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Dynamic cross-talk between PI3-kinase/Akt and Ras/ERK pathways in EGF receptor signaling that can affect drug sensitivity in tumor cells

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Abstract

Cell survival (PI3K/Akt) and mitogenic (Ras/ERK) signaling cascades rarely act as independent parallel pathways, rather they interact at different points and phases of signal propagation, generating positive and negative feedback loops. We explored the patterns of cross-talk between PI3K and ERK in various normal and cancer cells that were pretreated with wortmannin or U0126 inhibitors prior to stimulation with increasing EGF doses. Time-courses of total and phosphorylated ERK or Akt expression were detected by Multistrip Western blotting, a novel procedure used for concurrent comparison of signals derived from multiple blots. Quantitative analysis of immunoblots showed that PI3K enhanced and sustained ERK responses, whereas activated ERK suppressed PI3K activity in time- and EGF dose-dependent manner. The differential extent of such bidirectional reciprocal cross-talk could be attributed to diverse recruitment mechanisms of GAB family adaptor proteins, as suggested by immunoblot analyses of subcellular fractions and immunoprecipitates. Dynamic PI3K-ERK interactions also persist in tumors, making the activation patterns robust to individual perturbations, which can be linked to different drug sensitivity profiles. Nonetheless, combined inhibition of PI3K and ERK activities synergistically decreased tumor cell viability and growth, as measured by AlamarBlue assay.

Introduction

The epidermal growth factor receptor (EGFR) belongs to ErbB family of receptor tyrosine kinases. Upon binding to cognate EGF family of peptides, the autophosphorylated tyrosine residues on the C-terminal domain of dimerized receptor and the tyrosine phosphorylated receptor-substrates create the docking sites for Src homology 2 (SH2) domains-containing enzymes and adaptor/docking proteins without catalytic activity, but with multiple motifs to mediate protein-protein and protein-lipid interactions. By assembling different combinations of signaling molecules and by anchoring enzymes to the subcellular structures, where their protein or lipid substrates reside, docking proteins can direct a signal to different pathways and fine-tune cellular responses [1, 2].

EGF-evoked signals are generally transmitted to the Raf/MEK/ERK (also known as mitogen activated protein kinase (MAPK)) cascade through the GTPase Ras and Src family tyrosine kinases that also facilitate the activation of class I phosphoinositide 3-kinases (PI3K). PI3K phosphorylates phosphatidylinositol lipids to generate PtdIns(3,4,5)P_3, which is recognized by pleckstrin homology (PH) domains of various proteins, including the downstream effector serine/threonine kinase Akt. The translocation of ERK, Akt and their substrates into the cell nucleus leads to the expression of specific sets of genes that determine relevant biological responses to extracellular cues: namely, cell division, proliferation, differentiation, adhesion or migration, cytoskeletal rearrangements, changes in metabolism, DNA repair, survival or death (apoptosis). Signaling through the EGFR so governs critical physiological processes including cell-cycle progression, development, wound healing and oncogenesis [1, 3, 4, 5].

Abnormal expression and activity of EGFR and signaling components of mitogenic and pro-survival signaling pathways is common in different solid tumors [6] that display distinct drug sensitivity profiles. Depending on cellular context, these pathways can either stay individual and signal in parallel or may start to interact (cross-talk) with each other in a positive and/or negative manner at different stages of signal propagation [7]. An activation of anti-apoptotic and cell-cycle genes after repeated drug exposure contributes to chemoresistance, which is a major cause of treatment failure as well as poor prognosis in various human malignancies [8]. It is relevant to explore alternative therapeutic approaches to overcome drug resistance.

Research Objectives

We sought to evaluate the cross-talk between PI3K/Akt and Ras/MAPK signaling pathways upon acute exposure to increasing EGF doses, and to determine the efficacy of single and combined inhibition of these signaling pathways in a panel of EGF-responsive cancer cells.

