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Spatiotemporal Dynamics of Phosphorylation in Lipid Second Messenger Signaling*

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The plasma membrane serves as a dynamic interface that relays information received at the cell surface into the cell. Lipid second messengers coordinate signaling on this platform by recruiting and activating kinases and phosphatases. Specifically, diacylglycerol and phosphatidylinositol 3,4,5-trisphosphate activate protein kinase C and Akt, respectively, which then phosphorylate target proteins to transduce downstream signaling. This review addresses how the spatiotemporal dynamics of protein kinase C and Akt signaling can be monitored using genetically encoded reporters and provides information on how the coordination of signaling at protein scaffolds or membrane microdomains affords fidelity and specificity in phosphorylation events. ***Molecular & Cellular Proteomics 12: 10.1074/mcp.R113.029819, 3498–3508, 2013.***

The alteration of protein or lipid structure by phosphorylation is one of the most effective ways to transduce extracellular signals into cellular actions. Phosphorylation can alter enzyme activity, regulate protein stability, affect protein interactions or localization, or influence other post-translational modifications. A plethora of cellular processes, including cell growth, differentiation, and migration, are tightly regulated by phosphorylation. Cellular homeostasis is achieved by means of a precisely regulated balance between phosphorylation and dephosphorylation, and disruption of this balance results in pathophysiologies. Kinases and phosphatases are antagonizing effector enzymes that respond to second messengers and mediate phosphorylation/dephosphorylation events.

Two prominent lipid second messenger pathways are those mediated by diacylglycerol (DAG)[1] and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Fig. 1*A*). These membrane-embedded second messengers recruit effector kinases containing specific membrane-targeting modules to membranes, thus activating them. Specifically, DAG recruits C1-domain-containing proteins, notably protein kinase C (PKC), whereas PIP_3 recruits pleckstrin homology (PH) domain-containing proteins, such as Akt. Specificity and fidelity in signaling are often achieved via the compartmentalization of signaling on protein scaffolds and membrane microdomains, which can control the access of enzymes to particular substrates. In this review, we provide a brief background of the DAG and PIP_3 pathways and their effector kinases, PKC and Akt, respectively. We discuss sensors that have been developed to measure lipid second messenger levels and kinase activity at various subcellular compartments, the role of scaffolds and membrane microdomains in compartmentalizing signaling, and the consequences of dysregulation of second messenger signaling in disease.

Lipid Second Messengers—Lipid second messengers are signaling molecules produced in response to extracellular stimuli. Targeting enzymes and their substrates to the same membrane constrains them to a space of reduced dimensionality, thus increasing the apparent concentration of the signaling complex and the likelihood and amplitude of signaling (1).

DAG Pathway—Upon activation by agonists, receptor tyrosine kinases and G-protein-coupled receptors can activate phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) found at the plasma membrane into the second messengers DAG and inositol 1,4,5-trisphosphate (IP_3) (2). DAG recruits proteins that contain C1 domains, small globular DAG-binding domains, to membranes (3), whereas IP_3 freely diffuses inside the cell and binds to the IP_3 receptor at the endoplasmic reticulum. This releases another second messenger, Ca^{2+}, which induces further production of DAG at the Golgi (4). Two main classes of proteins that bind DAG are the protein kinases PKC and PKD and the Rac-GAPs chimerins; the affinity of their C1 domains for DAG can vary greatly and is discussed below in the context of PKC. DAG can also be generated from phosphatidic acid (PA) by phosphatidic acid phosphatases or sphingomyelin synthases. The removal of DAG, and thus termination of its signaling, is achieved by diacylglycerol kinases (DGKs) that convert it into PA (Fig. 1*B*).

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[1] The abbreviations used are: cPKC, conventional protein kinase C; DAG, diacylglycerol; DGK, diacylglycerol kinase; FRET, fluorescence resonance energy transfer; IP_3, inositol 1,4,5-trisphosphate; nPKC, novel protein kinase C; PA, phosphatidic acid; PDK-1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PHLPP, pleckstrin homology domain leucine-rich repeat protein phosphatase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PTEN, phosphatase and tensin homologue.

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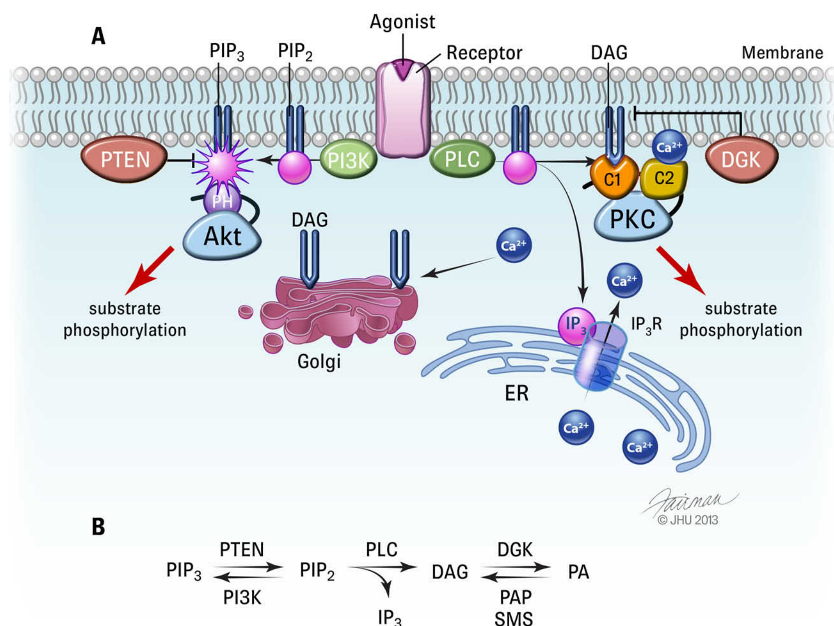

