MINIREVIEW

Current Perspectives on Akt Akt-ivation and Akt-ions

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The serine/threonine kinase Akt is an effector of PI3K-generated phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P3] and is a principle mediator of growth factor-induced signal transduction. Akt is activated through phosphorylation by specific kinases, and its activity is reduced directly by phosphorylation-site-specific phosphatases. In addition, Akt activity is effectively reduced by the action of phosphatases which dephosphorylate PI(3,4,5)P3, thereby reducing the levels of the essential lipid activators of PDK1 and Akt. The functions of Akt are pleiotropic and include regulation of cellular proliferation, differentiation, protein synthesis, and survival. Akt stimulates protein synthesis through actions on mTOR/p70S6K, and promotes survival by phosphorylating and inactivating pro-apoptotic molecules such as Ask1, Bad, Bax, and FoxO3a. Furthermore, loss of Akt decreases the intracellular ATP:AMP ratio, thus establishing a role for Akt in energy regulation. Three isoforms of Akt have been identified, and although redundant functions between isoforms exist, recent investigations have enumerated unique functions for each. Therefore, targeting specific Akt isozymes in a tissue- and context-specific fashion may lead to a greater understanding of Akt-mediated processes. Exp Biol Med 234:1264–1270, 2009

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Introduction

Akt (also known as protein kinase B) is a serine/threonine kinase downstream of phosphoinositide-3′-OH kinase (PI3K) and a member of the AGC family of protein kinases. Since first discovered in 1977 as the oncogene in the transforming retrovirus AKT8 (1), Akt has become the subject of intense research aimed at defining its involvement in cancer progression, metabolism, cellular growth and differentiation, and survival. Much has been learned about the regulation and actions of Akt since its cloning and identification of human homologues (2), yet much still remains to be elucidated. For example, there are three isoforms of Akt in mammals, and the functions of each have been shown to be redundant as well as unique depending on the tissue examined, stimulus, outcome (e.g. differentiation) or the conditions within the cellular milieu. Additionally, our knowledge of Akt regulation in terms of both activation and signal suppression is burgeoning, but further work must be accomplished to apply these findings in practice in humans; for instance, to attenuate cancer progression and metastasis, or to delay onset or minimize age-related sarcopenia. This review discusses factors involved in regulating Akt activation, select substrates of Akt involved in survival and growth, and isoform-specific functions of Akt.

Activation of Akt

Members of the PI3K class of enzymes generate phosphoinositol lipids that act as second-messengers in a number of intracellular signaling cascades, including the activation of Akt (3). PI3K catalytic enzymes are categorized into three classes by their structure, substrate specificity, and lipid products (4). Members of the Class IA PI3Ks (α, β, and δ) are heterodimers consisting of a 110

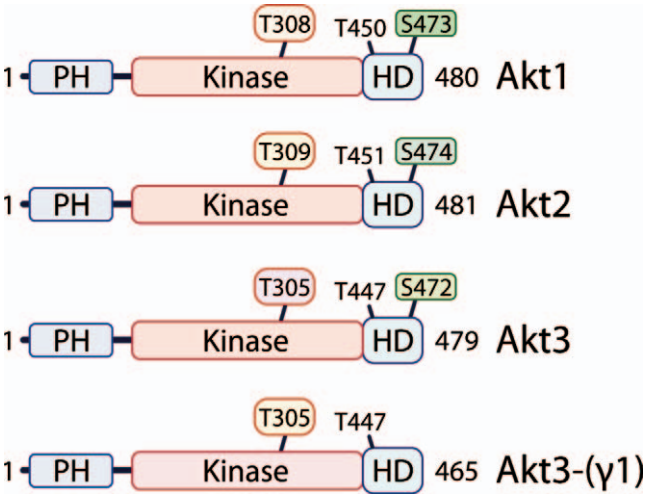

Figure 1. Comparison of human Akt isoform domain structures. Three isoforms of Akt exist in man that share approximately 80% amino acid sequence homology. Additionally, an alternative splice variant of Akt3 with a truncated hydrophobic domain (designated "Akt3-(γ1)" in this figure) has been identified (see text). All Akt isoforms possess an N-terminal PH domain responsible for phospholipid binding that is tethered to a catalytic region containing a threonine residue (T308 in Akt1) critical for activation of the enzyme. A C-terminal hydrophobic domain (designated "HD" in this figure) follows the catalytic domain and contains a serine residue (S473 in Akt1) important for full activation. Phosphorylation of a threonine residue in the turn motif (T450 in Akt1) by mTORC2 contributes to stability of the molecule. Numbers to the immediate left of the images designate the first amino acid, and numbers to the immediate right of the images represent the number of the most C-terminal residue as determined by comparative sequence analysis. A color version of this figure is available in the online journal.

