

PI3K/Akt and apoptosis: size matters

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Recent research has examined Akt and Akt-related serine–threonine kinases in signaling cascades that regulate cell survival and are important in the pathogenesis of degenerative diseases and in cancer. We seek to recapitulate the research that has helped to define the current understanding of the role of the Akt pathway under normal and pathologic conditions, also in view of genetic models of Akt function. In particular, we will evaluate the mechanisms of Akt regulation and the role of Akt substrates in Akt-dependent biologic responses in the decisions of cell death and cell survival. Here, we hope to establish the mechanisms of apoptosis suppression by Akt kinase as a framework for a more general understanding of growth factor-dependent regulation of cell survival.

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Structure of Akt kinases

The Akt gene product in mice is the cellular homolog of the *v-akt* oncogene transduced by AKT8, an acute transforming retrovirus in mice that was originally described in 1977 (Staal *et al.*, 1977). Research over the past decade has not only elucidated the regulation of Akt kinase by upstream signaling events, mainly as a consequence of activation of the second messenger phospholipid kinase phosphatidylinositol 3-kinase (PI3K), but also defined a role for Akt in promoting cell survival in a variety of apoptotic paradigms (Brazil and Hemmings, 2001; Scheid and Woodgett, 2001, 2003; Nicholson and Anderson, 2002). In 1991, three independent research groups cloned and characterized Akt kinases. The group of Philip Tsichlis identified *v-akt* as the gene transduced by rodent retrovirus AKT8 (Bellacosa *et al.*, 1991), and subsequently showed that its cellular homolog, then named *c-akt*, encoded the cytoplasmic serine–threonine protein kinase Akt in mice (Bellacosa *et al.*, 1993). Also, in 1991, two European research groups in Switzerland and England identified Akt and similar kinases when searching for novel kinases related to protein kinases A and C (Coffer and Woodgett, 1991; Jones *et al.*, 1991b). Thus, today Akt is frequently referred to as protein kinase **B** (= PKB, as a

kinase similar to protein kinases A and C), and has also been called RAC-PK in earlier reports (= a protein kinase related to protein kinases **A** and **C**).

All Akt serine-threonine kinases share a common structure that consists of an N-terminal regulatory domain resembling a pleckstrin homology (PH) domain (Franke *et al.*, 1994), a hinge region connecting the PH domain to a kinase domain with serine-threonine specificity (Ahmed *et al.*, 1993), and a C-terminal region required for the induction and maintenance of its kinase activity (Chan *et al.*, 1999). In mammals, three closely related isoforms of Akt are encoded by distinct genetic loci: Akt (that in fact is Akt1), Akt2 and Akt3. Whereas Akt (Akt1) is ubiquitously expressed at high levels with the exception of the kidney, liver and spleen (Coffer and Woodgett, 1991; Jones *et al.*, 1991b; Bellacosa *et al.*, 1993), Akt2 expression varies between different tissues, with higher expression levels in the muscle, intestinal organs and reproductive tissues (Jones *et al.*, 1991a; Konishi *et al.*, 1994). Akt3 is in turn expressed the highest in the brain and testis, and exhibits lower expression levels in intestinal organs and muscle tissue (Nakatani *et al.*, 1999a). Since different Akt isoforms are activated similarly and phosphorylate downstream substrates with equal specificity and efficiency, it has been postulated that the different Akt isoforms are functionally redundant (Franke, 2000).

Genetic mouse models of targeted deletion or altered function of specific isoforms have resulted in mutant animals. However, since animals with deleted expression of single Akt isoforms are viable and lack apparent developmental defects, these studies have raised an important question about the redundancy of Akt signaling and the potential of different Akt isoforms to compensate for the absence of other isoforms in cells. This apparent functional redundancy is particularly noteworthy in view of findings indicating that of all the known isoforms of human AKT, only human AKT2 and AKT3 are found to be pathologically amplified in human cancer, suggesting the specific involvement of these isoforms in the onset or propagation of cancer (Cheng *et al.*, 1996; Nakatani *et al.*, 1999b).

(Re)-discovery of a proto-oncogene

The original discovery of Akt kinase as the cellular homolog of a viral oncogene already implied its pivotal role in cell growth and survival that, since then, has been verified in many different cell systems (Brazil and

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Hemmings, 2001; Scheid and Woodgett, 2001). Oncogenic transformation that involves Akt has not been linked to any singular event, but rather to multiple causes that all result in a common end point of increased Akt activity and signaling strength through the Akt signaling cascade. As a result, all these changes lead to the Akt-dependent suppression of apoptosis and differentiation, and to increased cell cycle progression. The pathological conversion of the physiological pathway may be caused by multiple factors (see Figure 1) that also have the potential to cooperate. First, the extracellular factors that trigger the intracellular activation of Akt may be increased or the receptors that receive their signals may be altered or present in increased numbers (Bacus *et al.*, 2002; Basso *et al.*, 2002; Munster *et al.*, 2002; Pianetti *et al.*, 2002; Salh *et al.*, 2002). Second, the activity of intracellular mediators of Akt activation that include G-proteins, such as Ras and its regulators, second messenger-generating PI3K and other molecules, are increased either by oncogenic mutation or increased expression (Chang *et al.*, 1997; Jimenez *et al.*, 1998; Shayesteh *et al.*, 1999). Third, control mechanisms that regulate the signal leading to Akt activity are diminished by mutation or deletion. In particular, the cancer-

associated finding of frequent mutations and deletions in the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) also known as mutated in multiple advanced cancers 1 (MMAC1) or transforming growth factor β -regulated and epithelial cell-enriched phosphatase 1 (TEP1) has further supported the critical role of PI3K/Akt signal transduction in the control of cell growth and proliferation (Cairns *et al.*, 1997; Liu *et al.*, 1997; Risinger *et al.*, 1997; Maehama and Dixon, 1999). Finally, in mammary, ovarian, pancreatic and prostate cancer, the expression of Akt isoforms is enhanced (Cheng *et al.*, 1992, 1996; Bellacosa *et al.*, 1995; Nakatani *et al.*, 1999b; Graff *et al.*, 2000).

In summary, the increased activity and dysregulation of Akt kinases due to direct changes in Akt expression and indirect changes in modifiers of Akt activity result in increased signaling strength, and is a common feature of various forms of familiar and somatic tumors in humans (Cantley and Neel, 1999). Taken together, the results of these studies provide strong validation for combining a treatment approach that targets the Akt pathway in conjunction with apoptosis-inducing chemotherapeutics and, thereby, accelerate the drive to develop drugs that inhibit the Akt pathway. Compounds that target the pathway by inhibiting Akt kinase activity in which its ATP-binding site represents a defined point of attack are already under commercial development (Reed, 2003), but other drugs that target the regulatory domain of Akt have also been designed (Kozikowski *et al.*, 2003). Its enzymatic properties as a protein kinase add to the value of Akt as a potential drug target since, historically speaking, loss of function has been easier to achieve through pharmacological targeting than restoration of function, which would be required to compensate for the loss of PTEN or p53 function, for example. One caveat for targeting Akt, however, is the fact that except for its direct amplification, occurring mainly in ovarian tumors, none of the oncogenic factors involved in pathological Akt activation are truly specific. Thus, it is likely that some or all of the above changes in upstream signaling molecules will affect both Akt signaling and other parallel pathways that may contribute to the oncogenic changes associated with the finding of hyperactivated Akt. Moreover, when considering the importance of Akt for homeostasis of the organism, it is likely that pharmacological inhibition of Akt will have severe side effects for the function of several organ systems, including cardiac cells (Matsui *et al.*, 2003) and the nervous system (Yuan and Yankner, 2000). Consequently, the usability of potential Akt inhibitors for systemic treatment may be severely limited.

