKC, PKC-ε, or phosphorylation of DGK-α by Src is involved in the membrane association of these DGK isoforms (125, 127). Other isoforms (e.g., DGK-δ1 or DGK-ζ) may be negatively regulated by phosphorylation (106, 120). Overall, the precise mechanism by which phosphorylation affects membrane binding, and the differences observed among the various isoforms, require further study.

As indicated above, the agonist-induced translocation of DGKs appears to be the major mechanism responsible for regulating DGK activity. Davidson et al. (128) showed that gonadotropin-releasing hormone, acting via the type I gonadotropin-releasing hormone receptor, induces a translocation of DGK-ζ to the plasma membrane in association with Src kinase. DGK-θ translocates to the nucleus of fibroblasts in response to α-thrombin (114). Three other isoforms, DGK-α, DGK-ζ, and DGK-ι, have also been observed to translocate to the nucleus in response to agonist stimulation (6). Perhaps the strongest support for the notion that localization likely plays a key role in mediating a biological response is the observation that DGK-α shows an agonist-dependent localization. This isoform localizes to the plasma membrane of T-cells in response to activation of the T-cell antigen receptor (105) and to a perinuclear region in response to interleukin-2 (118, 129).

The role of agonist-dependent localization of DAGK

As noted above, at least four DGK isoforms (DGK-θ, DGK-ζ, DGK-α, and DGK-ι) translocate to the nucleus in response to agonists (6, 114). In view of the well-established role of PKC-α in mitogenic signaling pathways and its presence in the nucleus, one obvious role for nuclear DGK is to decrease nuclear DAG levels and, as a consequence, diminish nuclear PKC levels. This is particularly interesting given the evidence implicating nuclear DGK activity in the regulation of cell cycle progression. Support for this notion can be seen in studies using insulin-like growth factor. Insulin-like growth factor stimulates a transient increase in nuclear DAG levels followed by an increase in nuclear DGK activity in Swiss 3T3 cells. Inhibition of the DGK led to a sustained increase in intranuclear DAG levels and markedly potentiated the mitogenic effect of insulin-like growth factor. Translocation of DGK-α to a

perinuclear region in T-lymphocytes is critical to the regulation of interleukin-2-induced proliferation (118). Consistent with this, expression of the DGK-ζ that is retained at the nucleus reduced nuclear DAG levels and attenuated the growth of A172 cells (106). Recently, DGK-θ was shown to translocate to membrane and nuclear compartments in response to various agonists (114, 125, 130), but the regulation of this translocation and its physiological roles have not been established.

PERSPECTIVE

Localization of the SKs and DGKs is key to their signaling function. These enzymes have a number of similarities. They share a related catalytic site. This no doubt reflects the fact that these enzymes encounter their substrates in a similar manner at a membrane/cytosol interface. The nature of the membrane affects the catalytic activity of both enzymes. Both the substrates and the products of these enzymes are bioactive, so the action of these enzymes has the effect of altering a signaling balance rather than simply producing a second messenger. Finally, both the SKs and DGKs use signaling lipids as substrates and produce phosphorylated signaling lipids as products.

The concept that localization of signaling enzymes is important is not entirely novel. However, the SKs and DGKs provide an exciting opportunity to fully explore this concept, because localization is a dominant factor in their signaling capacity. Despite the advances summarized here, our understanding of the role of the localization of these enzymes is in its infancy. Three areas deserve further attention. First, we know little about the mechanism driving the translocation of either kinase to its site of action. Second, there is very little information about the site-specific levels of the various lipids affected by these enzymes. Third, we need to understand how this localization impinges on the downstream effectors of the phosphorylated lipids generated by these kinases. With this understanding, we will advance our picture of signaling pathways beyond two-dimensional arrows into the three dimensions of cellular architecture.

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