FIG. 1. **Diagram of diacylglycerol and PIP_3 signaling pathways.** *A*, agonist stimulation activates cell surface receptors such as receptor tyrosine kinases or G-protein-coupled receptors, which active phospholipase C (PLC) or phosphoinositide-3 kinase (PI3K), both of which act on phosphatidylinositol 4,5-bisphosphate (PIP_2). PLC hydrolyzes PIP_2 to produce diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 binds to the IP_3 receptor (IP_3R) at the endoplasmic reticulum (ER), releasing Ca^{2+}. DAG and Ca^{2+} recruit conventional protein kinase C (PKC) to the plasma membrane (via its C1 and C2 domains, respectively) and activate it. Ca^{2+} release also leads to the production of DAG at the Golgi. Diacylglycerol kinase (DGK) suppresses DAG-mediated signaling by converting DAG to phosphatidic acid (PA). Alternatively, PI3K phosphorylates PIP_2 into phosphatidylinositol 3,4,5-bisphosphate (PIP_3), which recruits Akt to the plasma membrane via its pleckstrin homology (PH) domain, where it gets activated. PTEN terminates PIP_3 signaling by converting it to PIP_2. *B*, enzymes involved in DAG and PIP_2 production and removal. PIP_2 is converted to PIP_3 by PI3K, whereas PTEN opposes this reaction by dephosphorylating PIP_3. PIP_2 can also be converted to DAG and IP_3 by PLC. DAG can then be removed by DGKs as it gets converted to PA. Phosphatidic acid phosphatases (PAPs) and sphingomyelin synthases (SMSs) convert PA back to DAG.

PIP_3 Pathway—The second messenger PIP_3 is generated upon the stimulation of receptor tyrosine kinases or G-protein-coupled receptors that activate phosphoinositide 3-kinase (PI3K) to phosphorylate PIP_2 (Fig. 1*A*). PIP_3 binds certain PH domains, recruiting enzymes such as Akt to the membrane (5). PIP_3 signaling is terminated by the tumor suppressor phosphatase and tensin homologue (PTEN), a lipid phosphatase that converts PIP_3 to PIP_2 (Fig. 1*B*).

Genetically Encoded Second Messenger Sensors—Second messenger levels can be measured in live cells, in real time, using genetically encoded sensors (6–11). The first generation of sensors comprised a fluorescent protein fused to a domain that specifically binds a second messenger (*i.e.* C1 or PH domain). Recruitment of the tagged domain to membranes containing the second messenger is monitored and used as a proxy for its levels (6, 10, 11). Recently, reporters containing two fluorescent proteins that undergo changes in fluorescence resonance energy transfer (FRET) as they change their distance or relative orientation upon binding of a second messenger have been developed (7–9). These reporters provide more quantitative data, as they rely on ratiometric measurements of two fluorophores as opposed to the translocation of a single fluorophore to membranes, thus minimizing artifacts resulting from cell movements, photobleaching, or variable cell thickness (12). Targeting of these second messenger sensors to particular subcellular compartments provides information on the kinetics and location of second messenger production.

Measuring DAG—One of the first DAG reporters comprised the C1 domain of PKCγ fused to a green fluorescent protein, and its agonist-evoked translocation to membranes served as a readout for DAG production (6). Reporters employing ratiometric measurements were later developed and contain a C1 domain and a FRET pair, such as the DAG reporters DAGR (Fig. 2*A*), which uses intermolecular FRET (7), and Daglas (Fig. 2*B*), which uses intramolecular FRET (8). These reporters can be targeted to specific subcellular compartments using a membrane localization sequence, allowing the detection of changes in DAG levels at various intracellular membranes. Such membrane-specific reports have revealed that the Golgi and endoplasmic reticulum have relatively high basal levels of DAG, whereas the plasma membrane lacks measurable basal DAG (9). DAG at the plasma membrane is produced following acute agonist stimulation, and this signaling is swiftly terminated (Fig. 3) as DGK converts DAG to PA (8, 13). In contrast, stimulated DAG levels at the Golgi are relatively sustained, whereas those at mitochondria are not detectably altered by ATP stimulation.