Materials and Methods

Cell lines: normal epithelial kidney (HEK293), mammary (MCF10A) and cancerous breast (MCF7, T47D, BT474), pancreatic (PL5, Capan-2), epidermoid (A431), bladder (T24), lung (A549) cells. **Stimulation**: serum-starved cells were preincubated with MEK inhibitor U0126 (5 M) or PI3K inhibitor wortmannin (WT, 100 nM) for 30 min before addition of varying EGF doses for indicated time intervals. **Immunoprecipitation (IP)** and **Ras-GTP assay**: typically, 4 mln cells were lysed [9] and total cell lysates (TCL) were incubated with 5 g of indicated 1° antibody (Ab) in the presence of protein A/G-agarose or with 30 l of Ras-RBD beads for 4 h at 4°C. The proteins from IP buffer and phosphate buffer saline-washed (2x/each) beads were released at 95°C in 4x LDS sample buffer. **Subcellular fractionation**: the isolation of soluble (Cyt) and particulate (Mem) fractions was carried out in digitonin-permeabilized cells (150 g/ml, 10 min) [9]. **Electrophoresis**: proteins were resolved by LDS-PAGE at 140V on 4-12% gradient Bis-Tris gels (Invitrogen) followed by transfer onto nitrocellulose membrane by conventional or **Multistrip Western blotting** (Fig.1) at 30V for 90 min. The membranes were kept in 4% bovine serum albumin blocking buffer for 1 h, incubated with primary Ab to phosphorylated or non-phosphorylated protein forms overnight, washed and treated with an appropriate 2°Ab for 1 h followed by final wash. Blocking, Ab dilution and washing solutions contained Tris-buffered saline with 0.5% Triton X-100. Chemiluminescent signals of bands were detected by ECL and quantified using KODAK Image Station 440CF software. Kinetic curves and charts were plotted in SigmaPlot. **Proliferation and viability** of cells cultured in EGF-supplemented serum-free medium in the presence of U0126, PI3K inhibitor LY249002 or their combination were assessed after 48 h by AlamarBlue oxidation-reduction indicator assay (BD Biosciences), based on conversion of blue non-fluorescent dye resazurin to pink highly fluorescent resorufin by mitochondrial enzymes. Fluorescence was measured in octuplets using microplate reader (BioTek) at wavelength set of 530Ex/590Em. **Gene silencing**: indicated cells were transfected with 100 nM siCONTROL non-targeting (NT) siRNA or pool of 4 GAB1 or Grb2 siRNA (Dharmacon) in antibiotics-free Opti-MEM medium using Amaxa's Nucleofector (Lonza) and Ingenio Electroporation solution (Mirus Bio) and seeded in 6-well plates. 6 h later the medium was supplemented with 10% FBS and antibiotics. At 72 h after transfection serum-starved cells were stimulated with indicated EGF doses. Alternatively, at 48 h after transfection, cells were treated with 5-fluorouracil (5-FU) (Sigma) in the presence of 0.5% FBS for 24 h prior to quantitative evaluation of cell viability and drug cytotoxicity by using fluorimetric AlamarBlue or colorimetric LDH release (Cayman Chemicals) assays. **Statistics**: values expressed as arbitrary units (AU) represent a mean ± SD of three signal measurements from at least three independent trials. Initial signal values were normalized to the appropriate loading control and then divided by background signals, reflecting protein activation fold over the basal levels. Student's t-test was used to determine the significant differences (when p<0.05) between each experimental group.

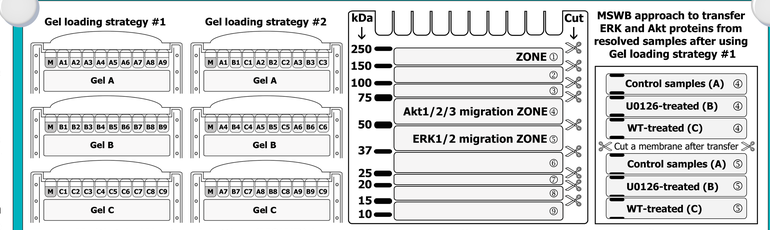

Fig. 1. Multistrip Western blotting (MSWB) is a modified immunoblotting procedure based on simultaneous electrophoretic transfer of proteins from multiple strips of polyacrylamide gels to single membrane sheet. In comparison with a conventional technique, MSWB can increase data output per single blotting cycle up to ten fold, enables concurrent viewing of multiple protein bands, comparison of kinetic curves, detection of up to nine different antigens from a same loading of sample, and substantially improves data accuracy by reducing immunoblotting-derived signal errors [10, 11].