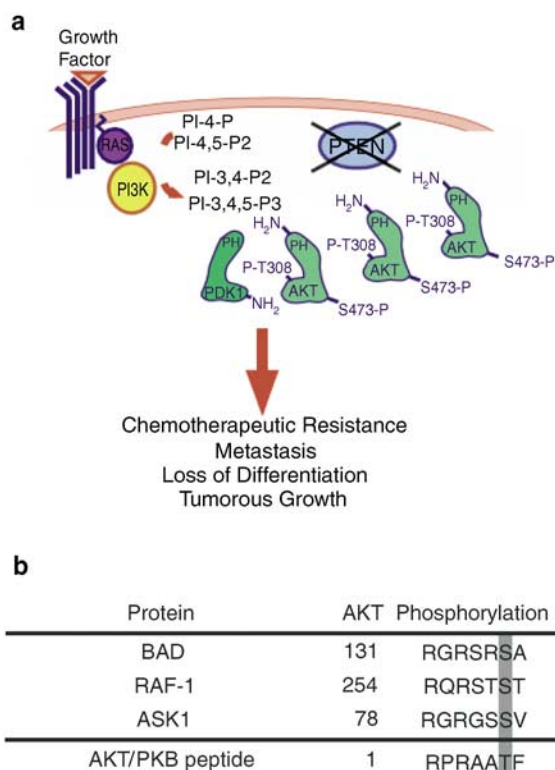


Figure 1 Activation of AKT in cancer leads to pathological cell responses through aberrant regulation of downstream substrates. **(a)** Oncogenic changes in Akt kinase signaling result in the conversion of physiological signal transduction pathways into pathological growth, loss of differentiation and apoptosis resistance. Frequent genetic changes in human tumors include the increased expression or activity of growth factor receptors, gene amplification of Akt isoforms, or mutational inactivation of PTEN. **(b)** The Akt-dependent phosphorylation sites of substrates with pathological implications have been depicted (Hosoi *et al.*, 1998; Biondi *et al.*, 2000; Page *et al.*, 2000; Yuan *et al.*, 2003)

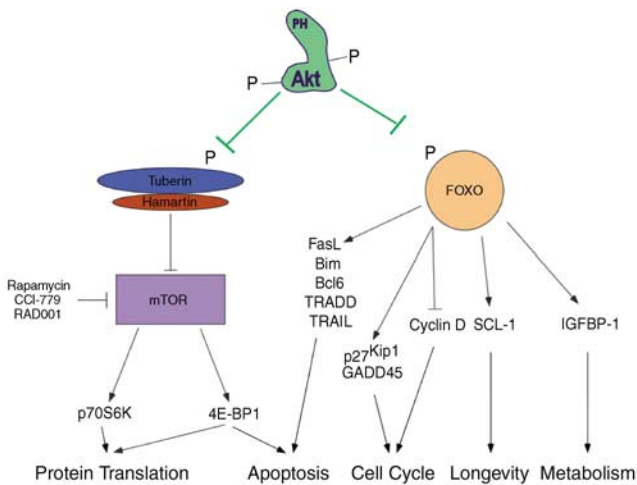


Figure 2 Evolutionary conserved pathways downstream of activated Akt regulate cellular homeostasis and transcription. In cooperation with the tumor suppressor hamartin (TSC1), the Akt substrate tuberin (TSC2) suppresses the activation of mammalian target of rapamycin (mTOR) in the absence of Akt activity. Akt phosphorylation results in decreased TSC2 expression, thus releasing the activity of mTOR toward p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which control protein translation and have also been implicated in apoptosis regulation (McManus and Alessi, 2002; Manning and Cantley, 2003). As a consequence of inhibiting transcription downstream of FOXO proteins, Akt activity facilitates cell cycle progression [e.g., by induction of Cyclin D (Ramaswamy *et al.*, 2002; Schmidt *et al.*, 2002) and inhibition of p27^{Kip1} (Medema *et al.*, 2000; Nakamura *et al.*, 2000) or growth arrest and DNA damage-inducible protein 45 (GADD45) (Tran *et al.*, 2002)], apoptosis suppression [e.g., by suppressing the expression of Fas-ligand (FasL) (Brunet *et al.*, 1999), tumor necrosis factor (TNF) receptor 1-associated death domain protein (TRADD) (Rokudai *et al.*, 2002) and TNF-related apoptosis-inducing ligand (TRAIL) (Modur *et al.*, 2002)] and metabolic responses [e.g., by the regulation of insulin-like growth factor-binding protein 1 (IGFBP1) expression (Rena *et al.*, 1999)]

interdependence within the signaling pathway (see Figure 2). Structural homologs of Akt kinases have been identified in many vertebrate and invertebrate species examined to date, and include Akt-related kinases in *Drosophila melanogaster*, *Caenorhabditis elegans* and *Dictyostelium discoides* (Franke *et al.*, 1994; Paradis and Ruvkun, 1998; Meili *et al.*, 1999). Genetic findings in *Drosophila* suggest a dual role for Akt, depending on the developmental stage. In early embryos, loss of Akt activity after overexpression of PTEN or dominant-negative (DN) PI3K results in ectopic apoptosis (Scanga *et al.*, 2000) and experiments using genetic mutants suggest a conserved role for Akt in apoptosis regulation and tumorigenesis (Staveley *et al.*, 1998). In contrast, at later developmental stages, the pathway primarily regulates cell size and does not affect apoptosis (Staveley *et al.*, 1998; Verdu *et al.*, 1999). These findings reinforce experimental evidence in mammalian cells and underscore the role for Akt in the regulation of cell growth and survival.

In *C. elegans*, genetic complementation groups have significantly contributed to the analysis and understanding of the Akt signal transduction pathway. A comparison of the Akt signaling pathways in *C. elegans*

and mammals indicates strong evolutionary conservation of the pathway (Paradis and Ruvkun, 1998). For example, the upstream regulatory molecules involved in activation of Akt, including the *DAF-18* gene (Ogg and Ruvkun, 1998), a structural homolog of PTEN that inhibits Akt signaling downstream of activated PI3K, *age-1*, a PI3K homolog gene, *pdk-1*, a phosphoinositide-dependent kinase 1 (PDK1) homolog gene (Paradis *et al.*, 1999) and other upstream components of the signaling cascade including insulin-like molecules (*ins-1*) and an insulin-like growth factor 1 (IGF1) receptor (*daf-2*) (Pierce *et al.*, 2001), are very similar. These studies in the nematode have not only provided a genetic model to complement biochemical studies of Akt signal transduction in vertebrate cells, but have also defined a functional end point for Akt signaling in the regulation of animal lifespan, suggesting some yet to be further clarified role for PI3K/Akt signal transduction in animal aging. Further support for a role of the Akt signaling cascade in longevity is obtained from studies in yeast, since the Sch9 protein kinase in *Saccharomyces cerevisiae* that has been implicated in longevity and stress resistance is also related to Akt, indicating that the conserved function for kinases such as Akt extends to the levels of single-celled organisms (Fabrizio *et al.*, 2001). It remains uncertain whether altered Akt activity in mammals impinges on animal lifespan. At least in animals where insulin-dependent metabolic responses are increased as a consequence of nutritional overload, the overall animal life expectancy is decreased in accordance with the genetic evidence in *C. elegans* (Finkel and Holbrook, 2000).

