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Dynamics of Akt activation during mouse embryo development: Distinct subcellular patterns distinguish proliferating versus differentiating cells

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ABSTRACT

Akt is a highly conserved serine–threonine protein kinase which has been implicated in a wide variety of cellular functions, from the regulation of growth and metabolism, to activation of pro-survival pathways and cell proliferation, and promotion of differentiation in specific cell types. However, very little is known about the spatial and temporal pattern of Akt activity within cells and whether this pattern changes as cells enter and proceed in their differentiation programs. To address this issue we profiled Akt activation in E8.5–E13.5 mouse embryos and in C2C12 cells. We used a commercial antibody against Akt, phosphorylated on one of its activating residues, Thr-308, and performed high resolution confocal imaging of the immunofluorescence in labeled embryos. We observe strong Akt activity during mitosis in the dermomyotome, the neuroepithelium and some mesenchymal cells. This burst of activity fills the whole cell except for heterochromatin-positive areas in the nucleus. A surge in activity during mitosis is also observed in subconfluent C2C12 cells. Later on in the differentiation programs of skeletal muscle and neural cells, derivatives of the dermomyotome and neuroepithelium, respectively, we find robust, sustained Akt activity in the cytoplasm, but not in the nucleus. Concomitantly with skeletal muscle differentiation, Akt activity becomes concentrated in the sarcomeric *Z*-disks whereas developing neurons maintain a uniform cytoplasmic pattern of activated Akt. Our findings reveal unprecedented cellular and subcellular details of Akt activity during mouse embryo development, which is spatially and temporally consistent with proposed functions for Akt in mitosis and myogenic and neural differentiation and/or survival. Our results thus demonstrate a subcellular change in the pattern of Akt activation when skeletal muscle and neural progenitor cells cease dividing and progress in their differentiation programs.

1. Introduction

The phosphoinositide3-kinase (PI3K)/Akt pathway is a ubiquitous signaling pathway with a multitude of well-established roles in proliferation, cell growth, differentiation, and survival (Brazil et al., 2004). Its primary effector, the serine threonine kinase Akt (also known as PKB) is the cellular homolog of murine thymoma virus akt8 oncogene (Bellacosa et al., 1991). There are three Akt isoforms (AKT1/PKBα, AKT2/PKBβ, AKT3/PKBγ) stemming from 3 distinct chromosomal loci, although with extensive amino acid sequence and domain structure homology among isoforms. Expression of the three isoforms overlaps in most mammalian tissues, and their homology may underlie a functional overlap. Nevertheless, Akt1 is the most ubiquitous isoform (although it is not expressed in kidney, liver, or spleen), Akt2 is most highly expressed in skeletal muscle, digestive and reproductive organs, and Akt3 is preferentially expressed in the brain and testis (Martelli et al., 2006).

Akt is activated when phosphorylated on two highly conserved regulatory motifs: Thr-308 (T308) in its catalytic domain, and Ser-473 (S473) in its hydrophobic motif (Alessi et al., 1996; Brazil and Hemmings, 2001). Signaling through tyrosine kinase receptors (e.g. EGF, insulin), integrins, and G protein-coupled receptors can activate PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-triphosphate lipids (PtdIns(3,4,5)P3). Akt is then recruited to the plasma membrane upon binding of its pleckstrin homology (PH) domain to PtdIns(3,4,5)P3, whereupon it is phosphorylated on T308 by PDK1 (Alessi et al., 1997), partially activating the enzyme, and on S473 by mTORC2 (Sarbassov et al., 2005), which completes the activation.

Activated Akt (pAkt) performs a multitude of functions within the cell which have been extensively studied, particularly in cancer models. In fact, cancer is an extreme phenotype of two common

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function of pAkt: aiding cell division, by promoting G1/S (Collado et al., 2000) and G2/M transitions (Shtivelman et al., 2002) and protecting cells from apoptosis (Liao and Hung, 2010). pAkt actively prevents apoptosis through (1) a negative feedback loop involving the degradation of p53 (Ogawara et al., 2002), (2) specific phosphorylation of Forkhead box transcription factors, which become exported to the cytoplasm, and unable to promote apoptosis (Brunet et al., 1999) or (3) phosphorylation and subsequent degradation of other Bcl-2 pro-apoptotic family members and their targets, such as Bad and caspase-9 (Datta et al., 1999). These roles in promoting proliferation and survival may also contribute to the documented role of activated Akt in epithelial-to-mesenchymal transitions (EMTs), particularly in the context of cancer, but also during embryogenesis (Larue and Bellacosa, 2005). Moreover, Akt activity has been implicated in promoting the actual EMT event itself. pAkt has been shown to activate Snail and Twist family members (Grille et al., 2003; Xue and Hemmings, 2013; Xue et al., 2012), key transcription factors known to promote the EMT program in a variety of situations, both in the embryo and in tumor progression (Nieto, 2011). Also noteworthy is the known and well-conserved role for Akt in cellular growth: the mammalian target of rapamycin (mTOR) is a serine–threonine kinase both upstream (as the mTORC2 complex) and downstream (as the mTORC1 complex) of Akt. mTOR regulates protein synthesis by phosphorylating targets such as the ribosomal p70S6 kinase and the eukaryotic initiation factor 4E binding protein (Fingar and Blenis, 2004). Akt is not only a major activator of mTORC1 in an oncogenic context (Sabatini, 2006), but is also a regular checkpoint sensor of nutrient availability for progression through the cell cycle. This is due to Akt also playing an important role in cellular response to insulin levels and glucose uptake: it has been shown that Akt activation causes the mobilization of glucose transporter 4 (Glut4) to the plasma membrane (Kohn et al., 1996) and that Akt2 associates specifically with Glut4 vesicles upon insulin stimulation (Calera et al., 1998). Activated Akt is then able to influence both the anabolism and catabolism of glucose within the cell: it enhances glycolysis in cancer cells (Elstrom et al., 2004), but it also phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β) (Cross et al., 1995), which no longer inhibits glycogen synthase to produce glycogen from available glucose-6-phosphate. It is possible that Akt and the canonical Wnt pathway synergize at this point, and β-catenin is released from the GSK3β-APC-Axin complex to the nucleus, where it stimulates transcription via LEF/TCF factors, although apparently different pools of cytoplasmic GSK3β are affected by Wnt and Akt (Grille et al., 2003).

