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Crosstalk between mitogenic Ras/MAPK and survival PI3K/AKT pathways: a fine balance

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ABSTRACT

Here we describe multiple levels of crosstalk between the PI3K/Akt and Ras/MAPK signalling pathways. Experimental data and computer simulations demonstrate that crosstalk is context-dependent, and both pathways can activate or inhibit each other. Positive influence of the PI3K pathway on the MAPK pathway is most effective at sufficiently low doses of growth factors, whereas negative influence of the MAPK pathway on the PI3K pathway is mostly pronounced at high doses of growth factors. Pathway crosstalk endows a cell with emerging capabilities for processing and decoding signals from multiple receptors activated by different combinations of extracellular cues.

Introduction

A cell in an organism is immersed in an ocean of growth factors and hormones, yet how signalling networks integrate multiple cues is largely unknown. Although individual signalling pathways have been extensively studied, the processing and integration of pathway responses by crosstalk is less understood. One plausible role of crosstalk is to achieve robust activation of key downstream targets by low, physiological doses of external stimuli. For instance, if a signal propagates through different branches that converge at a common target, the signalling responses along these branches will add to the overall target response [1]. Likewise, feedforward and feedback loops embracing interacting pathways make the input-output response of one pathway depend on the activity of the other, thereby creating context-dependent signalling output. Finally, pathway crosstalk generates a plethora of distinct spatiotemporal response patterns, which may facilitate an effective discrimination between combinations of extracellular cues and lead to different cell-fate decisions [2].

A diverse family of growth factors and other stimuli activate the mitogen-activated protein kinase (MAPK)[1] cascades and the phosphatidylinositol-3-kinase (PI3K)/Akt (Fig. 1A). Signalling by these pathways govern fundamental physiological processes, such as cell proliferation, differentiation, metabolism, cytoskeleton reorganization, and cell death and survival [3-6]. The MAPK and PI3K/Akt pathways are often mutated in cancer. Growth and survival of many cancer cells critically depends on aberrant signalling by these pathways, which are also involved in intensive crosstalk. Here we describe multiple levels of crosstalk interactions between the PI3K and MAPK signalling pathways. We present experimental data and computer simulations that show that crosstalk is context-dependent and mostly operational at low, physiological doses of growth factors, such as epidermal growth factor (EGF).

Organization and functions of Ras/MAPK signalling pathway

[1] Abbreviations: EGF - epidermal growth factor ; Shc - Src homology and collagen protein; SHIP2 - Src homology 2 domain–containing inositol phosphatase 2; PTEN - phosphate and tensin homologue; PDK1 - 3-Phosphoinositide-dependent kinase 1; Btk - Bruton's tyrosine kinase; IRS – insulin receptor substrate; GAB – Grb2-associated binder; Grb2 - growth factor receptor-bound protein 2; PKB – protein kinase B; DNA-PK - DNA-dependent protein kinase; ILK-1 - integrin-linked kinase-1; mTOR - mammalian target of rapamycin; ERK - extracellular signal-related kinase; MEK - MAPK kinase; MAPK - mitogen-activated protein kinase; STAT – signal transducer and activator of transcription; KSR - kinase suppressor of Ras; GSK3 - Glycogen synthase kinase-3; PH – plecktrin homology; PKC-protein kinase C; RKIP - Raf Kinase Inhibitory Protein