Mouse models of altered Akt function

Recent genetic studies in mice have further increased our understanding of Akt signal transduction in vertebrates, but they have also indicated that our present understanding of the Akt signaling cascades is incomplete. Two different *Akt* genes have been disrupted in the mouse germ line. The resulting animals are viable, but depending on which Akt isoform has been deleted, they either show significant retardation of growth and reduction of body weight (after *Akt1* deletion) (Chen *et al.*, 2001; Cho *et al.*, 2001b) or defects in the regulation of blood glucose levels following insulin stimulation (after knocking out the *Akt2* gene) (Cho *et al.*, 2001a). *Akt1*-null animals show certain organ-specific changes in apoptosis regulation, but the phenotypes of knockout animals are not as pronounced as expected when considering the pivotal role of Akt in signal transduction. The sole fact that both independent mutants are viable, with only a subtle difference in phenotype, may indicate the capacity of the three Akt isoforms to compensate for each other. In support of this idea, the phenotype of *Akt1* × *Akt2* double null animals shows severe dwarfism, atrophy of multiple organ systems (including the skin and skeletal muscle) and failed adipogenesis, and results in early neonatal lethality (Peng *et al.*, 2003). Still, with all the enumerated

defects, the animals develop normally during embryogenesis, and no significant increase of apoptosis induction is observed. One possibility for this is that Akt3 compensates for the loss of Akt1 and Akt2 functions. However, further studies will be required to determine whether additional mechanisms exist to compensate for the loss of Akt function or whether the most important function of Akt is indeed to control cell size, which is severely affected in these animals, and suggested both by these genetic studies in mice and by other experiments in invertebrate models including *D. melanogaster* (as outlined above). Candidate kinases to substitute for Akt function in addition to other Akt isoforms (i.e., Akt1 or Akt3 for deleted Akt2, Akt3 for deleted Akt1 and Akt2, etc.) exist and they include kinases that regulate similar substrates [such as serum- and glucocorticoid-induced protein kinase (SGK) (Brunet *et al.*, 2001)], or are even activated by similar upstream mediators [such as cytokine-independent survival kinase (CISK) (Liu *et al.*, 2000)].

Transgenic studies have also been performed by expressing mutant Akt from tissue-specific promoter systems. Studies using activated Akt1 in T cells have found increased apoptosis resistance, tumor formation and increased autoimmunity in aged transgenic animals (Jones *et al.*, 2000; Malstrom *et al.*, 2001). Studies using overexpression of activated Akt1 in mouse mammary glands or neural progenitor cells have observed a higher incidence of tumor formation, but only when Akt1 transgenic animals were crossed with animals carrying activated alleles of *K-ras* or animals expressing mutated middle T oncoprotein that leads to increased activation of Ras (Holland *et al.*, 2000; Hutchinson *et al.*, 2001). In the mammary model, apoptosis that physiologically occurs during the involution of the mammary gland when pups are weaned from their mother was suppressed, and confirmed the requirement of Akt activity for apoptosis suppression (Hutchinson *et al.*, 2001). Several other groups have also studied the importance of Akt in tumor models, including ovarian carcinoma (Orsulic *et al.*, 2002). In only one case, prostate-restricted activation of Akt in mice was shown to be sufficient to induce cell proliferation [prostatic intraepithelial neoplasia (PIN)], resulting in phenotypic changes (bladder obstruction) (Majumder *et al.*, 2003). In all models, however, a role for Akt in the regulation of cell size was observed. This may indicate that the Akt pathway is not selective to tumorigenesis, but rather acts mainly through the regulation of cell size and cell metabolism. Taken together, these data suggest that Akt1 expression by itself, in general, does not promote oncogenic transformation, even in systems where PI3K overexpression causes transformation (Aoki *et al.*, 1998; Zhao *et al.*, 2003). However, Akt1 expression is required to promote the tumorigenic properties of PTEN (Stiles *et al.*, 2002) and when Akt mutation and other oncogenic alterations coincide, aggressive tumors are formed. Thus, Akt resembles Bcl-2 which does not transform cells directly, but 'assists' oncogenes such as Ras through apoptosis suppression (Maestro *et al.*, 1999). Whether similar results can be obtained with

Akt2 or *Akt3* alleles remains an important question, also in light of findings that show their increased expression in tumors.

Akt: a direct downstream target of PI3K

Critical to the understanding of the regulation of Akt in cells was the finding that Akt kinase activity is induced following PI3K activation in various growth factor receptor-mediated signaling cascades (Burgering and Coffer, 1995; Franke *et al.*, 1995; Kohn *et al.*, 1995). PI3K phosphorylates phosphoinositides on the 3'-OH position of the inositol ring, and second messenger products of the kinase reaction in growth factor-stimulated animal cells are phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) and phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Rameh and Cantley, 1999). Findings of phosphoinositide-specific phospholipid phosphatases including PTEN further increase our understanding of second messenger signaling by phospholipids and the regulation of phosphoinositide metabolism in cells. The exact mechanism of Akt activation by PI3K has been studied thoroughly, and it is initiated by the binding of specific 3'-phosphorylated phosphoinositides to the Akt PH domain (Franke *et al.*, 1997b; Frech *et al.*, 1997). In contrast to PH domains in other molecules, which specifically bind to the PI3K product PIP3, the Akt PH domain binds to both PI-3,4-P2 and PIP3, and shows a relatively higher affinity for the binding of PI-3,4-P2. The binding specificities of different PH domains for different phosphoinositides have been confirmed by structural studies, and the specificity of the Akt PH domain for both PI-3,4-P2 and PIP3 may be relevant for achieving an extended signal of Akt activity in cells (Rameh and Cantley, 1999). An important consequence of the initial binding of Akt to the plasma membrane-bound phosphoinositides is the relocalization of the cytoplasmic Akt protein to signaling complexes at the plasma membrane. Deletion and mutation studies have found that the PH domain is critical for mediating this relocalization, in agreement with its ability to bind phospholipid second messenger molecules. The finding of increased activity of an N-terminal-deleted Akt mutant has been interpreted as an indication of intramolecular inhibition, but only recent evidence has suggested an interaction of N- and C-terminal regions of the Akt molecule (Calleja *et al.*, 2003). Oncogenic mutations of Akt that result in a constitutive, factor-independent activation include constitutive plasma membrane binding by N-terminal myristoylation, and also explain the constitutive activity of v-akt, by circumventing the initial translocation step (Franke *et al.*, 1995; Meier *et al.*, 1997).

Role of PDK1 in Akt activation

At the plasma membrane, phosphorylation of Akt at a threonine residue (residue threonine-308 in mouse Akt) in a flexible peptide loop in the kinase domain (T-loop) close to the catalytic core and at a second site within the

hydrophobic motif (HM) in the C-terminal tail (serine residue-473) potentially activates the enzyme. Mutations of these residues to non-phosphorylatable alanine residues inhibit AKT activation and activity, whereas substitution with charged aspartic acid or glutamic acid residues render the kinase constitutively active and uncoupled from upstream regulatory signals (Alessi and Cohen, 1998). The basic principles of AKT regulation are conserved by evolution in invertebrate and vertebrate animals alike (Scheid and Woodgett, 2000). The T-loop phosphorylation hereby resembles the phosphorylation step that is found in many AGC kinases [protein kinase A (PKA), G (PKG) and C (PKC)] and conferred by PDK1 (Alessi *et al.*, 1997; Stephens *et al.*, 1998). Once phosphorylated, the T-loop flips out of the active site and allows for access of both ATP and substrate. The requirement of PDK1 for phosphorylation of Akt and induction of its activity partially explains the importance of phosphoinositides in Akt activation in cells (Toker and Newton, 2000b). The role of phosphoinositides in determining Akt localization and PDK1 activity also explains the role of PI3K in Akt activation, where most factors that trigger PI3K activation also result in increased Akt activity.