kDa catalytic subunit and an 85, 55, or 50 kDa regulatory subunit (4, 5). PI3K activation leads to proliferation, survival, cell migration, and growth; however, abnormally increased PI3K signaling can promote aberrant proliferative signals that lead to cellular transformation (6–8). When bound to cognate ligands, receptor tyrosine kinases such as the insulin-like growth factor-I receptor undergo autophosphorylation, resulting in the recruitment of adaptor molecules including insulin receptor substrate (IRS) proteins (9, 10). PI3K regulatory subunits complexed to p110 catalytic subunits then bind IRS and convert plasma membrane-associated PI(4,5)P2 to PI(3,4,5)P3 (5). PI(3,4,5)P3 provides a phospholipid binding substrate for signaling effector molecules such as phosphatidylinositol-dependent kinase 1 (PDK1) and Akt (11). At least two identified phosphatases act to reduce PI(3,4,5)P3 levels: phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (12) and SH2-containing inositol 5′-phosphatase (SHIP) (13). PTEN hydrolyzes the phosphate at the D3 position on the inositol ring of PI(3,4,5)P3, thus generating PI(4,5)P2. SHIP proteins remove phosphates from the D5 position, thus generating PI(3,4)P2.

Akt exists as three isoforms in mammals (Akt1, Akt2, and Akt3), and each is transcribed from separate genes (14–17); additionally, a splice variant of Akt3 has been identified (18). Akt contains an N-terminal pleckstrin homology (PH) domain which interacts with PI(3,4)P2 and PI(3,4,5)P3 (19, 20), and a C-terminal domain that contains a hydrophobic domain (HD) with homology to other AGC kinases (21). Akt is activated by phosphorylation at two sites including one in the activation loop (A-loop) (T308, T309, T305 in Akt1, Akt2, and Akt3, respectively) in the catalytic domain, and one in the C-terminal HD (S473, S474, S472 in Akt1, Akt2, and Akt3, respectively) (21). The C-terminus of the Akt3 splice variant, however, lacks the analogous portion of the HD that contains S473, S474, or S472 present in the other Akt isoforms (Fig. 1). Phosphorylation at the turn motif (TM) at a highly conserved threonine residue in the carboxy-terminus results in increased stability of the molecule (22).

Phosphorylation at the A-loop is sufficient for activation of Akt, however full activation is achieved when both the A-loop and HD sites are phosphorylated (21). Akt can be activated through growth factor stimulation (21) and oxidative stress (23, 24). Phosphorylation of the A-loop in the catalytic domain is accomplished by PDK1 after recruitment to the cell membrane and binding with PI(3,4,5)P3 (11, 19, 25). HD phosphorylation has been shown to be mediated, under certain conditions, through actions of a number of factors including mammalian target of rapamycin complex 2 (mTORC2) (26), DNA-PK (27), lipid raft-associated elements (28), and autophosphorylation of Akt itself (29). However, growth factor-stimulated phosphorylation of Akt at the HD is considered to be mediated principally by mTORC2.

Direct de-phosphorylation of Akt remained an enigma until the discovery of PH domain leucine-rich repeat protein phosphatase 1 (abbreviated as PHLPP1) (30), and more recently, PHLPP2 (31). Both PHLPP1 and PHLPP2 were found to de-phosphorylate the HD site of Akt, but they did so in an Akt isoform-specific manner.

The mechanisms outlined above describe a general scheme for full activation for Akt in response to growth factor stimulation: generation of PI(3,4,5)P3 or PI(3,4)P2 by PI3K results in membrane recruitment of Akt and co-localization of PDK1. PDK1 phosphorylates Akt at the A-loop which results in conformational change of Akt allowing for phosphorylation of the HD by mTORC2. Akt then acts on downstream targets to initiate signaling cascades. Figure 2 presents these processes diagrammatically.