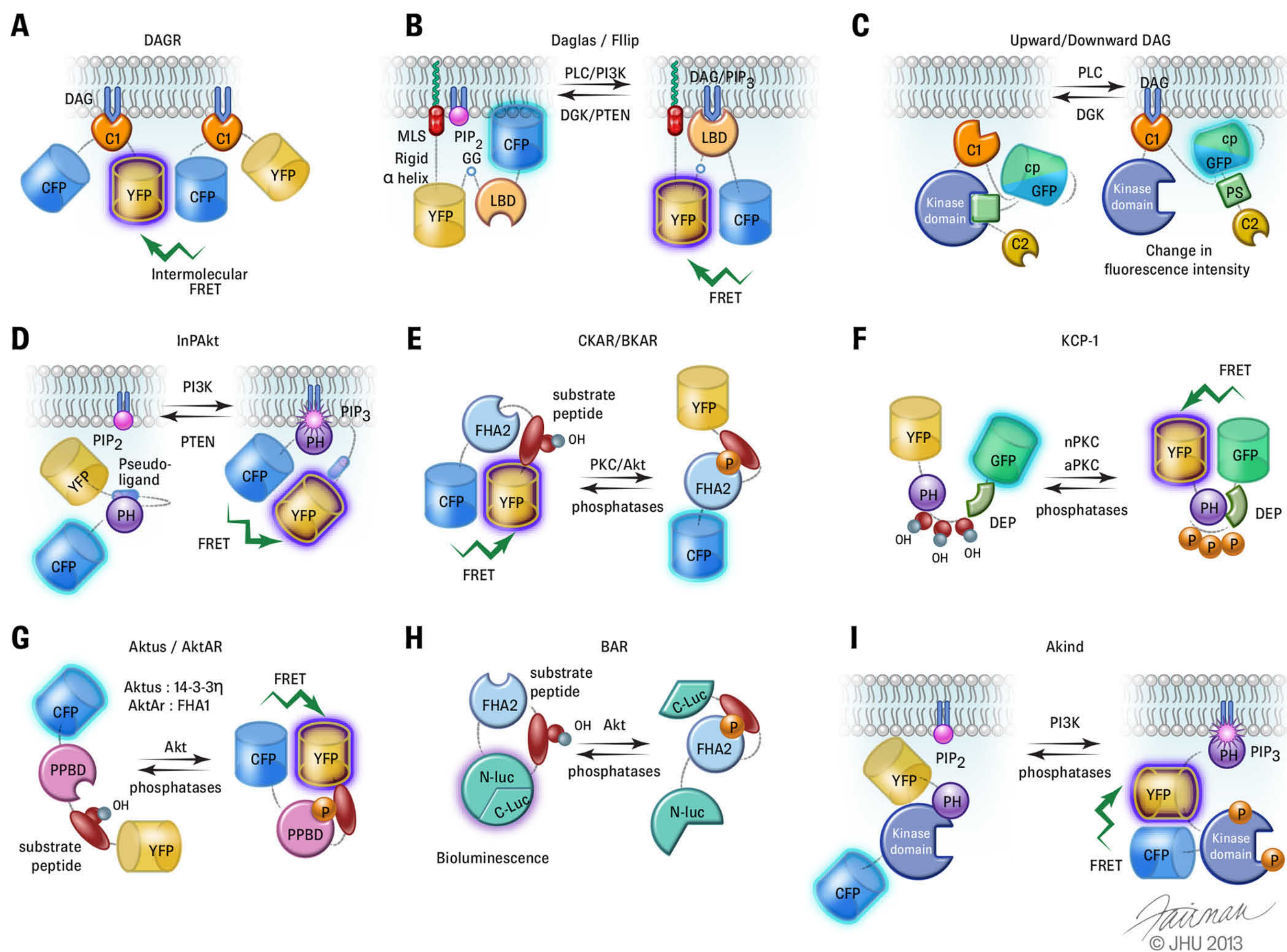

FIG. 2. **Genetically encoded reporters for measuring DAG and PIP_3 levels and PKC and Akt activities.** *A*, the diacylglycerol (DAG) reporter, DAGR, comprises a CFP–YFP FRET pair flanking a DAG-binding C1 domain. Translocation of the reporter to membranes upon elevation of DAG leads to intermolecular FRET. *B*, the DAG reporter Daglas and PIP_3 sensor Fllip are anchored to the membrane via a membrane localization sequence (MLS) and are composed of a FRET pair flanking a lipid-binding domain (LBD) (C1 domain for Daglas and PH domain for Fllip) and a glycine–glycine (GG) hinge. Engagement of the LBD to the membrane induces a conformational change in the reporter, increasing FRET efficiency. The FRET signal is reversed by enzymes that remove the second messengers (*i.e.* DGK and PTEN). *C*, Upward and Downward DAG are based on PKCδ and contain a circularly permuted GFP between the pseudosubstrate (PS) and the C1 domain. Binding of the C1 domain to DAG increases (for Upward DAG) or decreases (for Downward DAG) the fluorescence intensity by altering the conformation of the GFP β-barrel. *D*, InPAkt is a PIP_3 reporter comprising the PH domain of Akt, a pseudoligand that binds the PH domain with a lower affinity than PIP_3, and a FRET pair. Upon PIP_3 production, the PH domain releases the pseudoligand and binds PIP_3, increasing FRET. *E*, the PKC reporter, CKAR, and the Akt reporter, BKAR, are made up of an FHA2 domain, a PKC- or Akt-specific substrate peptide, and a FRET pair. When phosphorylated, the substrate sequence binds the FHA2 domain, and this conformational change results in a decrease in FRET. *F*, the nPKC and atypical PKC reporter KCP-1 is based on the PKC substrate pleckstrin. PKC phosphorylation at three sites between the PH and dishevelled–Egl-10–pleckstrin domains of pleckstrin results in a conformational change that increases FRET between enhanced YFP and GFP2. *G*, Aktus and AktAR measure Akt activity as phosphorylation of an Akt-specific substrate peptide causes it to bind a phosphopeptide-binding domain (PPBD) (14–3-3η for Aktus and FHA1 for AktAR), increasing FRET from CFP to YFP. *H*, the bioluminescent Akt reporter (BAR) contains a split luciferase, an FHA2 domain, and an Akt substrate peptide. Phosphorylation by Akt causes the split luciferase to dissociate, decreasing bioluminescence. *I*, Akind is based on Akt's kinase and PH domains. Upon recruitment of Akt to PIP_3-enriched membranes and its subsequent phosphorylation, Akt undergoes a conformational change that leads to an increase in FRET between the fluorophores.

