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Nuclear/cytoplasmic localization of Akt activity in the cell cycle

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Summary. The serine/threonine protein kinase Akt (also known as PKB) is a proto-oncogene and one of the most frequently hyperactivated kinases in human cancer. Its activation downstream of growth-factor-stimulated phosphatidylinositide-3′-OH kinase activity plays a role in the control of cell cycle, cell growth, apoptosis and cell energy metabolism. Akt phosphorylates some thousand downstream substrates, including typical cytoplasmic as well as nuclear proteins. Accordingly, it is not surprising that Akt activity can be found in both, the cytoplasm and the nucleus. Here we report the cell cycle regulation of nuclear and cytoplasmic Akt activity in mammalian cells. These data provide new insights into the regulation of Akt activity and have implications for future studies on the regulation of the wide variety of different nuclear and cytoplasmic Akt substrates.

Keywords: Akt – Cell cycle – Nucleus – Cytoplasm

Introduction

The insulin-signaling pathway has a central role in the regulation of cell growth in response to growth factors, cellular energy and nutrient levels. In this pathway insulin and insulin-like growth factors (IGFs) phosphorylate and activate their cognate tyrosine kinase receptors (INRs), triggering the recruitment and phosphorylation of insulin receptor substrates (IRSs), to generate a docking site at the membrane for the phosphatidylinositide-3′-OH kinase (PI3K). Phosphorylation of the membrane lipid phosphatidylinositol 4,5-biphosphate (PIP_2) by PI3K produces the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3), which activates the serine/threonine kinase Akt (also known as PKB). The tuberous sclerosis gene 2 product, tuberin, is phosphorylated by Akt, and through an unknown mechanism, this phosphorylation inhibits the ability of tuberin to act as a GTPase activating protein against Rheb (Ras homolog enriched in brain), which in turn regulates the mammalian target of rapamycin (mTOR). Accordingly, Akt is assumed to stimulate mTOR signaling by inhibiting the function of tuberin (Pan et al., 2004; Astrinidis and Henske, 2005; Corradetti and Guan, 2006).

mTOR regulates translation via S6K and 4E-BP1. S6K is considered to be involved in translation control of a small subset of mRNAs that contain a 5′-terminal oligopyrimidine tract. Cap-dependent translation is facilitated by mTOR phosphorylation and inactivation of 4E-BPs, which are suppressors of eIF4E. eIF4E regulates initiation of translation of specific mRNAs (Wullschleger et al., 2006). Thus, it is anticipated that mTOR localizes in the cytoplasm resulting in signals from the upstream components, PI3K and Akt, both of which also localize to the cytoplasm, to the downstream targets S6K and 4E-BP, also localized to the cytoplasm. However, nuclear eIF4E has been suggested to be involved in nuclear functions, including splicing (Lejbkowicz et al., 1992; Dostie et al., 2000). Beside regulation of translation, mTOR has also been implicated in the regulation of ribosome biogenesis, macroautophagy or transcription (Wullschleger et al., 2006). In fact, mTOR and its substrate S6K have been found to be localized to both, cytoplasm and nucleus, and cytoplasmic-nuclear shuttling of mTOR has been shown to be involved in rapamycin-sensitive signaling and translation initiation (Kim and Chen, 2000; Zhang et al., 2002; Bachmann et al., 2006). Very recently, it was demonstrated that the components (and their activities) of the entire PI3K-Akt-mTOR-S6K pathway are localized to both, cytoplasm and nucleus (Furuya et al., 2006).

Since Akt can potentially phosphorylate over 9000 proteins in mammalian cells (Martelli et al., 2006), it is not surprising that Akt activity can be detected in both, the

nucleus and the cytoplasm. It was the aim of this study to investigate the cell cycle regulation of nuclear and cytoplasmic Akt activity. The results reported here form the basis for a further understanding of the aspect in which cell cycle phase cytoplasmic and nuclear substrates can be regulated by this serine/threonine kinase.

Materials and methods

Cells, cell culture, flow cytometry

Rat1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate). The cultures were kept at 37 °C and 5% CO_2 and routinely screened for mycoplasma. For cell cycle synchronization Rat1 cells were starved in DMEM containing 0.1% serum for 72 h and restimulated by the addition of DMEM containing 10% FCS. For cytofluorometric analyses of DNA distribution cells were harvested by trypsinization and fixed by rapid submersion in ice-cold 85% ethanol. After overnight fixation at −20 °C, cells were pelleted by centrifugation and DNA was stained in an appropriate amount of staining solution containing 0.25 mg/ml propidium iodide, 0.05 mg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8. DNA distribution was analysed on a Beckton Dickinson FACScan using Modfit Analysis software.