Results

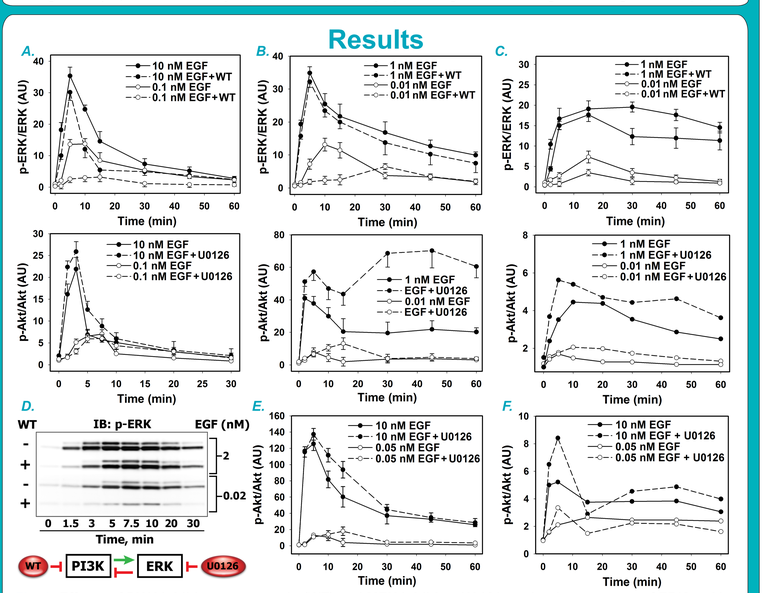

Fig. 2. Effects of PI3K inhibitor wortmannin (WT) and MEK inhibitor U0126 on the time-courses of ERK or Akt activation at increasing EGF doses in MCF7 (A), A431 (B), T47D (C), MCF10A (D), Capan-2 (E) and PL5 (F) cells.

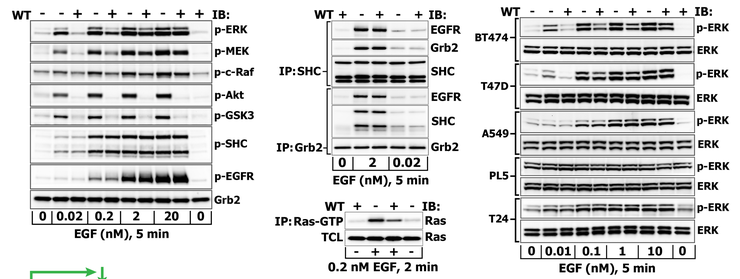

Fig. 3. PI3K inhibition does not affect the phosphorylation levels of EGFR, docking protein SHC (left panel) **and their binding to adaptor protein Grb2** (upper middle panel), **but reduces c-Raf/MEK phosphorylation** (left panel) **by decreasing Ras activity** (bottom middle panel) in MCF10A cells. Unlike in these and other cells that harbor wild-type Ras, **WT fails to suppress ERK phosphorylation** in A549, PL5 and T24 (right panel) **cells with activating Ras mutations**.

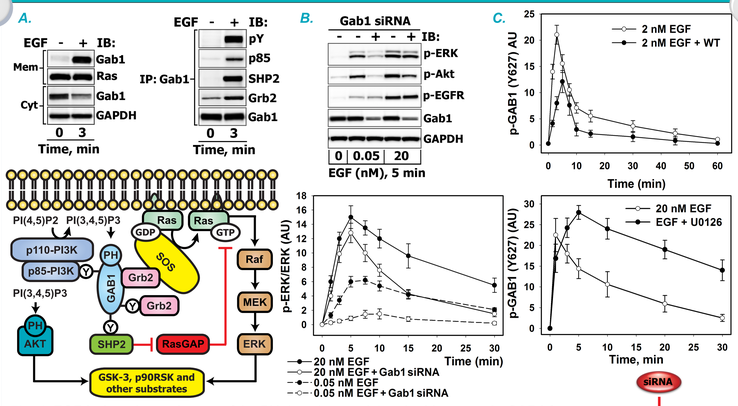

Fig. 4. EGF-induced recruitment of the PH domain-containing **docking protein GAB1** from the cytosol to the inner surface of the plasma membrane (**A**, left panel), **GAB1 tyrosine phosphorylation** and its **interaction with** the **p85 regulatory subunit of PI3K**, the protein tyrosine phosphatase **SHP2** and adaptor protein **Grb2** (**A, right panel**), correlate with the increase in phosphorylation levels of p90RSK and GSK3, the primary substrates of ERK and Akt kinases. ***B.*** **Silencing of GAB1 gene expression by small interfering RNA attenuates EGF-induced phosphorylation of Akt and ERK.** ***C.*** **GAB1 is a target of PI3K-mediated positive** (upper panel) and **ERK-mediated negative** (bottom panel) **feedback loops** in EGF signaling of most epithelial cells (HEK293 cells shown here). However, depending on cell type and kind of the ligand (e.g. EGF vs. HGF or Il-6), the properties of these feedbacks may vary dramatically.