Downstream of Akt: Survival, Growth, and Energy Homeostasis

A number of known and putative Akt targets have been identified thus far by virtue of their containing the essential Akt consensus motif (R-X-R-X-X-S/T-B) where X is any amino acid and B represents a bulky hydrophobic residue (32, 33). Among these targets are several pro-apoptotic molecules that are inactivated when phosphorylated by Akt,

thus demonstrating that Akt can act as a survival factor. There are many pro-apoptotic substrates of Akt including apoptosis-signal regulating kinase 1 (Ask1) (34), Bad (35), Bax (36), FoxO transcription factors (37–39), and human caspase-9 (40). Ask1 is a mitogen-activated protein (MAP) kinase kinase kinase that transmits pro-apoptotic signals through activation of downstream stress-activated protein kinases such as c-jun N-terminal kinase (JNK) and p38 MAPK (41). Phosphorylation of 14-3-3 by activated JNK promotes release of 14-3-3-bound Bad, Bax, and FoxO proteins, thus contributing to apoptosis through both mitochondrial-dependent (Bad and Bax) and -independent (FoxO3a) (42, 43). Each of these mechanisms will be explained in the following sections.

Phosphorylation of Ask1 at serine 83 by Akt has been shown to reduce Ask1 activation stimulated by oxidative stress, as well as apoptosis induced by Ask1 overexpression (34), thus providing evidence for a specific pro-survival role of Akt acting on Ask1. Interaction of Bad with outer mitochondrial membrane (OMM)-associated Bcl-2 and Bcl-XL leads to disruption of OMM integrity and cytochrome c release; hence, binding of Bad with Bcl-2 or Bcl-XL promotes apoptosis through the mitochondrial intrinsic death pathway. Phosphorylation of Bad at serine 136 by Akt leads to dissociation from Bcl-2/Bcl-XL and promotes association with cytosolic 14-3-3, thereby reducing pro-death signals (35). Another protein that disrupts OMM membrane integrity is Bax. Bax exists in the cytosol in a conformation permissive for actions by JNK and p38 MAPK (44); once acted upon by apoptotic stimuli, Bax undergoes a conformational change that permits insertion into mitochondrial membranes and oligomerization. However, prior phosphorylation of cytosolic Bax at serine 184 by Akt prevents this conformational change and reduces half-life, thereby impairing Bax-mediated apoptosis (36, 45).

Members of the FoxO family of transcription factors are structurally related proteins whose name is derived from the product of the *fork head* gene originally identified in *D. melanogaster* (46). FoxO transcription factors belong to a superfamily of Fox (Forkhead Box) proteins in a class defined as O (Other) which reflects disparities in DNA-binding domain sequences (47, 48). Among the FoxO proteins, FoxO3a has been shown to possess pro-apoptotic functions including regulation of FasL gene expression (49); however, FoxO3a-mediated apoptosis can be attenuated by Akt (39). FoxO3a is phosphorylated at three sites by Akt (T32, S253, S315), although S315 is preferentially phosphorylated by serum- and glucocorticoid-inducible kinase (SGK) (50). When phosphorylated by Akt at target residues, nuclear FoxO3a associates with nuclear 14-3-3; this association masks the nuclear localization sequence, and the complex is exported to the cytosol in an inactive state (51).

In addition to the target molecules described above, Akt can phosphorylate a number of other pro-death molecules to cause their inactivation, as well as oppose apoptosis indirectly by acting on proteins involved in transcription of pro-apoptotic genes (52). Altogether, it is clear that Akt plays a vital role in cell survival and is positioned at a point of convergence of a number of signaling pathways, balancing both pro-survival and pro-death signals.