The use of modified constructs of Akt (dominant negative, constitutively active (Manning and Cantley, 2007)) as well as the use of specific inhibitors of PI3K like wortmannin (Ferby et al., 1996), LY294002 (Vlahos et al., 1994), and, more recently, specific Akt inhibitors like triciribine (Evangelisti et al., 2011), has been paramount to advances in the knowledge of the effects of Akt activation in cells in vitro, again with a major emphasis on cancer cells. Furthermore, mouse mutants where single Akt isoforms were targeted for deletion have been produced (Yang et al., 2004): (1) Akt1 mutants show defects in placental vascularization, and animal growth; (2) Akt2 mutants are severely diabetic and insulin-resistant, with problems in growth and adipogenesis; and (3) Akt3 mutants have small brains. The single isoform mutants thus present distinct non-lethal phenotypes, revealing both a functional specificity of each isoform, as well as partial functional overlap, enabling the survival of each of the single mutants. Double mutants have more severe combined developmental defects and are either embryonic lethal or die post-natally (reviewed in Dummler and Hemmings, 2007).

In spite of all these advances in the study of Akt signaling, very little is known about the cellular pattern of Akt activity and how it is regulated during embryonic development: Is it enriched in specific cell types? Is it active during specific phases of a cell's life? Is its activity restricted to some sub-compartment(s) of the cells? In this study we aimed to characterize where and when Akt is active during mouse embryo development. We profiled Akt pathway activation in E8.5–13.5 embryos using a commercial antibody against Akt phosphorylated on residue T308 and used high resolution confocal imaging to characterize not only the cellular, but also the subcellular pattern of Akt activity in different cell types. Our data provide unprecedented detail of Akt activity in the mouse embryo. Furthermore, by focusing on the skeletal muscle and neural lineages, we identified distinct subcellular patterns of Akt activity in progenitor cells versus their differentiated derivatives. These observations suggest that Akt activity is differentially regulated, and may play distinct roles, in proliferating progenitors versus differentiated cells of these two lineages.

2. Material and methods

2.1. Embryos and cells

Wild-type mouse embryos were obtained from crossings of outbred Hsd:ICR(CD-1) mice (Harlan Interfauna Iberica). The day of the vaginal plug was designated embryonic day (E) 0.5. Embryos were staged as described by Houzelstein et al. (1999).

C2C12 cells (ATCC, CRL 1772; Blau et al., 1985) were cultured in GlutaMAX Dulbecco's modified Eagle Medium (DMEM), containing 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were cultured at 37 °C with 5% CO_2, and passaged every two days or when subconfluent, using 0.25% trypsin and 0.53 mM EDTA in phosphate buffered saline (PBS) until detachment from flask.

2.2. Immunofluorescence and confocal microscopy

Immunofluorescence in whole mounts, on thick cryostat (Bright Clinicut) sections (20–40 μm) and coverslip-seeded cells was performed as described (Bajanca et al., 2004). Incubations with primary and secondary antibody combinations were performed overnight for thick sections and for 24 h for whole mounts. Primary antibodies were anti-pan-pAkt T308 (ab38449; Abcam), anti-myogenin (F5D; Santa Cruz Biotechnology), anti-myosin heavy chain (F59; DSHB), anti-phospho-histone-H3 (05-806; Millipore) and anti-neurofilament (N5264; Sigma), all diluted 1:100 in PBS with 1% bovine serum albumin (BSA, Sigma). The anti-pAkt antibody is a pan antibody which recognizes consensus sequences common to Akt1, Akt2, and Akt3, phosphorylated on threonine residues corresponding to T308 in Akt1. Secondary antibodies were Alexa Fluor 568-conjugated anti-mouse IgG (A-11019; Molecular Probes), and Alexa Fluor 488-conjugated anti-rabbit IgG (A-11070; Molecular Probes), both F(ab)2 fragments, diluted 1:1000 in PBS with 1% BSA. Negative controls were PBS with 1% BSA, followed by incubation in secondary antibodies. We also used a rabbit antibody against Myf5 (SC-302; Santa Cruz), the same Ig type as the rabbit anti-pAkt antibody, to test for unspecific labeling of a primary antibody. Actin cytoskeleton was stained with Alexa Fluor 568-conjugated phalloidin (A-12380; Molecular Probes). Some embryos and sections were stained with ToPro3 (T3605; Molecular Probes) diluted 1:1000 in PBS with 1% BSA and with 10 μg/ml ribonuclease A (55674; Calbiochem) to visualize nuclear DNA. Whole mount samples were dehydrated in methanol and cleared in methyl salicylate (Martins et al., 2009), while thick sections and cells were mounted in 0.5% n-propyl gallate in PBS-glycerol. Confocal images were obtained in a Leica SPE confocal microscope.