More recently, a sensor has been engineered to simultaneously measure two second messengers in the same cell. The Green Upward or Downward DAG (Fig. 2*C*) was paired with a Ca^{2+} sensor (not shown) for the concomitant measurement of DAG and Ca^{2+} (9). Both of these sensors are based on fluorescent proteins that undergo changes in fluorescence intensity upon the binding of second messengers. The DAG sensor (based on PKCδ) and the Ca^{2+} sensor (based on calmodulin and a calmodulin-binding domain (14)) are linked by a peptide that, upon cleavage, produces equal amounts of the two reporters as distinct peptides (15). Considering that DAG and Ca^{2+} are often co-elevated, measuring these sec-

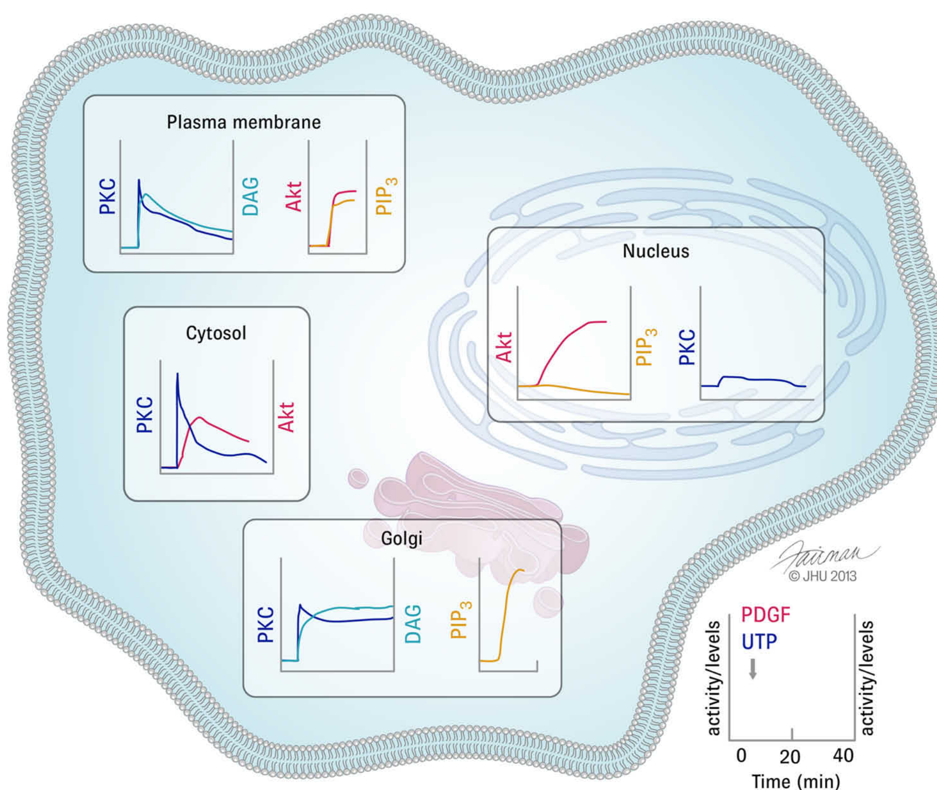

FIG. 3. **Schematic diagram displaying DAG and PIP_3 levels at various intracellular membranes and the PKC and Akt activity they induce at these locations.** UTP stimulates rapid but short-lived production of DAG at the plasma membrane. In contrast, stimulated DAG levels at the Golgi are relatively sustained, leading to more sustained PKC activity at the Golgi than at the plasma membrane. Typically cPKCs are recruited to the plasma membrane because their Ca^{2+}-regulated C2 domain selectively recognizes PIP_2, which is enriched at the plasma membrane. In contrast, nPKCs signal primarily at the DAG-enriched Golgi (111). The nucleus shows little UTP-induced PKC activity, whereas the cytosol has the greatest, although transient, UTP-stimulated PKC activity. Upon PDGF stimulation, PIP_3 is more rapidly generated at endomembranes such as the Golgi than at the plasma membrane. PDGF-induced Akt activity rapidly increases at the plasma membrane, whereas in the cytosol the kinetics of activation are slower. The nucleus contains high but delayed Akt activity, despite the lack of PIP_3 production at the nuclear membrane, suggesting that active Akt translocates to the nucleus where there is little phosphatase suppression.

ond messengers concomitantly gives a more complete view of signal transduction and has the advantage of providing a direct comparison of second messenger production downstream of the activation of various receptors with different agonists. However, because these reporters rely on changes in fluorescence intensity instead of a FRET ratio, care should be taken when using them, as their readout is dependent on the absolute concentration of the sensor, and intensity changes resulting from focus drift or cell movements can occur during imaging.