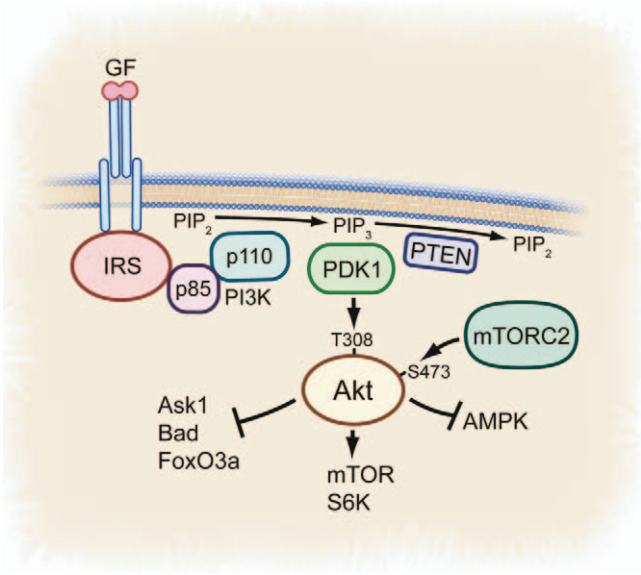

Figure 2. Regulation of Akt activation. Tyrosine kinase growth factor receptor-induced activation of Akt is initiated upon growth factor (GF) ligand binding to its cognate receptor. Recruitment of PI3K (p85 regulatory subunit complexed with p110 catalytic subunit) to the receptor at the membrane occurs after binding of adaptor molecules, including IRS docking proteins, to the intracellular subunits of receptors. PI3K, in close proximity to PI(4,5)P2 (PIP_2) then phosphorylates the D3 position of the inositol ring generating PI(3,4,5)P3 (PIP_3). PIP_3 then recruits PH-domain-containing molecules including PDK1 and Akt to the lipid bilayer (Akt recruitment not shown for clarity), allowing for binding to PIP_3, and phosphorylation of Akt at the A-loop (T308 in Akt1) by PDK1. Reconversion of PIP_3 to PIP_2 is promoted by PTEN, which reduces available PIP_3 for PDK1 and Akt binding, thus terminating growth factor signals. Phosphorylation of Akt at the HD (S473 in Akt1) occurs through the actions of the mTORC2 complex, which contains Rictor, SIN1, and mTOR. Activated Akt can then act on substrates in the cytosol to promote survival, growth and energy homeostasis, or translocate to the nucleus and phosphorylate target molecules such as FoxO3a. A color version of this figure is available in the online journal.

The mammalian target of rapamycin (mTOR) responds to nutrient or growth factor inputs, depending on its association with one of two adaptor molecules, either Raptor or Rictor (53). One principle effector of mTOR action is p70S6K, an AGC kinase family member whose actions promote growth and survival (54, 55). Raptor-bound mTOR, when also bound in complex with GβL (referred to as mTOR complex 1, or mTORC1) is generally considered the rapamycin-sensitive p70S6K kinase (56), but Rictor-bound mTOR (the complex, in association with GβL and SAPK interacting protein 1 (SIN1) (57), being referred to as mTORC2) has been shown to phosphorylate rapamycin-resistant mutants of p70S6K (58). Current models suggest that Akt positively regulates mTOR by acting on mTOR-inhibitory molecules such as proline-rich Akt substrate of 40

kDa (PRAS40) and tuberous sclerosis complex 2 (TSC2) (53, 59–61). mTORC2 is capable of phosphorylating Akt at the HD site (26, 57), as well as controlling folding and stability of the enzyme (22, 62); thus, there exists positive feedback signaling between mTOR and Akt.

AMP-activated protein kinase (AMPK) acts as an intracellular energy sensor (63) that is sensitive to Akt signaling. In a recent study, decreased ATP levels were observed in *Akt1*$^{-/-}$ *Akt2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) as compared to wild-type cells (64), suggesting that depletion of Akt affects energy charge. This reduced energy charge was associated with increased AMPK. Since AMPK can inactivate mTOR through direct phosphorylation of TSC2 (59), these findings suggest the existence of a mechanism to reduce growth and translation during low-energy states secondary to Akt depletion.