Recent structural studies have been powerful tools in clarifying the molecular interactions at both the PH (Thomas *et al.*, 2002) as well as the C-terminal HM domain (Yang *et al.*, 2002), which is present in numerous AGC kinases and plays a dual regulatory role. It acts as a docking site for PDK1 and this docking is essential for the activation of the T-loop (Frodin *et al.*, 2000, 2002; Biondi *et al.*, 2002). Furthermore, the HM also acts as an allosteric regulator of Akt kinase activity (Yang *et al.*, 2002). By associating with the so-called PKC-related kinase-2-interacting fragment (PIF) pocket (Balendran *et al.*, 1999), it stabilizes the N-lobe of the enzyme, thus increasing its kinase activity 10-fold. Experiments investigating the mechanism of Akt activation by phosphorylation form the rationale for a model in which PI3K-generated lipids recruit both Akt and PDK1 to the plasma membrane, where the serine-473 residue of the HM domain of Akt is then phosphorylated (Biondi and Nebreda, 2003). As a result of adding the phosphate group, the increased negative charge of the HM domain facilitates its interaction with PDK1 by means of the PIF pocket. This protein-protein interaction stabilizes and activates PDK1 in order to phosphorylate threonine-308. Once threonine-308 has been phosphorylated, the HM domain of Akt can interact with its own PIF pocket and by competition for PDK1 binding release PDK1. Unfortunately, this elegant model has already been challenged with the fact that competition for the PIF pocket of PDK1 after addition of a peptide structurally related to PIF (PIF-tide) does not prevent Akt phosphorylation.

A tail tale of two PDKs

The affinity of the HM for the PIF pocket is greatly increased by the concomitant phosphorylation of sites

analogous to serine-473 in other AGC kinases, but not in Akt itself (Biondi *et al.*, 2001). It is not fully understood how this second phosphorylation step occurs in Akt. Experimental data exist to support both autophosphorylation (Toker and Newton, 2000a) as well as the existence of distinct serine kinases, including integrin-linked kinase (ILK)-1 (Persad *et al.*, 2000). In keeping with the established nomenclature, a distinct phosphoinositide-dependent kinase 'PDK2' activity has been proposed to explain the phosphorylation of the C-terminal residue. Experimental evidence suggests that PDK1 can phosphorylate the C-terminal residue *in vitro* in the presence of PIF peptide, raising the possibility that PDK1, under certain conditions, may pose as PDK2 kinase by undergoing an unprecedented specificity switch after the initial phosphorylation of the catalytic site residue in Akt (Balendran *et al.*, 1999; Biondi *et al.*, 2000). Studies of the PDK1-deficient mouse embryonic fibroblast strongly support the importance of PI3K-dependent PDK1 activation for the induction of Akt activity in cells (Williams *et al.*, 2000). Genetic models in *C. elegans* and *D. melanogaster* also underscore the importance of PDK1 activity for Akt signaling *in vivo*, and suggest that our current model of PI3K/PDK1-dependent activation of Akt is accurate (Cantley and Neel, 1999). Most recently, biochemical activities for potential serine-473 kinase have been isolated and shown to be distinct from PDK1 and ILK, but further studies are required for the identification of their molecular composition (Hill *et al.*, 2002; Hodgkinson *et al.*, 2002). Still, no additional PDK-type kinase that can explain the PDK2-like activity in cells has been cloned.

PDK1-independent mechanisms of Akt activation

Alternative mechanisms of Akt activation, which are independent of PDK1 and sometimes do not even involve PI3K, exist. One such PI3K-independent mechanism involves ILK1 (Yoganathan *et al.*, 2000). Coexpression of Akt with ILK1 in cells induces phosphorylation of Akt on serine-473, and expression of an ATP-binding site mutant form of ILK1 together with Akt interferes with cell-survival signaling and with Akt-dependent oncogenesis in prostate cancer cells (Persad *et al.*, 2000). The assumption that ILK1 phosphorylates Akt on the C-terminal residue serine-473 is an attractive explanation to link Akt signal transduction to those cell-matrix interactions that have significant consequences for cell survival (Attwell *et al.*, 2000; D'Amico *et al.*, 2000; Persad *et al.*, 2000). However, the ability of ILK1 to function not only as an adaptor molecule, but also as a protein kinase, is still controversial after alignment of the sequence of ILK1 with that of other kinases (Lynch *et al.*, 1999). In particular, ILK1 is missing residues that are found to be essential for catalyzing phosphotransfer reactions in other kinases (Lynch *et al.*, 1999). Finally, the characterization of a non-ILK serine-473 kinase activity in lipid rafts shows that it is not the only kinase capable of fulfilling this role (Hill *et al.*, 2002).

A growing number of studies have reported mechanisms of PI3K-independent induction of Akt activity. Ca^{2+} /calmodulin-dependent kinase kinase (CaM-KK) was reported to phosphorylate Akt on threonine-308 independently of PI3K and trigger Akt-dependent survival responses (Yano *et al.*, 1998). The model of CaM-KK-dependent activation of Akt is attractive, also in view of the activation of Akt by *N*-methyl-D-aspartate (NMDA) (Bhave *et al.*, 1999; Lafon-Cazal *et al.*, 2002; Sutton and Chandler, 2002), but little additional evidence applicable to other AGC family kinases has been found to support this model (Imai *et al.*, 2003). PKA itself is a substrate for PDK1-dependent phosphorylation, but it also activates Akt in a PI3K-independent manner by a mechanism that does not result in Akt phosphorylation on serine-473 (Sable *et al.*, 1997; Filippa *et al.*, 1999). Other reports have shown PI3K-independent activation of Akt by oxidative stress (Konishi *et al.*, 1996) and mitogen-activated protein kinase activated protein (MAPKAP) kinase-2, a stress-activated kinase that is able to phosphorylate Akt on serine-473 *in vitro* (Clifton *et al.*, 1996). Recent studies, however, contradict earlier findings by emphasizing the importance of growth factor signaling and PI3K activity in stress-mediated Akt activation (Shaw *et al.*, 1998). Finally, several different heat-shock proteins (HSPs) including HSP25/27 and HSP90 associate with Akt, suggesting a role in the reported activation of Akt following exposure of cells to heat shock and other stresses, perhaps by altering the accessibility of Akt to inactivating protein phosphatases (Konishi *et al.*, 1997; Sato *et al.*, 2000). Further experiments with HSP inhibitors, performed by Basso and coworkers, in addition to confirming previous results, also indicated that HSPs may play a role in protecting Akt from proteasome-mediated degradation (Basso *et al.*, 2002).

Negative regulators of PI3K/Akt signaling

Phosphorylation by upstream kinases is required for complete Akt activation and is important for the maintenance of its activity. Thus, it is not surprising that several protein phosphatases are able to inactivate Akt by dephosphorylation. Nonspecific inhibition of endogenous phosphatase activity efficiently activates Akt (Andjelkovic *et al.*, 1996) and phosphatase activity is involved in mediating the effects of extracellular stresses on Akt signal transduction (Meier *et al.*, 1998; Chen *et al.*, 1999). Dephosphorylation of Akt is also observed after increased ceramide levels, possibly due to inhibition of PI3K or other upstream signaling molecules (Zhou *et al.*, 1998; Zundel and Giaccia, 1998). Insights into the importance of phosphatases for the regulation of Akt activity have also been obtained from the study of phosphatases that dephosphorylate phosphoinositide products of PI3K. Of these, the src homology-2 (SH2) domain-containing inositol phosphatases SHIP-1/2 and the PTEN phosphatase are critical mediators in determining Akt activity. SHIP-1 depho-

phorylates inositides and phosphoinositides on the 5'-position and regulates B-cell/myeloid cell function (Aman *et al.*, 1998; Astoul *et al.*, 1999; Liu *et al.*, 1999), whereas SHIP-2 controls the intracellular levels of Akt-activating phosphoinositides in non-hematopoietic cell types (Taylor *et al.*, 2000).