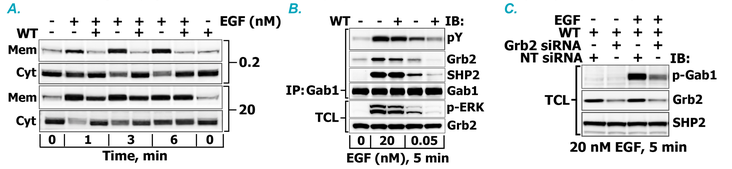

Fig. 5. A. At higher EGF dose, PI3K activity becomes only partially required to initiate GAB1 translocation, and not necessary to maintain GAB1 accumulation at the plasma membrane. B. Tyrosine phosphorylation of GAB1 and its binding to Grb2 and SHP2 proteins are less affected by PI3K inhibition at higher EGF dose. C. siRNA-mediated downregulation of Grb2 endogenous levels reduces PI3K-independent phosphorylation of GAB1.

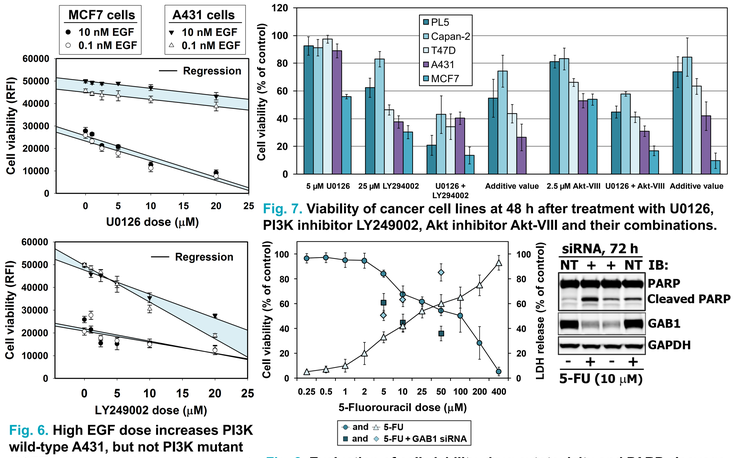

Fig. 7. Viability of cancer cell lines at 48 h after treatment with U0126, PI3K inhibitor LY249002, Akt inhibitor Akt-VIII and their combinations.

Fig. 6. High EGF dose increases PI3K wild-type A431, but not PI3K mutant MCF7 cell survival in the presence of MEK and PI3K inhibitors. RFI, relative fluorescence units.

Fig. 8. Evaluation of cell viability, drug cytotoxicity and PARP cleavage at 24 h after cell treatment with IC20, IC30 and IC50 value doses of 5-FU antimetabolite in GAB1 siRNA-transfected PL5 adenocarcinoma cells.

Conclusions

1. PI3K activation potentiates EGF-induced ERK phosphorylation, whereas enforced activation of ERK downregulates PI3K activity in time-dependent manner.

2. The strength of reciprocal interaction between the PI3K/Akt and Ras/MAPK signaling pathways inversely depends on EGF dose.

3. PI3K influences the activation of the MAPK cascade downstream of the EGFR/SHC/Grb2-SOS, but upstream or at the level of small GTPase Ras.

4. Our findings indicate that docking protein GAB1 is an important node coupling cell survival and mitogenic signaling pathwhays. Its contribution to ERK activation is most pronounced at physiologicaly low EGF doses, when GAB1 membrane targeting mainly depends on PI3K activity, and signal flow through the canonical EGFR/SHC/Grb2/SOS route is relatively weak.

5. Our data suggest, that upon treatment with MEK/ERK inhibitory agents, higher EGF doses may increase the survival of those cells, which provide relatively stronger negative feedbacks from ERK to PI3K.

6. Simultaneous targeting of MEK/ERK and PI3K/Akt pathways is particularly efficient in suppressing pancreatic tumor cell growth.

7. Suppression of endogenous GAB1 levels by siRNA significantly increases the cytotoxic effects of antitumor drug 5-fluorouracil in PL5 pancreatic adenocarcinoma cells.

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