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AKT/PKB Signaling: Navigating Downstream

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The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. Aberrant loss or gain of Akt activation underlies the pathophysiological properties of a variety of complex diseases, including type-2 diabetes and cancer. Here, we review the molecular properties of Akt and the approaches used to characterize its true cellular targets. In addition, we discuss those Akt substrates that are most likely to contribute to the diverse cellular roles of Akt, which include cell survival, growth, proliferation, angiogenesis, metabolism, and migration.

The serine/threonine kinase Akt/PKB has emerged as a critical signaling node within all cells of higher eukaryotes and as one of the most important and versatile protein kinases at the core of human physiology and disease. Since its discovery as an oncogene within the mouse leukemia virus AKT8 (Bellacosa et al., 1991; Staal, 1987) and as a homolog of protein kinase C (Jones et al., 1991), there have been many exciting breakthroughs elucidating the mechanism of upstream regulation of Akt (summarized in Figure 1). Several excellent recent reviews have covered the molecular details of Akt regulation and its role in human disease (such as Bellacosa et al., 2005; Engelman et al., 2006). Here, we focus on signaling downstream of Akt, with an emphasis on direct phosphorylation targets of the kinase and its bona fide cellular functions.

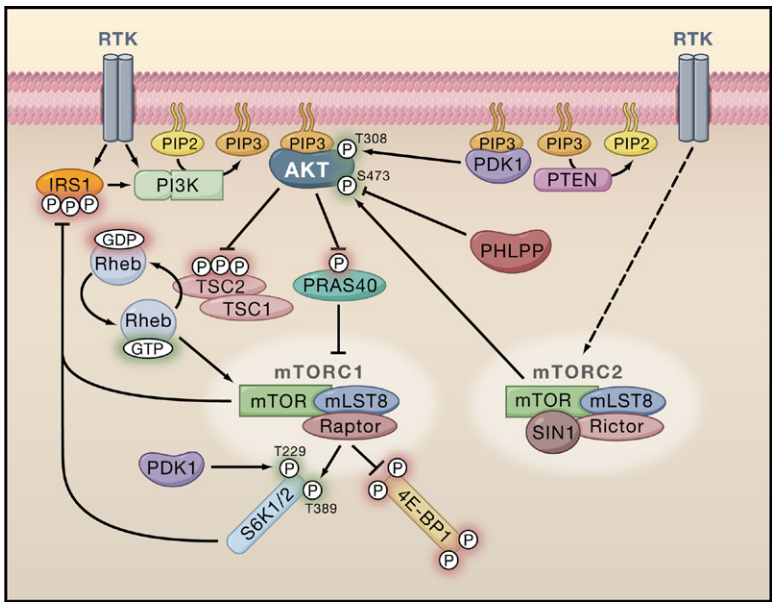

Figure 1. Upstream Activation of Akt by Growth Factors

Also depicted is the complex relationship between Akt signaling and mTOR. Activated receptor tyrosine kinases (RTKs) activate class I phosphatidylinositol 3-kinase (PI3K) through direct binding or through tyrosine phosphorylation of scaffolding adaptors, such as IRS1, which then bind and activate PI3K. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), in a reaction that can be reversed by the PIP3 phosphatase PTEN. AKT and PDK1 bind to PIP3 at the plasma membrane, and PDK1 phosphorylates the activation loop of AKT at T308. RTK signaling also activates mTOR complex 2 (mTORC2) through a currently unknown mechanism, and mTORC2 phosphorylates Akt on the hydrophobic motif S473, which can be dephosphorylated by the S473 phosphatase PHLPP. Akt activates mTOR complex 1 (mTORC1) through multisite phosphorylation of TSC2 within the TSC1-TSC2 complex, and this blocks the ability of TSC2 to act as a GTPase-activating protein (GAP) for Rheb, thereby allowing Rheb-GTP to accumulate. Rheb-GTP activates mTORC1, which phosphorylates downstream targets such as 4E-BP1 and the hydrophobic motif on the S6 kinases (S6Ks; T389 on S6K1). PDK1 phosphorylates the activation loop on the S6Ks (T229 on S6K1) in a reaction independent of PDK1 binding to PIP3. Akt can also activate mTORC1 by phosphorylating PRAS40, thereby relieving the PRAS40-mediated inhibition of mTORC1. Once active, both mTORC1 and S6K can phosphorylate serine residues on IRS1, which targets IRS1 for degradation, and this serves as a negative feedback mechanism to attenuate PI3K-Akt signaling. See text for references to recent reviews detailing Akt regulation and mTOR signaling.