Measuring PIP_3—Changes in PIP_3 levels can be assessed by monitoring the agonist-dependent relocalization of fluorescently tagged PIP_3-selective PH domains to membranes (10, 12). However, because these methods monitor translocation to all membranes, pools of PIP_3 at specific membranes cannot be readily monitored. Thus, more quantifiable FRET-based PIP_3 sensors that can be targeted to particular subcellular localizations have been developed. For example, an indicator for phosphoinositides based on Akt's PH domain, InPAkt (Fig. 2*D*), showed that the plasma membrane contains basal levels of PIP_3 that are maintained through a balance between PI3K and phosphatases, whereas the nucleus has no detectible production of this second messenger (16). Another PIP_3 sensor, Fllip (Fig. 2*B*), comprising the PH domain of GRP1 and a FRET pair, is anchored to a membrane via a membrane localization sequence (17). This reporter revealed that platelet-derived growth factor (PDGF) stimulates greater production of PIP_3 at the Golgi and endoplasmic reticulum than at the plasma membrane, and that PIP_3 is generated at these endomembranes by endocytosed receptor tyrosine kinases that activate a localized pool of PI3K.

PKC and Akt as Effectors—PKC and Akt, effector kinases of DAG and PIP_3, respectively, phosphorylate myriad downstream targets (reviewed in Refs. 18–20), many of which have been identified through phosphoproteomic screens (21–23).

PKC—The PKC family consists of nine genes that are divided into three categories based on the domain structure of the enzymes they encode, and hence the second messengers they require for activation. Both conventional PKCs (cPKCs) (α, β, γ) and novel PKCs (nPKCs) (δ, ε, θ, η) bind DAG through tandem C1 domains; cPKCs also bind membranes in a Ca^{2+}-dependent manner through a C2 domain. Atypical PKCs

(ζ and ι/λ) bind neither DAG nor Ca^{2+} and are regulated by protein–protein interactions through a PB1 domain (24). cPKCs and nPKCs are constitutively phosphorylated at three conserved phosphorylation sites termed the activation loop, turn motif, and hydrophobic motif (25). These phosphorylations are necessary for proper PKC folding, and thus for its activation; for PKCα, these modifications occur with a half-time of 5 to 10 min following biosynthesis (26). The activation loop is phosphorylated by the phosphoinositide-dependent kinase PDK-1 (27–29), an event that triggers two tightly coupled phosphorylations at the turn and hydrophobic motifs (25, 30). mTORC2 is required to initiate the phosphorylation cascade, and in cells lacking mTORC2, PKC is not phosphorylated and thus is shunted for degradation (31–33); however, the mechanism of this regulation is unknown. The hydrophobic motif is autophosphorylated by an intramolecular reaction *in vitro*, but whether this is the mechanism of modification in cells or whether it is the direct target of another kinase such as mTORC2 remains controversial (33, 34). When first translated, PKC is in an open conformation, with the autoinhibitory pseudosubstrate out of the active site (35). Upon phosphorylation, PKC matures into a catalytically competent, but inactive, species that is maintained in an autoinhibited (closed) conformation in which the pseudosubstrate occupies the substrate-binding cavity. Upon intracellular Ca^{2+} release and DAG production, cPKCs are recruited to membranes through their Ca^{2+}-sensitive C2 domain. This relocalization reduces the dimensionality in which the C1 domain has to probe for its membrane-embedded ligand, DAG, thus increasing the effectiveness of this search by several orders of magnitude (36). Binding of one of PKC's C1 domains to DAG provides the energy necessary to expel the pseudosubstrate from the substrate-binding site, allowing the phosphorylation of downstream targets. For some isozymes, such as PKCδ, the second C1 domain (C1B) is the major binder (37). The affinity of C1 domains for DAG is toggled from low to high by a single residue within the DAG binding cavity: a Trp at position 22 confers an affinity for DAG that is 2 orders of magnitude higher than that conferred by a Tyr at that position (38). Consequently, nPKCs, which contain a high-affinity C1B domain, can respond to DAG alone, whereas cPKCs, which have a low-affinity C1B domain, require the elevation of Ca^{2+} concomitantly with DAG production in order to become activated. Reporters such as the Green Upward or Downward DAG (Fig. 2*C*) paired with a Ca^{2+} sensor (9), described above, would be useful tools for discerning which agonists solely activate cPKCs *versus* nPKCs.

The amplitude of PKC signaling is diligently balanced through its phosphorylation state (controlling its steady-state levels), the presence of its lipid second messenger, DAG (acutely controlling activity), and, for cPKCs, Ca^{2+} levels. Thus PKC activity can be antagonized via direct dephosphorylation by protein phosphatases such as the PH domain leucine-rich repeat protein phosphatase (PHLPP) or by removal of the lipid second messenger through phosphorylation by the lipid kinase DGK (Fig. 1) (39). The peptidyl-prolyl isomerase Pin1 was recently shown to be necessary for cPKC dephosphorylation and degradation following agonist activation, as it isomerizes the phosphorylated turn motif of cPKCs, thus facilitating PKC dephosphorylation (40).