There are at least six distinct MAPK signalling pathways, which are named according to their terminal tier kinases: the extracellular signal-related kinases (ERK1/2), the c-Jun amino-terminal kinases/stress-activated protein kinases (JNK1/2/3 or SAPKs), the p38 MAPK, ERK3/4, ERK5 and ERK7/8 [5-6]. In the best-studied Ras/MAPK pathway, growth factor-induced ERK1/2 (further referred to as ERK) activation involves the recruitment of the cytosolic growth factor receptor-bound protein 2 (Grb2) to the plasma membrane. Grb2 binds to tyrosine phosphorylated receptors directly or via the docking protein Shc (Src homology and collagen protein). A subset of Grb2 molecules is constitutively associated with the proline-rich domain of the Son of Sevenless protein (SOS), which is a guanine exchange factor (GEF) for the small G protein Ras [5]. In addition, Grb2 molecules can be involved in activation of the phospholipase D2 (PLD2), which generates phosphatidic acid that interacts with the pleckstrin homology (PH) domain of SOS [7]. This further facilitates SOS recruitment to the plasma membrane where SOS catalyzes a transformation of inactive GDP-bound Ras into active GTP-bound Ras. Active RasGTP stimulates multiple downstream effectors, including PI3Ks (thereby creating one point of Ras/MAPK → PI3K crosstalk), RalGDS and serine/threonine kinase Raf, which is the first kinase of the three-tiered MAPK/ERK cascade. The major substrates of Raf are cytosolic kinases MEK1/2, which subsequently phosphorylate ERK on two conserved Thr and Tyr residues within the ERK activation loop. Activated ERK then phosphorylates a plethora of nuclear and cytoplasmic substrates [5-6]. The temporal activation patterns of MAPK cascade is also modulated by scaffolding proteins (e.g. RKIP, KSR, MP1), phosphatases (e.g. MKPs) and various feedbacks [5-6, 8]. In many cell types, the localisation, strength and duration of ERK signalling control cellular programs of embryogenesis, proliferation, differentiation and apoptosis. Aberrant activation of the Ras/MAPK pathway correlates with cancer progression and metastatic tumor cell growth [5-6].

Organization and functions of PI3K/Akt signalling pathway

The p85 regulatory subunit of PI3K stabilizes and protects p110α subunit from degradation, but at the same time inhibits its catalytic activity. Consequently, in resting cells PI3K is inactive. Upon extracellular stimuli, interactions of the SH2 domains of p85 with phosphorylated tyrosine residues of receptors, non-receptor tyrosine kinases and/or adaptor proteins relieve auto-inhibition of PI3K and recruit it to the inner surface of the plasma membrane, where PI3K activity is further modulated by

RasGTP and SFKs [4, 9]. Activated class I PI3Ks generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a second messenger, which is subsequently converted into $PI(3,4)P_2$ or $PI(4,5)P_2$ by PI-5- or PI-3-phosphatases SHIP2, PTEN and others [4].

Generation of membrane-associated phospholipids recruits a battery of signalling molecules that have lipid-binding domains, such as the PH domain, including non-receptor tyrosine kinases (e.g., Btk family kinases), guanine exchange factors (e.g. Vav, Tiam-1, Sos), GTPase-activating proteins (e.g. RasGAP), lipid kinases (e.g. PLC-γ), adaptor/docking/scaffolding proteins (e.g. IRS, Grb7, intersectin families) and serine/threonine kinases such as PDK1 and protein kinase B (PKB/Akt) [10-12]. PDK1 acts upstream of a major downstream effector of PI3K (Akt), protein kinase C (PKC) isoforms, serum- and glucocorticoid-induced protein kinase (SGK) and p70 ribosomal S6 kinase (p70S6K) [11, 13]. In addition, PDK1 activates PAK1 [14] and protein kinase N/protein kinase C-related kinase (PKN/PRK) that are effectors of Rho family GTPases. As described below, the phospholipid-mediated recruitment of multiple proteins not only facilitate signal propagation through the PI3K/Akt pathway, but also affect complex processes of Ras and Raf activation, thereby creating another point of PI3K → Ras/MAPK crosstalk.

Full Akt activation requires dual phosphorylation on Ser-473 by mammalian target of rapamycin (mTOR)-Rictor complex (mTORC2), ILK-1 or DNA-PK and on Thr-308 by PDK1 [15]. Active Akt dissociates from the membrane and translocates to the cytoplasm and the nucleus where it phosphorylates multiple target proteins, implicated in the regulation of apoptosis, DNA repair, metabolism, protein synthesis and cell division [3-4, 13, 16]. Activation of Akt stimulates angiogenesis and induces epithelial-mesenchymal transition characterized by the morphological changes, production of metalloproteinases, loss of cell-to-cell adhesion and increased invasive cell migration [3, 16-17].