Nuclear and cytoplasmic fractionation

Adherent cells were washed twice with PBS, collected by scraping and pelleted by centrifugation. Cell pellets were lysed in 5 packed cell volume buffer F1 containing 20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchlorid, 10 μg/ml trypsininhibitor) for 2 min at room temperature and subsequent incubation on ice for 10 min. Thereafter NP-40 was added at a final concentration of 1% (v/v) and lysates were homogenized by passing through a 20-gauge needle for three times. Nuclei were pelleted by centrifugation at 600 *g* for 5 min at 4 °C and supernatant containing cytoplasmic proteins was collected and stored at −80 °C. Remaining nuclei were washed three times in buffer F1 containing 1% NP-40. During the last wash nuclei were stained with trypan blue and microscopically examined for number, purity and integrity. The nucleic pellets were lysed in buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, supplemented with protease inhibitors (see above) by repeated freezing and thawing. Supernatants containing soluble nucleic proteins were collected by centrifugation at 25000 *g* for 20 min and stored at −80 °C (Rosner et al., 2007).

Immunoblotting

For preparing lysates cells were washed with PBS, collected by scraping and lysed in buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchlorid, 10 μg/ml trypsin inhibitor by repeated freezing and thawing. Supernatants were collected by centrifugation and stored at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumine as the standard. Proteins were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein (Rosner and Hengstschläger, 2004). For immunodetection antibodies specific for the following proteins were used: phospho Akt (S473) (Cell Signaling), Akt (Cell Signaling), α-tubulin (Ab-1, Calbiochem), topoisomerase IIβ (Santa Cruz). Signals were detected with appropriate HRP-conjugated secondary antibodies and the enhanced chemiluminescence method.

Results

For cell cycle synchronisation logarithmically growing Rat1 cells were serum starved (ss) and restimulated with serum. Flowcytometric analyses of DNA distributions revealed that 0.1% serum starvation for 72 h caused an increase of G0/G1 cells from 55 to 80% and a decrease of S phase cells from 30 to 12%. In logarithmically growing Rat1 cells 15% of the cells were in G2/M phase and after serum starvation 8% G2/M cells were observed. Between 6 and 9 h after restimulation with serum the amount of G0/G1 cells decreased from 83 to 75% and the amount of S phase cells increased from 8 to 25%. These data indicate that cells start to pass the G1 to S phase transition during this time period. Fifteen hours after restimulation 32% of Rat1 cells were in G0/G1 phase and 68% of the cells were in S phase (Fig. 1A). These findings allow the conclusion that serum restimulation of Rat1 cells is a useful biological approach to analyse cell cycle regulations.

Western blot analyses of total protein lysates of the different cell cycle fractions demonstrated that Akt activity (represented by the amount of Akt phosphorylated at S473) is heavily downregulated upon serum starvation, whereas the Akt protein levels are not affected. Accordingly, Akt activity is post-translationally regulated during the cell cycle. Immediately, within 15–30 min, after restimulation with serum Akt activity is induced. The Akt activity peaks between 1 and 3 h after serum treatment and is downregulated again before the cells pass the G1 to S phase transition. During replication (compare 15 h time point) the level of Akt activity is comparable to the activity observed 24 h after restimulation. From the flowcytometrical analyses it can be concluded that 24 h after serum restimulation Rat1 cells already started to grow logarithmically again (Fig. 1B, the first two rows).

The purity of the obtained nuclear and cytoplasmic protein extracts was investigated by analysing the expression of α-tubulin (cytoplasmic) and topoisomerase IIβ (nuclear). This biochemical fractionation was found to be clean (Fig. 1B, the rows below). By Western blot analyses we found the levels of cytoplasmic Akt activity to be much higher than those of nuclear origin. In fact, nuclear Akt activity was only detectable on longer exposures. To allow a better visualization of the different nuclear and