The PTEN gene was originally identified as a tumor-suppressor gene and is frequently affected by germline and somatic mutations in human cancer (Myers and Tonks, 1997; Cantley and Neel, 1999). Although substrate specificity predictions based on structural comparisons initially concluded that PTEN may be a phosphotyrosine-specific phosphatase, in an influential study, Maehama and Dixon showed that PTEN is a phosphoinositide phosphatase that efficiently dephosphorylates PI3K products at the 3'-position (Maehama and Dixon, 1998). Subsequently, many papers analyzed the expression and function of PTEN in human cancer cell lines and in primary human tumors (Myers and Tonks, 1997; Cantley and Neel, 1999). Since PTEN dephosphorylates the phosphoinositide products of PI3K that otherwise trigger the activation of Akt and other signaling pathways downstream of activated PI3K, inactivating mutations or loss of PTEN expression lead to increased levels of PI3K products in cells. These increased levels of second messenger molecules result in a constitutive signal leading to enhanced Akt activity and to increased cell cycle progression, apoptosis resistance and oncogenic transformation (Cantley and Neel, 1999). These findings explain the tumor-suppressor activity of PTEN: PTEN counteracts the pro-oncogenic effects of elevated PI3K activity by decreasing the intracellular levels of Akt-activating phospholipids (see also Figure 1). Additional support for the importance of PTEN in normal cell function is obtained from transgenic animal studies (Di Cristofano *et al.*, 1998; Stambolic *et al.*, 1998) and from studies that examine the function of PTEN in controlling the threshold of Akt signaling in *C. elegans* (Ogg and Ruvkun, 1998).

Interacting proteins that determine Akt activity

Other Akt-interacting proteins include proto-oncogenic proteins such as T-cell lymphoma (TCL-1), which has been recently shown to bind to the Akt PH domain and to amplify PI3K-dependent signaling through complex formation with Akt or by altering Akt localization in cells (Laine *et al.*, 2000; Pekarsky *et al.*, 2000). These molecules are intriguing because their interaction with Akt is isoform-specific: both TCL-1 and the related mature T-cell proliferation (MTCP-1) primarily bind to Akt1 and not Akt2 (Pekarsky *et al.*, 2000; Kunstle *et al.*, 2002). The consequence of TCL-1 binding appears to be homo- or hetero-oligomerization of Akt (Laine *et al.*, 2000). Furthermore, it was suggested that this hetero-oligomerization may facilitate the transphosphorylation of Akt (Laine *et al.*, 2002). The exact contribution and relative importance of this novel pathway to Akt regulation have yet to be determined. Recently, exam-

ination of Akt in the pathways regulating kainate-induced neurotoxicity has revealed Jun kinase (JNK)-interacting protein (JIP1) as yet another interaction partner to affect Akt activation (Kim *et al.*, 2002a). This interaction is similar to the interaction between Akt1 and TCL-1 in that the binding of Akt1 is greatly enhanced in comparison to the binding to the Akt2 isoform, thus suggesting that some specificity downstream of the different Akt isoforms may be attained by the preferential binding to their interacting proteins. In contrast, even though JIP1 has been implicated in regulating Akt1 activity (Kim *et al.*, 2003), Akt1 kinase activity is irrelevant in the functional suppression of JIP1-mediated neurotoxicity (Kim *et al.*, 2002a).

Other interacting molecules that regulate Akt activity include the 27 kDa protein carboxy-terminal modulator protein (CTMP) that binds to the C-terminus of Akt in a region including the HM and C-terminal serine phosphorylation site (Maira *et al.*, 2001). Akt and CTMP form an endogenous complex at the plasma membrane, and it was shown that the effect of CTMP binding was to reduce the phosphorylation of serine-473, resulting in a decrease in Akt activity. Furthermore, when expressed in AKT8-transformed mink lung cells, CTMP expression resulted in a phenotypic regression of these transformed cells to the wild type (Maira *et al.*, 2001). Another two-hybrid assay from Marc Montminy's group has resulted in the identification of the homolog 3 of *Drosophila* tribbles (TRB3) protein as an inhibitory regulator of Akt signaling (Du *et al.*, 2003). TRB3 is comparable to CTMP in that its binding to the Akt kinase domain blocks Akt activation by limiting access to critical phosphorylation sites, in this case the T-loop residue in the catalytic core. Considering the high degree of homology between Akt isoforms in the kinase domain, in contrast to TCL-1 or JIP1, TRB3 interacts with and blocks both Akt1 and Akt2 (Du *et al.*, 2003).

Apoptosis suppression by Akt

An important function of activated PI3K in cells is the inhibition of programmed cell death (Yao and Cooper, 1995), and Akt is a good candidate for mediating these PI3K-dependent cell-survival responses. The initial evidence to show that Akt acts as an anti-apoptotic signaling molecule was observed in cerebellar granule neurons after trophic factor withdrawal (Dudek *et al.*, 1997), and in fibroblasts after forced expression of c-Myc (Kauffmann-Zeh *et al.*, 1997). Subsequent work in many laboratories has established the principle role of Akt in the regulation of cell survival in several cell types, consistent with its ubiquitous expression pattern. Akt has been implicated as an anti-apoptotic in many different cell death paradigms, including withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation and treatment of cells with chemotherapeutic drugs and ischemic shock (Franke *et al.*, 1997a; Downward, 1998). Multiple studies supporting the role of Akt in apoptosis suppression have connected Akt to

cell death regulation either by demonstrating its down-regulation following pro-apoptotic insults, or by using gene-transfer experiments that transduce both activated, anti-apoptotic and inactive, pro-apoptotic mutants of Akt.

Taken together, these observations suggest that Akt may play a critical role both in the function of cancer cells and in the pathogenesis of degenerative diseases. By promoting the cell survival of mutated, damaged or transformed cells even under adverse conditions, Akt can promote cancer cell growth by protecting cells from apoptosis, which would otherwise be eliminated by programmed cell death. To experimentally prove the importance of Akt kinases in oncogenic transformation, in a seminal paper, Peter Vogt and colleagues demonstrated that a transformed cellular phenotype could be reverted to normal when using a cell model for PI3K-dependent oncogenesis as long as dominant-negative mutants of Akt were expressed concomitantly (Aoki *et al.*, 1998). Akt is also likely to play a significant role in degenerative diseases, where excessive or inappropriate cell death occurs possibly because proper trophic factor support is lacking. The relevance of Akt signaling in neurodegenerative disease is supported by studies that examine its activity and function in Alzheimer's disease models *in vitro* (Hong and Lee, 1997; Wehl *et al.*, 1999). A role for Akt has also been suggested in other models of human degenerative diseases, including cardiac failure (Matsui *et al.*, 1999) and other cardiovascular diseases where there is increased and chronic loss of cells (Reed and Paternostro, 1999).

Targets downstream of Akt kinase

The first molecular insights into the function of Akt were derived from studies directed at its role in insulin-dependent metabolic responses. When searching for kinases that could regulate glycogen synthase kinase-3 (GSK3), the groups of Brian Hemmings and Phil Cohen realized that Akt inhibited GSK3 activity by direct phosphorylation of an N-terminal regulatory serine residue downstream of insulin-activated PI3K (Cross *et al.*, 1995). GSK3 is also involved in consequences of Akt signaling that are not primarily related to metabolic responses (Cross *et al.*, 1995). Moreover, GSK3 is also involved in regulating proliferative and anti-apoptotic pathways (Ikeda *et al.*, 1998; Pap and Cooper, 1998), and in regulating cell cycle progression by phosphorylation of Cyclin D1 (Diehl *et al.*, 1998).