Crosstalk between Ras/MAPK and PI3K/Akt signaling pathways

Traditional schemes of growth factor, hormone and cytokine receptor signalling networks display PI3K/Akt and Ras/MAPK as two independent, parallel pathways. However, there are multiple crosstalk points between these two pathways, whose coordinated action determines the cell fate (Fig. 1B). There are 802 interactive proteins involved in PI3K-mediated signalling [18] and more than 2,000 interactions related to the MAP-family kinases [19] where at least 284 proteins are the components of endogenous ERK1 complexes [20]. In view of such broad interactomes, it is not surprising that the

PI3K/Akt and Ras/MAPK pathways influence each other at different stages of signal propagation, both negatively and positively, resulting in dynamic and complex crosstalk.

PI3K-mediated regulation of ERK responses. PI3K affects MAPK signalling at multiple nodes of the Ras/ERK pathway (Fig. 2). A major crosstalk node is at the level of $PI(3,4,5)P_3$-mediated RasGEF and RasGAP signalling. Generation of $PI(3,4,5)P_3$ by PI3K induces the plasma membrane recruitment of the PH-domain-containing Grb2-associated binding partner (GAB), insulin receptor substrate (IRS) and Grb7 scaffolding proteins, which are subsequently phosphorylated on multiple tyrosine residues by membrane receptors and non-receptor tyrosine kinases. Tyrosine phosphorylated GAB and IRS bind p85-PI3K, thereby bringing additional PI3K molecules to the plasma membrane and creating positive feedback [21-22]. Phosphorylated GAB interacts with a number of molecules, including Shc, PLC-γ, Grb2, Crk, SHIP, STAT3, STAT5, c-Src, ERK and SHP2 (SH2 domain containing tyrosine phosphatase 2) [23]. The recruitment of Grb2-SOS complexes to the plasma membrane by GAB, IRS and SHP2 amplifies Ras activation [24-26]. GAB1 association with SHP2 increases SHP2 phosphatase activity, manifested by dephosphorylation of SHP2 substrates, such as RasGAP, c-Src (on inhibitory Tyr527 site) and the binding partners of c-Src-inactivating kinase CSK. Resulting activation of c-Src and Ras leads to up-regulation of ERK signalling [23, 27].

Other PI3K → ERK crosstalk nodes include PI3K-induced Raf and MEK stimulation, although some of these interactions also amplify Ras signalling. Positive PI3K-GAB-PI3K feedback further increases $PI(3,4,5)P_3$ production and stimulates the Rac/Cdc42/PAK signalling pathway [3, 28-29]. PAK then phosphorylates Raf on Ser388, which is required for Raf activation, and also increases Raf association with MEK [30-31]. In addition, PAK-mediated MEK1 phosphorylation on Ser-289 increases the association between MEK1 and ERK2 [32].

Similarly, Grb7 is recruited to the plasma membrane in PI3K-dependent manner. Subsequent association of Grb7 (via the SH2 domain) with activated focal adhesion kinase (FAK) or with RasGTP (via the Ras-associating (RA) domain of Grb7), promotes Rac/Cdc42/PAK and MAPK signalling [33-34]. Interactions with tyrosine-phosphorylated receptors, GAB1, $PI(3,4,5)P_3$ and Rac GTPases activate PLCγ [35]. PLCγ produces the second messenger diacylglycerol (DAG), leading to activation of protein kinase C (PKC) isoforms that influence the MAPK/ERK cascade at the levels of Raf, MEK and ERK [36-38]. PDK1 can increase the MAPK/ERK responses by activation of PKCs [11] and PAK1 [14]. In addition, PDK1 phosphorylates MEK on Ser222 and Ser226 residues, which are essential for full activation [39].

While crosstalk interactions resulting from PI3K activation and mediated by GAB/IRS/Grb7, PAK and PDK1 activate the Ras/MAPK pathway, Akt and its downstream effectors, mTOR and p70S6K, negatively affect ERK signalling (Fig. 2). Upstream or at the level of Ras, a negative feedback control occurs via serine phosphorylation of GAB and IRS proteins, resulting in a decrease of their tyrosine phosphorylation levels and impaired ability to sustain or amplify ERK phosphorylation [21, 40-41]. In addition, Akt-dependent phosphorylation of c-Raf on Ser259 and Rac/Cdc42 on Ser71 can interfere with Raf membrane recruitment and Raf activation by PAK, respectively [42-43].