Akt—Akt, also known as PKB because of its homology to PKA and PKC, is a serine/threonine kinase that promotes cell growth and survival (41). The three Akt isozymes (Akt1, Akt2, and Akt3) contain an N-terminal PH domain that mediates PIP_3-dependent membrane recruitment. Akt is maintained in an inactive conformation by an interaction between the PH and kinase domains, and unlike PKC, Akt is directly activated by phosphorylation at its activation loop and hydrophobic motif following agonist-dependent recruitment to membranes (42). Thus, Akt phosphorylation at these sites can be used as a proxy for activity under certain conditions (43). Akt is co-translationally phosphorylated at the turn motif by mTORC2 (contrasting with the post-translational modification of PKC), an event that is not necessary for function but increases the stability of the protein (44). PIP_3 recruits Akt to the plasma membrane via its PH domain, unmasking the kinase domain to permit phosphorylation of the activation loop by PDK-1 (45) and subsequent phosphorylation of the hydrophobic motif. mTORC2 facilitates hydrophobic motif phosphorylation (46, 47), possibly by assisting in unmasking the kinase domain for phosphorylation. Indeed, manipulations that displace the PH domain effectively bypass the requirement for mTORC2 for phosphorylation of the hydrophobic motif (but not the turn motif) (48). Phosphorylated Akt is locked in an active conformation and can disengage from the membrane and relocalize to other intracellular regions, such as the nucleus (49), to phosphorylate diverse substrates (19). Akt signaling is terminated by the hydrolysis of PIP_3 to PIP_2 by PTEN or by direct dephosphorylation of the activation loop by protein phosphatase 2A or of the hydrophobic motif by PHLPP (50–52). PHLPP1 dephosphorylates Akt2 and Akt3, whereas PHLPP2 dephosphorylates Akt1 and Akt3 (52), suggesting that these isozymes are differentially compartmentalized, likely via the unique PDZ (PSD-95, disheveled, and ZO1) ligand of each PHLPP isozyme (53). Interestingly, stoichiometric quantification of Akt phosphorylation at the activation loop and hydrophobic motif sites using LC-MS revealed that in untreated T cells, less than 1% of Akt is phosphorylated at both of these sites, and a low level of Akt phosphorylation is sufficient to contribute to tumorigenesis (54), highlighting the importance of keeping Akt activity low for maintaining cellular homeostasis.

Identifying PKC and Akt Substrates—A number of PKC and Akt substrates have been identified through biochemical methods, and several phosphoproteomic screens have been devised to identify new substrates and novel roles of these kinases. For example, a functional proteomic screen identified enhancer of mRNA decapping 3 as a substrate of Akt that

regulates the mRNA decay and translation repression pathways downstream of insulin signaling (21), and another identified a number of chaperone proteins and protein disulfide isomerases as potential Akt substrates in rat mesangial cells, suggesting that Akt might regulate chaperone function (22). An *in vivo* quantitative phosphoproteomics study employing stable isotope labeling by amino acids in cell culture implicated a highly deregulated kinase network composed of PKC, PAK4, and SRC in squamous cell carcinoma, but not in papilloma (55).

Vast efforts combining phosphorylation sites identified *in vivo* though mass spectrometry, *in vitro* through protein microarrays, and computationally through prediction algorithms have uncovered more substrates, setting the foundation for the development of human phosphorylation network maps (23, 56). However, despite efforts to enrich for physiologically relevant phosphorylation events by accounting for subcellular localization and scaffolding, these networks still have shortcomings, as the algorithms predict some false positives and miss a large fraction of known phosphorylation sites.

Kinase Activity Reporters—Genetically encoded reporters allow the visualization of the spatiotemporal dynamics of kinase activity in individual cells. These reporters can be targeted to various subcellular localizations and to protein scaffolds to measure localized activity, which can be more physiologically relevant than bulk activity in the cytosol.

PKC—Because cPKCs and nPKCs are constitutively phosphorylated at the C-terminal sites, and because the phosphate at the activation loop does not modulate activity once the C-terminal tail is phosphorylated (25), their activity cannot be measured with phosphorylation-specific antibodies, as is done for most other kinases. However, PKC activity can be monitored using activity reporters such as the C kinase activity reporter (Fig. 2*E*), which is composed of a CFP–YFP FRET pair flanking a PKC-specific substrate and an FHA2 phosphothreonine-binding domain (7). Upon phosphorylation of this substrate by PKC, the reporter undergoes a conformational change that decreases FRET. As phosphorylation of the reporter is reversible (*i.e.* phosphatases can dephosphorylate the reporter), it provides a real-time readout of PKC activity.

C kinase activity reporter has been targeted to various intracellular locations to enable specific monitoring of PKC activity at these regions. Using these targeted reporters, Gallegos *et al.* (13) found that the activation of PKC with the agonist UTP leads to rapid and relatively sustained PKC activity at the Golgi, driven by the persistence of DAG at this membrane (Fig. 3). UTP-dependent PKC activity in the cytosol is, however, quickly terminated by phosphatases, and activity in the nucleus is low because of high phosphatase suppression in this compartment. The mitochondria also have little UTP-stimulated activity; however, using a mitochondrially targeted PKCδ-specific activity reporter, Mito-δCKAR, Zheng *et al.* (57) revealed that PKCδ translocates to, and is active at, the outer membrane of mitochondria upon stimulation with phorbol esters, and that its intrinsic catalytic activity is required for its interaction with the mitochondria.

The Schultz lab has developed a reporter for nPKCs and atypical PKCs, KCP-1 (Fig. 2*F*), that is based on the PKC substrate pleckstrin (58). This reporter does not utilize a phosphopeptide-binding domain; rather, phosphorylation of residues between its PH and dishevelled–Egl-10–pleckstrin domains causes a conformational change in the reporter, resulting in a change in FRET. Thus, interactions between the phosphorylated sites and other endogenous proteins are reduced.