Substrate Specificity

The three Akt isoforms (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ) have extensive homology to protein kinases A, G, and C within their kinase domains and are, therefore, members of the AGC kinase family. Following the identification of a residue on the kinase GSK3 as the first direct target of Akt in cells (Cross et al., 1995), groundbreaking experiments with peptides containing variants of this sequence defined the minimal recognition motif of Akt as R-X-R-X-X-S/T-B (Alessi et al., 1996b), where X represents any amino acid and B represents bulky hydrophobic residues. The critical requirement for R residues at both the −5 and −3 positions (that is, 5 and 3 residues, respectively, N-terminal to the phospho-acceptor site) on peptides efficiently phosphorylated by Akt distinguishes the substrate specificity of Akt from that of two other mitogen-stimulated AGC kinases, RSK (MAPKAP-K1) and S6K1 (p70S6K), which can better tolerate K at these positions. In these "peptide-bashing" experiments, more subtle Akt preferences were also uncovered for other residues surrounding the phosphorylation site (such as a preference for T at −2). Structural insights into the molecular interactions dictating the substrate selectivity of Akt have been provided by a high-resolution crystal structure of Akt bound to this GSK3 peptide substrate (Yang et al., 2002).

Further details of the preferred substrate specificity of Akt have been obtained using peptide library screening (Hutti et al., 2004; Obata et al., 2000), which provides an unbiased, systematic, and quantitative score for all 20 amino acids at each site within seven residues N-terminal and C-terminal to the phospho-acceptor site. This approach allows the identification of residues selected both for and against by the kinase of interest at each position surrounding the phosphorylation site. A bioinformatics program called Scansite (http://scansite.mit.edu; Yaffe et al., 2001) has provided the ability to search protein databases with the matrix of data obtained from such screens, rather than a single consensus sequence, and has been instrumental in identifying new Akt substrates and narrowing down target residues on suspected substrates. However, this approach needs to be used with caution, as it is only useful for identifying candidate phosphorylation sites. The sheer number of high-quality Akt sites within the proteome, in addition to the overlap in substrate specificity with other AGC kinases, illustrates the need for a rigorous demonstration of the direct in vivo phosphorylation of a given candidate target by Akt.

It remains possible that there are unknown sequence contexts or macromolecular interactions within cells that might allow Akt to phosphorylate motifs other than the minimally required R-X-R-X-X-S/T. Currently, however, there are no rigorously demonstrated and independently confirmed Akt substrates that fall into this category. Therefore, in the discussion of substrate characterization and the role of specific substrates below, we consider the presence of an R-X-R-X-X-S/T motif to be essential.

Defining Criteria for Bona Fide Akt Substrates

Here we review criteria that, in our view, should be met to define an in vivo Akt substrate. We recognize the limits of such standards, as it is possible that the same site phosphorylated by Akt in one cell type under one condition might be phosphorylated by additional kinases in other settings. However, given the increasing complexity of Akt signaling and its importance in human physiology and disease, it is worth evaluating the experimental paradigms currently used to define a direct downstream target of Akt. Our discussion is specific to Akt, but the criteria are similar to that put forward for other protein kinases (Frame and Cohen, 2001). Many approaches have been used to demonstrate phosphorylation of sites on target proteins within cells. However, no single method is sufficient; rather, both Akt loss- and gain-of-function approaches are needed. In the end, it is essential to demonstrate that phosphorylation of a candidate site on an endogenous substrate is induced by specific physiological stimuli that activate endogenous Akt and that the phosphorylation is lost upon Akt inactivation.

In Vitro Phosphorylation

Demonstration of direct in vitro phosphorylation of a candidate R-X-R-X-X-S/T site by Akt is a necessary step in defining an Akt substrate. Phosphorylation of the full-length protein of interest should be demonstrated, rather than fragments or motifs fused to other proteins (e.g., GST). Phosphate incorporation at the relevant site must be demonstrated. However, Akt is a somewhat promiscuous kinase in vitro and will phosphorylate most R-X-R-X-X-S/T sites and some R-X-X-S/T sites if given enough time and substrate. Therefore, in vitro phosphorylation, although necessary, is not sufficient for defining a new phosphorylation site for Akt.

Phosphorylation-Site Readout

In order to confirm candidate phosphorylation sites detected using bioinformatics and/or in vitro approaches, one first needs a reliable and specific readout of the phosphorylation event in question. This can occasionally be detected as a shift to a slower migrating form on denaturing polyacrylamide gels. However, it is rare that phosphorylation of an Akt site causes such a shift, which is usually indicative of a proline-directed site (i.e., S/T-P), and Akt poorly phosphorylates such sites (Alessi et al., 1996b; Hutti et al., 2004). Mass spectrometry techniques and in vivo [^{32}P]-orthophosphate labeling followed by two-dimensional phospho-peptide mapping are indispensable tools for identifying in vivo phosphorylation sites. However, due to their time-consuming and low-throughput nature, these approaches are somewhat less useful as an experimental readout when characterizing cellular conditions and kinases responsible for the phosphorylation. Antibodies specific for a phosphorylated motif (i.e., phospho-substrate antibodies) have been extremely valuable for identifying and characterizing new Akt substrates over the past five years (e.g., Kovacina et al., 2003; Manning et al., 2002; Sano et al., 2003). These antibodies are raised against degenerate