Other kinases besides Akt have been implicated in GSK3 regulation (Shaw and Cohen, 1999). The identification of GSK3 as an Akt substrate, however, also helped to define the Akt phosphorylation motif (Alessi *et al.*, 1996). By systematically permutating the amino-acid sequence surrounding the Akt phosphorylation site in GSK3, Alessi *et al.* (1996) derived an optimal peptide sequence for Akt phosphorylation. Indeed, most of the known Akt substrates contain a phosphorylation site similar to that found in GSK3. To date, many

additional Akt substrates have been identified, and even more have been predicted and await experimental verification (Yaffe *et al.*, 2001). Although recent research has confirmed the accuracy of the original phosphorylation motif (Obata *et al.*, 2000), the power of predicting possible downstream substrates solely based on a phosphorylation motif remains limited, since factors other than the primary peptide sequence such as intracellular protein localizations or access to the phosphorylation site may also influence kinase–substrate interactions.

Substrate regulation by Akt-dependent phosphorylation

Even if it is unlikely that all the potential targets are indeed regulated by AKT *in vivo*, dozens of substrates have already been confirmed *in vitro* and more are still being added to the list (Datta *et al.*, 1999; Brazil and Hemmings, 2001; Scheid and Woodgett, 2001). Due to the increasing complexity of signaling downstream of AKT kinase and the existence of alternative pathways with the ability to regulate targets in similar fashion (Liu *et al.*, 2000), the focus in the field is shifting away from the identification of novel substrates to the validation of known substrates in a relevant context, such as the chemotherapeutic resistance in cancer cells with activated Akt (Hosoi *et al.*, 1998; Ng *et al.*, 2000; Page *et al.*, 2000; Yuan *et al.*, 2003).

Without reviewing all these substrates in detail, they can be classified into two general categories (here, called group I and group II) depending on the consequences of Akt-dependent phosphorylation for their function. For group I substrates, Akt phosphorylation either increases (group Ia) or decreases (group Ib) the activity of substrates with intrinsic enzymatic activity. For group II substrates, Akt phosphorylation increases the affinity of the substrate for interaction with 14-3-3 proteins. 14-3-3 proteins are abundantly expressed in the cytoplasm and specifically bind phosphoserine-threonine-containing polypeptides, thus retaining phosphorylated Akt substrates in the cytosol (Franke and Cantley, 1997).

Examples of Akt phosphorylation events that inhibit substrate enzymatic activity include the inhibition of different GSK3 isoforms (Cross *et al.*, 1995). Correspondingly, examples of downstream enzymes that are activated by Akt include the activation of 6-phosphofructo-2 kinase (PFK2) (Deprez *et al.*, 1997) and nitric oxide synthase (NOS) (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; Michell *et al.*, 1999). In addition, specific kinases in mitogen-activated protein kinase (MAPK) and p38/JNK-type stress-regulated kinase cascades including Raf-1, B-Raf, mixed lineage kinase-3 (MLK3) and apoptosis signal-regulating kinase 1 (ASK1) are functionally inhibited following Akt-dependent phosphorylation (Rommel *et al.*, 1999; Zimmermann and Moelling, 1999; Guan *et al.*, 2000; Kim *et al.*, 2001; Barthwal *et al.*, 2003). It is not clear, however, whether Akt phosphorylation directly inhibits the activity of these kinases, or whether Akt determines their cellular function indirectly by affecting their

cellular localization and/or their interaction with accessory molecules. For the regulation of the serine–threonine kinase Raf-1, Akt-dependent inhibition plays an important role in the cellular coordination and integration of different branches of Ras-dependent signaling, especially in relation to cell differentiation (Scheid and Woodgett, 2000) and during the cellular response to chemotherapeutic treatment (Graff *et al.*, 2000; Yuan *et al.*, 2003).

Akt substrates with metabolic functions

Other Akt substrates are involved in downstream metabolic consequences of insulin stimulation, and include the enzyme PFK2 (Deprez *et al.*, 1997) and winged-helix forkhead-related transcription factors (Guo *et al.*, 1999; Nakae *et al.*, 1999). In *C. elegans*, the forkhead-related transcription factor DAF16 is a substrate of Akt in an insulin-like receptor pathway that is involved in the regulation of the exit from the *dauer* stage (Paradis and Ruvkun, 1998). Akt activity in insulin-dependent responses is also involved in the regulation of translocation and/or expression of glucose transporters (GLUTs) including GLUT1 or GLUT4 (Kohn *et al.*, 1996; Barthel *et al.*, 1999), and in the translocation of hexokinase to the mitochondria that captures intracellular glucose by phosphorylation (Gottlob *et al.*, 2001). These metabolic functions of Akt are related to its function in cell survival, since contrary to the pro-survival proteins of the Bcl-2 family, survival downstream of Akt directly relies on glucose metabolism, particularly at the first step of committing cells to glycolysis where it does not require *de novo* protein synthesis (Gottlob *et al.*, 2001; Plas *et al.*, 2001). Furthermore, Akt has also been implicated in the regulation of phosphodiesterase by direct phosphorylation of phosphodiesterase 3B (PDE3B) (Wijkander *et al.*, 1998), into the induction of NOS activity (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; Michell *et al.*, 1999), and in several other biologic functions that are related to metabolic control. Overall, these data suggest that the distinction between the regulation of metabolic processes and cell death regulation may be difficult, since Akt not only modifies cell-survival responses by phosphorylating specific substrates which are easily measured, but also induces more subtle changes in cellular homeostasis. These additional functions of Akt are of fundamental relevance for the maintenance of the cellular machinery, and the production and availability of nutrients and other building blocks necessary for the execution of basic cellular functions (Plas *et al.*, 2002).

Substrates of Akt that regulate protein synthesis

Recent studies have suggested that tuberin (TSC2) is regulated by Akt-dependent phosphorylation and, thus, constitutes another tumor-suppressor gene with direct implications for Akt signal transduction, in addition to

PTEN and p53. Reduced TSC2 function alternates with loss of hamartin (TSC1) and is frequent in tuberous sclerosis (TSC), an autosomal dominant tumor disease affecting 1 in 6000 individuals who present with a large number of benign tumors (Cheadle *et al.*, 2000). Another genetic disorder, lymphangioleiomyomatosis (LAM), is also associated with mutations in the TSC2 gene (Krymskaya, 2003). However, TSC rarely leads to highly malignant tumors, as they are observed after PTEN loss-of-function. Still, it is unclear whether this paradoxical difference stems from the relative position of these tumor suppressors within the Akt cascade, where PTEN is upstream and TSC2 downstream of Akt, or whether the loss of TSC2 is somehow self-restricted so that it fails to support more malignant phenotypes of the resulting tumors. Tumor growth in the *Tsc2*^{-/-} Eker rat model provides additional evidence for the relevance of Akt-mediated regulation of mammalian target of rapamycin (mTOR) activity for the oncogenic properties of the PI3K pathway (Kenerson *et al.*, 2002). In addition, treatment of cells with rapamycin and rapamycin-analogous pharmacological inhibitors such as CCI-447 (Wyeth-Ayerst) and RAD001 (Novartis) recapitulates the inhibitory action of TSC1/TSC2 on mTOR activity, and is very efficient at inhibiting growth arrest and inducing apoptosis in PTEN- or p53-deficient cells (Aoki *et al.*, 2001; Neshat *et al.*, 2001; Podsypanina *et al.*, 2001; Shi *et al.*, 2002). In fact, these inhibitors have proven so effective that they are now the focus of phase I/II clinical studies and are excellent examples for cancer treatments directed at the pathway that regulates cell size through nutrient uptake and protein synthesis (Potter *et al.*, 2003).