Akt—A number of FRET-based Akt reporters have been developed, most of which measure the phosphorylation of a synthetic substrate of Akt in live cells; these include Aktus, AktAR (Fig. 2*G*), and BKAR (Fig. 2*E*) (59–61). Aktus has low sensitivity and requires overexpression of Akt, whereas BKAR and AktAR can sense endogenous Akt activity. Because activated Akt can disengage from the membrane and diffuse to other subcellular locations, targeting these reporters to various subcellular compartments is particularly useful. BKAR targeted to the plasma membrane revealed that phosphatase suppression of Akt is low at this location (60). The phosphatase suppression of Akt activity is greater in the cytosol than it is at the plasma membrane, so Akt is more rapidly inactivated in the cytosol (Fig. 3), most likely by protein phosphatases that dephosphorylate Akt's substrates as opposed to Akt itself. Conversely, the nucleus has low phosphatase suppression of Akt, so once Akt diffuses to the nucleus its activity is much more sustained (60). AktAR (61) has a greater dynamic range for detecting Akt activity than BKAR and was used to measure Akt activity in plasma membrane microdomains (addressed below).

Whereas fluorescent reporters can measure rapid signaling kinetics at subcellular levels, bioluminescent reporters have the advantage of producing their own light and therefore bypass issues with autofluorescence, photobleaching, and tissue damage from the excitation light (62). A bioluminescent Akt reporter, BAR, was engineered using a split luciferase, an Akt specific substrate, and a phosphopeptide-binding domain (Fig. 2*H*). This reporter has the capability to measure Akt activity in a noninvasive manner *in vivo* (63). In addition to Akt activity reporters that measure the phosphorylation of a synthetic substrate by Akt, reporters that measure conformational changes of Akt induced by its translocation and phosphorylation (and thus activity state) have also been developed (64–66). The Akt indicator Akind (57), comprising the PH and catalytic domains of Akt and a FRET pair (Fig. 2*I*), was used to visualize Akt translocation to and activity at lamellipodial protrusions. Conformational changes attendant to the phosphorylation of Akt result in an increase in FRET. The use of another reporter of Akt action, ReAktion (not shown) (58), led to the proposal that activation loop phosphorylation of Akt decreases its membrane binding affinity, thus allowing disengagement from the membrane and relocalization to other

cellular compartments. One advantage of these reporters is that both the activity and the translocation of Akt can be measured concurrently. However, the reporters themselves can phosphorylate endogenous substrates of Akt and could theoretically displace Akt from its scaffolds, thus potentially perturbing the system more than the introduction of an exogenous Akt substrate.

Localized Signaling and the Importance of Scaffolding—Protein scaffolds coordinate and allow specificity and fidelity by compartmentalizing kinases and their downstream substrates, as well as the phosphatases that can rapidly terminate the signal (67). Even though lipid second messengers acutely regulate the activity of PKC and Akt, scaffolds can mediate their access to particular substrates.

PKC Scaffolds—Although multiple PKC isozymes respond to the same second messengers, there is some specificity in their function and signaling, mediated in part by their cell-specific pattern of expression, their differential affinities for certain lipids, and by protein scaffolds. PKC is anchored to numerous protein scaffolds through interactions mediated by its regulatory domain, its pseudosubstrate, or, in the case of PKCα and the atypical PKCs, a PDZ ligand. The first PKC scaffolds were identified by Mochly-Rosen and colleagues as receptors for activated C kinase, which are proposed to selectively bind active PKC and enhance its activity toward substrates anchored at that location (68, 69). A kinase anchoring proteins (AKAPs), which were first identified as PKA scaffolds (70), also anchor PKC in proximity to its targets, but in its inactive state, thus enabling rapid downstream signaling upon PKC activation. For example, AKAP-Lbc coordinates PKCη and PKA to phosphorylate PKD and release it from the scaffold (71), whereas AKAP79 functions as a scaffold for PKC, PKA, and protein phosphatase 2B at the postsynaptic density in neurons (72). One critical aspect of scaffolding is that it can alter the pharmacological profile of tethered kinases, which could have clinical implications for drug design. For example, through the use of targeted PKC activity reporters, Scott and colleagues found that PKC bound to the AKAP79 scaffold is refractory to active site inhibitors, but not allosteric ones. Similarly, the processing phosphorylation of cPKCs by PDK-1 is refractory to active site inhibitors of the co-scaffolded PDK-1 (73).

PKCα, -ζ, and -ι also bind PDZ-domain-containing scaffolds through their distinct PDZ ligands. In the case of PKCα, scaffolding by its PDZ ligand is required for cerebellar long-term depression (74). One likely PDZ-domain-binding partner involved in this is protein interacting with Cα kinase (PICK1), which interacts specifically with the PDZ ligand of PKCα (75, 76). Interestingly, PICK1 can have opposing effects on PKCα function in neurons, where it can act as either a mediator or an inhibitor of the phosphorylation of downstream targets. PICK1 targets activated PKCα to synapses to phosphorylate the glutamate receptor subunit GluR2, leading to its endocytosis (77), but it can also act as a barrier to phosphorylation of the metabotropic glutamate receptor mGluR7a by PKCα (78). More recently, a family of Discs large homolog scaffolds that interact with the PDZ ligand of PKCα to facilitate cellular migration was also identified (79).