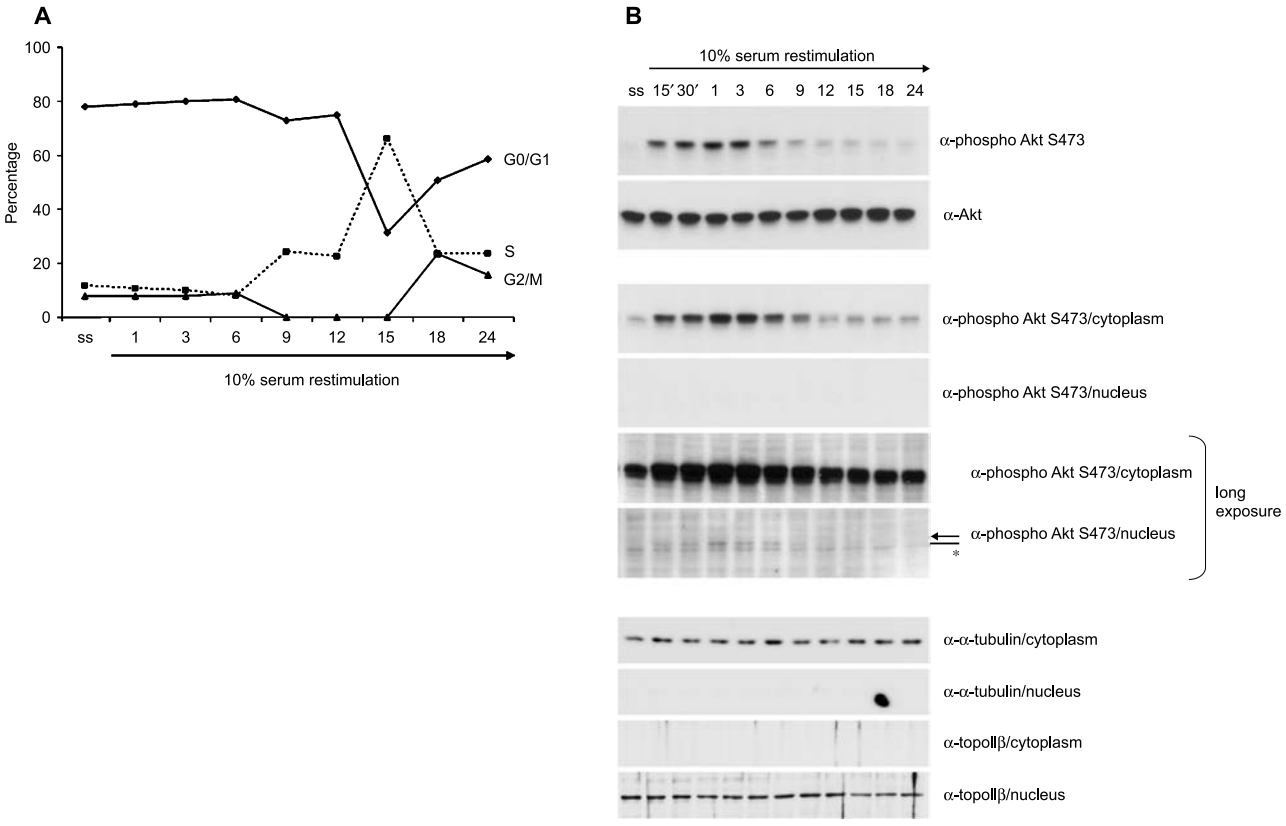

Fig. 1. Cell cycle regulation of nuclear and cytoplasmic Akt activity. **A** Logarithmically growing Rat1 cells were arrested by serum starvation for 72 h (ss). At the indicated time points of serum restimulation (given in hours) cells were harvested and cytofluorometrically analysed for DNA distribution. **B** In addition, at the indicate time points after serum restimulation total protein extracts were prepared and phospho-Akt S473 (representing Akt activity) and Akt was analysed by Western blotting. Furthermore, fractions containing cytoplasmic or nucleoplasmic proteins were isolated. Each fraction was analysed for Akt activity via Western blotting. Shorter and longer exposures are presented to allow a better visualization of the different nuclear and cytoplasmic protein fluctuations (the asterisk indicates an unspecific band). Purity of fractions was proven by co-analysing tubulin (cytoplasmic) and topoisomerase II (nuclear)

cytoplasmic protein fluctuations longer and shorter exposures are presented for both, nuclear and cytoplasmic Akt activity levels (Fig. 1B). In both, the cytoplasm and the nucleus, serum-induced re-entry into the cell cycle immediately triggered a strong induction of Akt activity. The cell cycle regulation of Akt activity, highest levels between 1 and 3 h after restimulation, downregulation before the G1/S transition, and low levels during replication, was very comparably in the cytoplasm and in the nucleus.