The exact consequences of the Akt-dependent phosphorylation of TSC2 remain controversial, since some data point to a decrease in the stability of the TSC1/TSC2 complex following phosphorylation by Akt (Inoki *et al.*, 2002; Potter *et al.*, 2002), which has not been repeated by other groups (Dan *et al.*, 2002; Manning *et al.*, 2002). The Akt-dependent decrease in the stability of TSC2 may be mediated by proteasomal degradation, and is facilitated by disruption of the stabilizing TSC1/TSC2 complex (Dan *et al.*, 2002; Inoki *et al.*, 2002). Akt-dependent proteasomal degradation has also been observed for other Akt targets such as FOXO3a (Plas and Thompson, 2003), and phosphorylation may enhance the degradation of p27^{Kip1} (Mamillapalli *et al.*, 2001). Alternative regulatory mechanisms of Akt-dependent TSC2 regulation involve 14-3-3 proteins (Li *et al.*, 2002b; Liu *et al.*, 2002; Nellist *et al.*, 2002; Shumway *et al.*, 2003), but findings in this field still remain contentious since the presumed 14-3-3 binding site does not match well with other 14-3-3 sites, including those on the Bcl-2 antagonist of cell death (BAD) and FOXO transcription factors (Manning and Cantley, 2003). However, it is clear that as a result of TSC2 phosphorylation by Akt, mTOR activity is depressed from the inhibitory control of the TSC1/TSC2 complex. Consequently, p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) are activated by

mTOR (Inoki *et al.*, 2002; Tee *et al.*, 2002). The role of 4E-BP1 during rapamycin-induced apoptosis has been underscored by a recent study in rhabdomyosarcoma cells in which apoptosis was induced downstream of 4E-BP1-mediated JNK activation in p53-deficient cells (Huang *et al.*, 2003). Similar to the DAF-16-related FOXO substrates of Akt, the pathway leading to Akt-dependent phosphorylation of TSC2 is conserved by evolution. More specifically, the enhanced cell growth after overexpression of Akt in proliferating cells of *Drosophila* is suppressed by expression of a TSC2 mutant lacking the Akt phosphorylation site (Potter *et al.*, 2002) and regulated in a TSC2-dependent pathway downstream or parallel to Akt (Gao and Pan, 2001; Potter *et al.*, 2001; Tapon *et al.*, 2001). Presently, a major question that still remains is whether the apparent growth deficiencies of Akt-mutant mice are also caused by a loss of TSC2 function.

A BAD kinase makes good

One of the first targets of Akt identified with direct implications for regulating cell survival is the pro-apoptotic Bcl-2-family member BAD. When it is not phosphorylated, BAD will inhibit Bcl-2 and other anti-apoptotic Bcl-2 family members by direct binding (Gajewski and Thompson, 1996). Once phosphorylated, however, the phosphoserine residues of BAD form affinity-binding sites for 14-3-3 molecules, thus localizing phosphorylated BAD to the cytosol and effectively neutralizing its pro-apoptotic activity (Zha *et al.*, 1996). In addition, phosphorylation of BAD changes its affinity for interaction with Bcl-2 molecules, an idea that is supported by the observation that a BAD mutant resembling a constitutive phosphorylation event is unable to inhibit Bcl-2 function, even though it is not bound to 14-3-3 proteins (Wang *et al.*, 1999). Two of the phosphorylated serine residues in BAD that are involved in mediating its interactions with 14-3-3 resemble an Akt phosphorylation motif (group II substrate). Two studies initially demonstrated that Akt was able to phosphorylate BAD, both in IL-3-dependent myeloid cells (del Peso *et al.*, 1997) and in fibroblasts and primary neuronal cells (Datta *et al.*, 1997). These studies suggested that Akt was directly involved in the regulation of survival by phosphorylation of BAD (Franke and Cantley, 1997).

Additional studies have subsequently linked Akt activity to BAD phosphorylation (Blume-Jensen *et al.*, 1998; Kulik and Weber, 1998; Yano *et al.*, 1998; Zundel and Giaccia, 1998; Mok *et al.*, 1999; Pastorino *et al.*, 1999), but other studies have pointed out a dissociation of Akt activity and BAD phosphorylation in certain signaling models (Scheid and Duronio, 1998; Craddock *et al.*, 1999; Hinton and Welham, 1999). In fact, a recent report suggests that kinases other than Akt actually mediate BAD phosphorylation in some IL-3-dependent cell models (Harada *et al.*, 1999). Today, the list of kinases that have been shown to regulate BAD phosphorylation includes CaM-KK, MAPK kinase

(MEK), PKA, PKC, p21-activated kinases (PAK1 and PAK4), CISK and p90 ribosomal S6 kinase (RSK) (Scheid and Duronio, 1998; Bonni *et al.*, 1999; Harada *et al.*, 1999; Scheid *et al.*, 1999; Tan *et al.*, 1999; Bertolotto *et al.*, 2000; Liu *et al.*, 2000; Lizcano *et al.*, 2000; Schurmann *et al.*, 2000; Shimamura *et al.*, 2000). Not all of these kinases phosphorylate BAD on the 14-3-3 binding sites. Indeed, an additional phosphorylation site that is not involved in binding to 14-3-3 proteins is present in BAD (Tan *et al.*, 2000). Still, its phosphorylation facilitates binding to 14-3-3 proteins, presumably by forcing the BAD protein into an optimal protein conformation for 14-3-3 binding (Datta *et al.*, 2000). Thus, several different signaling pathways that lead to BAD phosphorylation exist. Phosphatases such as calcineurin that inhibit Akt-dependent cell survival by dephosphorylation of BAD also have been identified (Wang *et al.*, 1999).

Accordingly, apoptosis regulation by BAD phosphorylation has emerged as a prime example of a physiological mechanism that integrates kinase cascades with apoptosis regulation. Our understanding of cell death regulation by BAD phosphorylation has been significantly increased by recent studies from the Greenberg laboratory that have expressed phosphorylation-site mutant Bad in a *Bad*-null genetic background in mice (Datta *et al.*, 2002). The finding of a lowered threshold for apoptosis induction in various animal tissues expressing mutant Bad is a direct *in vivo* confirmation of ideas originally proposed by Martin Raff, namely, that apoptosis is in fact the default cellular predisposition (Raff, 1992) unless, however, it is prevented by pathways such as Akt that reprogram and redirect the death pathways toward survival (Bergmann, 2002).

Other substrates of Akt in apoptosis regulation

Although the mechanism of BAD phosphorylation provides an elegant model of apoptosis regulation, it is not the only mechanism by which Akt can promote cell survival. One reason for this is the fact that Akt expression is fairly ubiquitous, whereas BAD expression itself is more restricted (Kitada *et al.*, 1998). Furthermore, several cell types that do not express BAD protein, but in which Akt still efficiently suppresses apoptosis, have been examined. Additional targets of Akt in cell survival have been postulated and some candidate protein substrates have already been identified and characterized (Datta *et al.*, 1999).

One set of targets may include proteins involved in the mitochondrial pathway of apoptosis. Akt not only plays a role in keeping cytochrome *c* in the mitochondria (Kennedy *et al.*, 1999), but its activity also inhibits the response of cells to cytochrome *c* after it is released from the mitochondria. Although caspase-9 appears to be a target of Akt in human cells and may explain cytochrome *c* resistance (Cardone *et al.*, 1998), it is not the only, or most important, target since Akt-dependent cytochrome *c* resistance is observed in other

vertebrate cell models in which caspase-9 lacks an Akt phosphorylation site (Fujita *et al.*, 1999; Zhou *et al.*, 2000). A second subset of Akt substrates that is, like BAD, regulated by phosphorylation-dependent binding to 14-3-3 proteins includes the forkhead-related family of mammalian transcription factors. The *C. elegans* forkhead-related DAF-16 protein transduces insulin receptor-like signals in a PI3K/Akt-homologous signaling cascade (Paradis and Ruvkun, 1998) and regulates the expression of the SCL-1 protein implicated in longevity and stress resistance (Ookuma *et al.*, 2003). In mammalian cells, forkhead-related transcription factors are retained within the cytoplasm when phosphorylated and bound to 14-3-3. Loss of Akt activity results in their dephosphorylation and translocation to the nucleus, where they then induce transcriptional events (Eder *et al.*, 1998; Biggs *et al.*, 1999; Datta *et al.*, 1999; Guo *et al.*, 1999; Kops *et al.*, 1999; Nakae *et al.*, 1999; Rena *et al.*, 1999; Tang *et al.*, 1999). It has been suggested that transcriptional events depending on forkhead-related transcription factors are involved in pro-apoptotic responses. Indeed, several studies have already demonstrated how Akt inhibits the transcription of specific pro-apoptotic genes by retaining forkhead-related protein in the cytoplasm (Datta *et al.*, 1999; Tang *et al.*, 1999). Furthermore, studies in primary human tumors and cell lines suggest that nuclear exclusion of forkhead-related transcription factors is frequent and often associated with oncogenic transformation (Graff *et al.*, 2000; Jackson *et al.*, 2000; Nakamura *et al.*, 2000; Hutchinson *et al.*, 2001).