Akt Scaffolds—Akt translocation to different intracellular regions is contingent on which upstream pathway is activated, partially because of the scaffolding proteins that direct its signaling. For example, the stimulation of endothelial cells with insulin leads to the activation of Akt and its translocation to both the Golgi and mitochondria, whereas stimulation with 17β-estrodiol leads to translocation of Akt to the Golgi but not mitochondria (59). Only a few Akt scaffolds have been identified thus far, but it is becoming more apparent that different receptors use different complexes to direct Akt activity (80). For example, Akt kinase-interacting protein 1 was identified as a scaffold for PI3K/PDK-1/Akt that associates with activated EGF receptors (81). This scaffold is necessary for Akt phosphorylation by PDK-1 downstream of EGF signaling. Conversely, Akt scaffolds can also attenuate Akt signaling by scaffolding it in proximity to its phosphatases. β-arrestin 2 scaffolds Akt and its activation loop phosphatase, PP2A, in response to G-protein-coupled receptor stimulation in dopaminergic neurotransmission (82), and β-arrestin 1 scaffolds Akt1 and its hydrophobic motif phosphatase, PHLPP2, downstream of receptor tyrosine kinases (83). Considering that the three Akt isozymes have some overlapping expression, are activated downstream of a multitude of receptors, have numerous cellular functions, and show specificity in their dephosphorylation by PHLPP, an abundance of Akt scaffolds potentially await identification.

Membrane Microdomains—Lipid rafts, which are cholesterol- and sphingolipid-rich microdomains, not only compartmentalize signaling complexes, but also increase signal transduction by aggregating particular signaling complexes in a small area (1).

PKC—DAG and its effector kinase PKC are often enriched in specialized lipid rafts that form membrane invaginations called caveolae (84, 85). For example, activation of the adenosine A1 receptor in cardiomyocytes causes PKCε and PKCδ to translocate to caveolae. PKCα is also enriched within caveolae through interactions with caveolin, which inhibits its activity, or with the serum deprivation response protein (86, 87).

Akt—Akt signaling not only differs at various subcellular localizations, but also varies within microdomains of a particular membrane. The Akt reporter AktAR (Fig. 2*G*) was preferentially targeted to different plasma membrane microdomains such as lipid rafts (using the N-terminal region of Lyn kinase) and non-raft regions (using the Kras CAAX motif) to analyze the spatiotemporal dynamics of Akt activity (61). These reporters demonstrate that Akt residing in lipid rafts is activated more potently and with faster kinetics than non-raft Akt. PDK-1 is enriched in membrane microdomains, partially accounting for the increased Akt activity in this compartment (88, 89).

Membrane microdomains provide sheltered environments in which signaling is protected from immediate termination. For example, PI3K gets activated and produces PIP_3 in lipid rafts where Akt is recruited, enabling Akt to be rapidly and specifically activated by PDK-1 (90). However, PTEN, which terminates PIP_3 signaling, is primarily found in non-raft microdomains, allowing PIP_3 levels to be temporarily maintained in lipid rafts, underscoring the importance of spatially separating kinases from phosphatases. Disturbing this spatial segregation of negative and positive regulators of a pathway can lead to disease states.

Dysregulation of Lipid Signaling in Disease—Dysregulation of the PIP_3 and DAG signaling pathways leads to numerous pathophysiologies, such as inflammation, cardiovascular disease, diabetes, neurodegeneration, and cancer (91). Imbalances in these pathways are caused by mutations, gene amplifications or deletions, chromosomal translocations, or epigenetic changes of genes in these pathways. In general, enzymes that promote signaling such as PKCι, Akt, and the catalytic subunit of PI3K have been identified as oncogenes (92–94), whereas those that terminate signaling—PTEN, PHLPP, and DGK—have been implicated as tumor suppressors (53, 95–100).

Although mutations in Akt are uncommon, Akt signaling is often elevated in cancer. Indeed, the PI3K pathway is one of the most frequently up-regulated pathways in cancer (101–103). Mutations that inactivate PTEN or hyperactivate the catalytic subunit of PI3K are very common and induce constitutive Akt signaling. Interestingly, a functional proteomics study using a reverse-phase protein lysate array revealed that in breast cancers, Akt activation loop and hydrophobic motif phosphorylation strongly inversely correlates with PTEN levels, underscoring the importance of PTEN regulation of Akt activity (104).

Mutations in receptors upstream of DAG and PIP_3 can also lead to enhanced signaling. For example, certain EGF receptor mutants constitutively associate with the PI3K/PDK-1/Akt scaffold Akt kinase-interacting protein 1, leading to increased Akt signaling in lung cancer (105). The involvement of some of these genes in cancer is further substantiated by their frequent amplification or deletion in tumors. For example, genes encoding PKCι, Akt1, Akt2, and the catalytic subunit of PI3K are amplified in various cancers (92, 94, 106–109), whereas those encoding PTEN and PHLPP are commonly deleted (110).

CONCLUSIONS

Extracellular stimuli lead to the rapid but transient production of localized pools of DAG and PIP_3 at discrete subcellular locations. The kinetics of the production and removal of these second messengers vary at different intracellular regions, leading to unique signaling signatures at each location. Protein scaffolds and membrane microdomains play key roles in signaling by providing ways to mediate the action of promiscuous enzymes. As we enter the frontier of localized signaling, reporters that measure lipid second messenger levels and kinase activity at the diverse signaling platforms afforded by intracellular membranes, membrane microdomains, and scaffolds will become increasingly sought after as a means to precisely measure activity downstream of particular ligands and receptors. Moreover, our knowledge of the human phosphorylation network will continue to expand, as kinase–substrate relationships are systematically being tackled.

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