In summary, the here obtained data demonstrate mammalian Akt activity to be strictly cell cycle regulated on a post-translational level. Akt activity is much lower in the nucleus than in the cytoplasm. However, in both compartments Akt activity is heavily downregulated in G0 arrested cells, immediately upregulated upon growth factor stimulation and donwregulated again before cells enter S phase.

Discussion

The serine/threonine protein kinase Akt plays a role in the control of cell cycle, cell growth, apoptosis and cell energy metabolism. Hyperactivation of Akt is a hallmark of a wide variety of different human cancers. Akt can potentially phosphorylate over 9000 substrates in mammalian cells including typical cytoplasmic as well as nuclear proteins, what makes it not surprising that Akt activity can be detected in both, the nucleus and the cytoplasm (Furuya et al., 2006; Martelli et al., 2006; Wullschleger et al., 2006).

A major role of Akt is assumed to be its potential to stimulate mTOR signaling by phosphorylating and inhibiting the function of tuberin (Pan et al., 2004; Astrinidis and Henske, 2005; Corradetti and Guan, 2006; and see Introduction).

However, phosphorylation by Akt has also been shown to be able to regulate protein localization via affect-

ing either nuclear import or nuclear export. For example, Akt has been demonstrated to regulate the nuclear/cytoplasmic localization of the cyclin-dependent kinase inhibitor p27 (Fujita et al., 2002; Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002).

Also, tuberin was earlier shown to be localized to both, the cytoplasm and the nucleus. Tuberin is a multifunctional protein, which is involved in the regulation of cell size, cell cycle, translation, transcription, and cell differentiation. A wide variety of proteins, implicated in different regulations, have been demonstrated to interact with tuberin. Beside typical cytoplasmic proteins, such as e.g. all isoforms of the mammalian 14-3-3 protein family, tuberin also was shown to bind to nuclear proteins, such as e.g. the cell cycle molecules cyclin B1 or cyclin-dependent kinase 1 (Wienecke et al., 1996; Lou et al., 2001; Rosner et al., 2004; Astrinidis and Henske, 2005; Corradetti and Guan, 2006). Accordingly, it was interesting to investigate the role of Akt phosphorylation in tuberin localization. Here it is important to note that although Akt activity is known to be induced upon mitogenic stimulation (Wullschleger et al., 2006; this report) tuberin protein amounts are constant during the cell cycle (Soucek et al., 1997; Miloloza et al., 2000). We recently reported that high amounts of ectopic activated Akt increase the phosphorylation of tuberin S939 and T1462 and downregulate nuclear localization of endogenous tuberin as well as of ectopic tuberin. Downregulation of phosphorylation of tuberin via a dominant negative Akt mutant mediated the opposite effects on the localization of endogenous tuberin. A TSC2 mutant, which cannot be phosphorylated on S939 and T1462, showed increased nuclear localization. These findings demonstrated Akt phosphorylation to induce cytoplasmic tuberin localization (Rosner et al., 2007).

We further found already earlier that the phosphorylation of tuberin by Akt is higher in the cytoplasm than in the nucleus (Rosner et al., 2007). Our here reported finding that the levels of Akt activity are much higher in the cytoplasm than in the nucleus are in perfect agreement with this earlier observation. Furthermore, we reported earlier that in logarithmically growing cells with high Akt activity tuberin is predominately localized to the cytoplasm. In G0 arrested cells Akt activity is downregulated and a significant proportion of tuberin can be detected in the nucleus. Upon mitogenic restimulation into the cell cycle Akt activity is induced and nuclear localization of tuberin is downregulated. These findings were in perfect agreement with our model of Akt-upregulated cytoplasmic tuberin localization (Rosner et al., 2007). However, the question whether Akt mediated phosphorylation in logarithmically growing cells and upon restimulation can occur in both compartments or exclusively in the nucleus or in the cytoplasm remained unclear. The here reported cell cycle analysis of nuclear and cytoplasmic Akt activity demonstrates that tuberin can be targeted by Akt in both compartments. This is only one example how the here reported data can provide further insights into the regulation and localization of Akt-mediated control on each of its thousands of substrates.

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