Isoform-Specific Actions of Akt

Three Akt isoforms have been identified, and murine gene disruption models demonstrate that each isoform possesses a distinct function (65). Mice lacking Akt1 have reduced body size (66), mice lacking Akt2 show abnormal glucose homeostasis and a diabetic phenotype (67, 68), and mice lacking Akt3 have diminished brain size (69, 70). In mammary tumor cells, Akt1 or Akt2 knockdown had opposing effects on differentiation (71), while in melanoma cells, Akt3 was found to be the principle Akt isoform involved in tumor progression (72). Furthermore, in differentiating skeletal muscle cells, Akt2 mRNA, protein, and activity were found to be upregulated, and overexpression of Akt2 prevented apoptosis in response to serum deprivation, suggesting that Akt2 acts to reduce apoptosis during myogenic differentiation (73). Although these data demonstrate discrete functions for Akt isoforms, there is also accumulating evidence for overlapping actions. For example, each Akt isoform contributed to phosphorylation of GSK3α and GSK3β in 3T3-L1 adipocytes (74). Additionally, knockdown of individual Akt isoforms in H157 cells demonstrated distinct roles for Akt3 acting on p27 and Akt2 acting on GSK3α, but redundant roles for all three Akt isoforms in regulating FoxO1, GSk3β, and TSC2 (31). Moreover, in Akt1/Akt2 double knockout MEFs (DKO MEFs), Akt3 alone was sufficient to prevent cell death until reduced to only 20% of the initial level (75). Reduction of Akt3 through increased concentrations of siRNA, however, potentiated apoptosis in response to several pro-apoptotic stimuli in DKO MEFs. This latter study underscored that very limited amounts of total Akt, even of a single isoform, can promote survival under non-stressed conditions; but that under stress, cells require a higher threshold of Akt to remain viable. Together, these studies illustrate the complexity of Akt regulation and specificity, and demonstrate that Akt isoforms possess both distinct and functionally redundant actions in growth, metabolism, and cell survival.

Conclusions and Future Outlook

Much has been learned about Akt regulation and function since it was first discovered, yet much still remains to be elucidated. For instance, what elements control intracellular localization of Akt? Akt activation depends on proximity to PI(3,4)P2 and PI(3,4,5)P3 in cell membranes and it is important to define those factors within the cell that are responsible for membrane recruitment. Interaction of the PH domain with phosphoinositide lipids is essential for activation, but identification of the processes and molecules involved in executing this specifically targeted relocation is of importance. It then follows that the location of Akt in unstimulated cells is of interest in order to define movement patterns of Akt after stimuli.

Defining intracellular localization also extends to isoform-specific locations within the cell, as well as isoform-specific activation in response to stimuli. What are the determining factors that contribute to activation of Akt isoforms in response to growth factors? Why is one isoform preferentially activated over another? One answer may be that Akt isoforms are expressed at different levels in a given cell-type. If availability of one isoform is greater than another, then it may be a matter of proximity to membrane-located signaling complexes. For example, if Akt1 is the most highly expressed Akt isoform in a cell, then it would logically follow that there would be more Akt1 available for recruitment to membranes than Akt2 or Akt3. It is also possible that inactive Akt may be localized to specific intracellular compartments or areas within the cell, possibly near the membrane, that contain increased densities of a particular isoform.

In addition to activation of Akt, it is important to further characterize existing known phosphatases as well as to discover new methods to de-activate the enzyme. The identification of isoform-specific de-phosphorylation of Akt by PHLPP1 and PHLPP2 was a groundbreaking discovery, and further research in defining the mechanisms of action and regulation of these two phosphatases is warranted.

An exciting and promising line of research involves the development of Akt isoform-specific small molecule inhibitors. Some inhibitors exert their effects by preventing Akt from being activated due to changes in ternary structure. For instance, an Akt-specific inhibitor commonly referred to as "Akti-1/2" has recently been developed that can bind to the PH domain and/or hinge region on Akt1 or Akt2 thereby preventing activation (76). This inhibitor can also bind to activated Akt and inhibit phosphorylation of substrates. However, Akti-1/2 can also inhibit Akt3 in 3T3-L1 adipocytes and L6 myotubes (77), as well as in C2C12 myoblasts (Matheny and Adamo, unpublished observations) at micromolar concentrations. Although Akti-1/2 is not specific for Akt isoforms in some cell lines at specific concentrations, this compound still possesses great potential under conditions where all three Akt isoforms can act in a redundant manner. In any case, intense research in the

development of Akt isoform-specific inhibitory compounds is ongoing (78).

In conclusion, Akt is at the crossroads of a number of intracellular pathways and is a key signaling intermediate in growth and survival. Much has been learned thus far with respect to regulation and signaling of Akt, yet much remains to be discovered; specifically, the localization of Akt isoforms in response to various stimuli in different tissues, as well as isoform-specific actions of Akt on target substrates.

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