Interestingly, Akt-mediated transcriptional controls of ERK activation can be positive. This regulation involves multiple protein phosphatases (MKPs) that dephosphorylate ERK. MKP expression and activities are positively regulated by ERK, p38-MAPK and glycogen synthase kinase-3 (GSK-3) [44-45]. Suppression of p38 MAPK and GSK-3 by Akt [4, 46] downregulates MKP expression and therefore can prolong the period of time when ERK is phosphorylated.

ERK-mediated regulation of the PI3K/Akt pathway. Active ERK can influence the PI3K/Akt pathway via several interaction routes (Fig. 2). One mechanism involves the modulation of the tyrosine phosphorylation level and/or half-life of GAB and IRS scaffolding proteins by ERK-mediated phosphorylation at serine and threonine residues. For instance, ERK phosphorylates GAB1 on several serine residues that are adjacent to p85-PI3K binding sites (three YXXM motifs) [47]. Depending on type of stimulation, this results in decreased (e.g., EGF signalling) or increased (e.g., HGF signalling) levels of GAB-p85-PI3K complexes, which correlates with PI3K activity due to positive PI3K-$PI(3,4,5)P_3$-GAB-PI3K feedback [21, 48-50]. Likewise, IRS-1 and FRS-2 phosphorylation on serine and threonine residues mediated by ERK or its downstream kinases, such as p70S6K, decreases their binding to p85-PI3K and thereby decreases PI3K activity by disrupting positive feedback [51-52].

Both ERK and its kinase substrate 90 kDa ribosomal protein S6 kinase 1 (p90RSK) phosphorylate and inhibit GSK-3 [53]. Since GSK-3 is a negative regulator of PI3K antagonist PTEN [54], activation of ERK alleviates PTEN inhibition, thereby decreasing $PI(3,4,5)P_3$ levels. This disrupts positive feedback loops via $PI(3,4,5)P_3$-binding proteins, thereby decreasing PI3K activity. Additional crosstalk mechanisms include FAK dephosphorylation on Tyr397 following ERK-mediated FAK phosphorylation on Ser910 [55]. This can disrupt the formation of the FAK complexes with p85-PI3K, c-Src, Grb7 and Grb2 and eventually decrease the activation levels of c-Src, PI3K, Rac/Cdc42/PAK and Ras/MAPK [9, 34].

While ERK-mediated phosphorylation of GAB/ IRS/FRS scaffolding proteins and kinase substrates serves as negative feedback loops to PI3K, RasGTP-induced stimulation of PI3K creates a positive, growth-factor induced loop [4, 9]. The E3-ubiquitin ligase and multifunctional scaffolding protein c-Cbl can also serve as a point of crosstalk between PI3K/Akt and Ras/MAPK pathways (Fig. 2). Serine/threonine phosphorylation of c-Cbl by PKCs [56] inhibits c-Cbl tyrosine phosphorylation thus affecting its interactions with numerous SH2-domain containing proteins [57]. This can result in delayed ubiquitylation and proteasomal degradation of receptors and other c-Cbl-associated-proteins (e.g. PI3K, Vav and SFKs), consequently enhancing Ras/ERK signalling. On the other hand, c-Cbl binding to both the SH2 and SH3 domains of p85 subunit of PI3K facilitates PI3K activation [57-58]. This process may be hindered due to c-Cbl sequestration by Sprouty proteins [59], whose expression levels, in turn, are positively regulated by ERK [60].

Context-dependent crosstalk

The experimental findings reviewed above suggest that in most cellular systems, PI3K positively regulates the Ras/MAPK cascade, facilitating maximal ERK responses to physiologic stimuli, whereas activated ERK, in turn, negatively controls the PI3K/Akt pathway (Fig. 3 and [21, 48]). Yet, multiple signalling routes and nodes involved in Ras/MAPK - PI3K/Akt crosstalk make it context-dependent, and in some cells, activation of Raf, MEK and/or ERK is enhanced by PI3K inhibition [61-63], and PI3K activity is decreased following MAPK inhibition [50]. Experimental data and mathematical modelling demonstrate that crosstalk depends dramatically on the concentrations of growth factors and the levels of receptors and scaffolding proteins, such as GAB and IRS [21, 64].