Akt-dependent inhibition of stress-regulated kinase cascades

Another important class of Akt targets are proteins involved in the stress-activated protein kinase (SAPK) cascades. Growing experimental evidence points to a close functional relationship between the Akt survival pathway and SAPK/MAPK cascades that are activated by various cellular stresses and linked to apoptosis. Increased Akt activity has been shown to suppress the p38/JNK pathways in a number of cell systems (Berra *et al.*, 1998; Cerezo *et al.*, 1998; Okubo *et al.*, 1998). Recently, it has been shown that ASK1 is regulated by Akt and contains an Akt-specific phosphorylation site (Kim *et al.*, 2001). Thus, ASK1 is likely to be one of the points of convergence between PI3K/Akt signaling and stress-activated kinases cascades, although probably not the only one. For example, Akt also phosphorylates and regulates MLK3 (Barthwal *et al.*, 2003), and the small G protein Rac1 (Kwon *et al.*, 2000), other activators of the JNK pathway. However, the role of ASK1 has been underscored by a recent report, in which ASK1 activity in rhabdomyosarcoma cells was increased during rapamycin-induced apoptosis unless p53-dependent expression of p21^{Cip1/WAF1} blocked ASK1 activity (Huang *et al.*, 2003).

Integration of PTEN and p53 function at the level of Akt

The interphase between p53 tumor-suppressor signaling and Akt activity extends far beyond the implications of ASK1 in mediating the p53-dependent suppression of apoptosis following JNK activation. One interaction between the pathways is mediated by the ubiquitin ligase mouse double minute homolog 2 (Mdm2) or its human homolog HDM2 that are induced by p53 and block p53 function on the level of repression of its transcriptional function, proteasome-mediated degradation or nuclear export, and act as oncogenes in metastatic tumors of the breast and prostate (Mayo and Donner, 2002). Two alternative mechanisms of Akt-dependent regulation of Mdm2/HDM2 have been described, and they include the induction of Mdm2 nuclear import (Mayo and Donner, 2001) or the regulation of Mdm2/HDM2-ubiquitin ligase enzymatic activity (Zhou *et al.*, 2001b; Ashcroft *et al.*, 2002; Ogawara *et al.*, 2002). Furthermore, Mdm2 and Akt form phosphorylation-independent complexes (Zhou *et al.*, 2001b) and additional implications of these interactions for other p53-dependent processes have been recently examined in prostate and cervical tumor cell lines (Lin *et al.*, 2002).

Notably, the crosstalk between the Akt and p53 pathways extends to many levels and includes both Akt activators and Akt targets, making this interaction an extremely interesting area of research. Thus, the upstream PTEN tumor suppressor regulates p53 protein levels not only by affecting Akt activity, but also by direct interaction where physical interaction between p53 and PTEN stabilizes p53 expression and enhances its transcriptional activity (Freeman *et al.*, 2003). Conversely, based on the finding of a p53-binding site within the PTEN promoter, Stambolic *et al.* (2001) have presented evidence for a regulation of PTEN transcription by p53. In summary, several lines of evidence indicate a functional crosstalk between the PTEN and p53 tumor suppressors that may partly explain why somatic mutations in p53 and PTEN are found to be mutually exclusive (Kurose *et al.*, 2002).

The interactions between the Akt and p53 pathways also extend to p53 targets and include the cell cycle regulator protein p21^{Cip1/WAF1} that was shown to be stabilized after activation of the Akt pathway by direct phosphorylation and GSK3 inactivation (Rossig *et al.*, 2001; Li *et al.*, 2002a; Rossig *et al.*, 2002). Also, Li and coworkers showed that the phosphorylation of p21^{Cip1/WAF1} by Akt inhibited its interaction with proliferating cell nuclear antigen (PCNA) and promoted the assembly of the cyclin D1: cyclin-dependent kinase 4 (CDK4) complex (Zhou *et al.*, 2001a). Akt also phosphorylates the cell cycle inhibitor p27^{Kip1}, which in turn is a target gene of FOXO transcription factors. Phosphorylation of p27^{Kip1} induced its cytoplasmic localization, similar to the regulation of p21^{Cip1/WAF1} by Akt, and results in its functional inhibition (Liang *et al.*, 2002; Shin *et al.*, 2002; Viglietto *et al.*, 2002). The findings of p21^{Cip1/WAF1}

and p27^{Kip1} phosphorylation link the Akt pathway to cell cycle regulation. Thus, the potential of these proteins to act as oncogenes (Blagosklonny, 2002) and the correlation of increased Akt-dependent phosphorylation of p27^{Kip1} in aggressive breast cancer (Blain and Massague, 2002; Clarke, 2003) make them very attractive targets in search for novel cancer therapeutics.

Conclusions

When considering the current understanding of all the signals leading to and from Akt, we face a growing complexity that is in part compounded by the intersection of different signaling cascades. Many substrates of Akt are shared with other kinases that have similar specificities, but alternative activation mechanisms. Moreover, signals originating from activated Akt do not simply lead to changes in the biological activity of a few specific downstream substrates, but affect whole signaling cascades, including changes in the activity of transcription factors.

In mammalian cells, known points of convergence between different signaling cascades include BAD, GSK3 and forkhead-related transcription factors. In invertebrate genetic models of signal transduction cascades, however, findings of overlapping and intersecting pathways are rare (Noselli and Perrimon, 2000). It thus remains likely that some of the current complexity of PI3K/Akt signal transduction in vertebrates may be an artifact of *in vitro* studies. The growing number of Akt substrates discovered also complicates our understanding. It is, therefore, important to determine which of these substrates are genuine and directly relevant, and how Akt independently regulates each one. As it stands now, it appears that multiple substrates are phosphorylated simultaneously in any given system, suggesting that Akt regulates multiple downstream functions in parallel. How cells coordinate these multiple activities of Akt, however, remains a mystery.

Pharmacological inhibitors of Akt are likely to become important tools for examining Akt function in cells and modulating Akt-dependent downstream cell responses. Recent studies that have identified endogenous inhibitors of PI3K/Akt signal transduction have significantly fueled and accelerated the effort to identify and characterize synthetic small-molecule compounds (Kim *et al.*, 2002b; West *et al.*, 2002; Chang *et al.*, 2003). Such reagents, together with a more detailed analysis of Akt signal transduction *in vivo*, should help to clarify the role of Akt in transducing survival signals.

Finally, the identification of novel isoform-specific binding partners of Akt has channeled our interest and that of other groups (Brazil *et al.*, 2002) towards investigating the binding partners of Akt and the formation of Akt-dependent protein complexes for novel drug targets. These may be more selective targets and better suited for inhibiting only a part of the spectrum of Akt-dependent substrates and biologic responses.

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