For instance at saturating EGF doses, PI3K inhibition by wortmannin only slightly attenuates ERK phosphorylation in A431 human epidermoid carcinoma and MCF7 human mammary carcinoma cells (over the 2 - 60 min response period), whereas PI3K inhibition dramatically decreases ERK phosphorylation at low, physiological EGF doses (Fig. 3A, upper and middle panels). In MCF10A normal mammary epithelial cells stimulated with moderate EGF doses, wortmannin decreases ERK phosphorylation in the early and late time points of signal propagation, but the peak of activation (7.5-10 min) does not differ from that of control cells (Fig. 3A, lower panel). This decreasing sensitivity of ERK phosphorylation to PI3K inhibition with increasing EGF doses is also observed in HEK293, HeLa, T47D, BT-474 and other cell lines (Fig. 3B and unpublished observations). Thus, the positive control

exerted by PI3K activation on the Ras/MAPK pathway is most effective at low EGF doses, and these data are also supported by computational modelling (Fig. 3D-E and [21, 64]).

On the contrary, the negative influence of the Ras/MAPK pathway on the PI3K/Akt pathway is more pronounced at high EGF doses (Fig. 3D). In fact, inhibition of ERK phosphorylation by the same concentration of the specific MEK inhibitor U0126 causes larger increases in Akt activity at higher EGF doses (Fig. 3C and [21]). Importantly, the Ras/MAPK - PI3K/Akt crosstalk effects depend on both signal strength (EGF dose) and the stimulation time. Thus, the analysis of the entire time-course of ERK and Akt activation at varying signal strength conditions and respective inhibitors is required to characterise interactions between the pathways. Judging about crosstalk on the basis of a single EGF dose and one time point can be misleading, since it might suggest that the Ras/MAPK pathway is uncoupled from PI3K/Akt pathway (Fig. 3). However, in some cases crosstalk cannot be detected due to activating mutations within different signalling branches. For example, wortmannin treatment does not suppress EGF-induced ERK phosphorylation in Ras-mutant PL-5, A549 and T24 cells (Fig. 3B and unpublished observations).

Concluding remarks

Pathway crosstalk endows a cell with emerging capabilities for signal processing and decoding, thereby adapting the cellular behaviours to the combinatorial variety of external cues and conditions. Here we showed that interactions between mitogenic (Ras/ERK) and survival (PI3K/Akt) pathways, which involve multiple signalling nodes and routes, generate context-dependent responses to growth factor stimulation. Crosstalk changes the dynamic topologies of signal propagation networks downstream of cell-surface receptors, leading to amplification or attenuation of key target protein activities. It was recently shown that Ras/ERK - PI3K/Akt crosstalk can mediate insulin-EGF interactions, leading to amplification of mitogenic signalling by insulin at physiological concentrations of growth factors [64]. Importantly, crosstalk leads to activation of compensatory signalling, allowing cancer cells to evade apoptosis if only the ERK cascade or the PI3K pathway are targeted therapeutically. Combined inhibition of PI3K and MAPK proved to be more efficient in suppressing cancer cell growth and viability than targeting the components of each pathway alone [46, 62].

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Figure legends

Figure 1. **A**. Schematic diagram of PI3K/Akt and Ras/MAPK cell signaling pathways. PA – phosphatidic acid, PS – phosphatidylserine. **B**. Levels of signalling network where the PI3K/Akt (orange lines) and Ras/MAPK (purple lines) signaling pathways may influence each other.

Figure 2. Flow-chart of representative PI3K-MAPK interactions. Black arrows and red lines with blunt-ends show activating and inhibitory interactions, respectively, mediated by posttranslational modifications, such as phosphorylation and ubiquitination. Solid lines with circular ends represent protein-protein interactions.

Figure 3. Changes in the time-courses of ERK (**A**) and Akt (**C**) activation upon PI3K inhibition by wortmannin (WT) (**A**) or MEK inhibition by U0126 (**C**) at different EGF doses in different cell lines (as indicated). **B.** Changes in dose-dependence of ERK activation upon WT treatment. **D.** Scheme depicting crosstalk between the PI3K/Akt and Ras/MAPK pathways at low and high EGF doses. Prevailing feedback loops are shown by thick lines. **E.** Computer simulations of WT effects on ERK activation at two different doses of EGF (1 and 20 nM). Phosphorylated ERK fraction was calculated at 5 min stimulation in the absence (red bars) or presence (green bars) of WT. See references [21, 64] for further details.