Program No. 946.1

Abstract

Understanding the interactions that occur among the distinct signal transduction pathways, triggered by activation of the prolactin receptor (PRL-R), is essential in studying the pathogenesis of metastatic breast cancer. Quantitative measurements of phosphorylation/activation patterns of the proteins by Multi-strip Western blotting of immunoprecipitates or total cell lysates shows that PRL induces the tyrosine phosphorylation of adaptor proteins of insulin receptor substrate (IRS) and Shc families as well as concurrently activates Src family kinases (SFKs)/focal adhesion kinase (FAK), Janus kinase/signal transducer and activator of transcription (JAK/STAT), phosphoinositide-3-kinase (PI3K)/Akt and Ras/mitogen-activated protein kinase (MAPK) signaling pathways in T47D and MCF-7 human breast cancer cells, derived from the patients with infiltrative ductal carcinoma and expressing different amounts of PRL-R. A specific blockade of SFK/FAK, JAK2/STAT5, PI3K/PDK1/Akt, Rac/PAK or Ras regulatory circuits revealed that (1) PI3K/Akt signaling pathway crosstalks with c-Raf/MEK/ERK cascade and positively regulates PRL-induced ERK1/2 phosphorylation; (2) PI3K-mediated ERK1/2 activation via c-Raf occurs regardless of signaling downstream of STAT5, Akt and PKC, but depends on activities of JAK2, c-Src and FAK; (3) activated PRL-R largely utilizes a PI3K-dependent Rac/PAK pathway rather than the canonical Shc/Grb2/SOS/Ras route to initiate and sustain ERK1/2 signaling. These findings suggest that by interconnecting diverse signaling pathways PLR may enhance proliferation, survival, migration and invasiveness of breast cancer cells.

THE ROUTES OF ERK ACTIVATION IN PROLACTIN-STIMULATED BREAST CANCER CELLS

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Introduction

Prolactin (PRL), a hormone secreted by the pituitary gland and to a lesser extent by other tissues, is involved in critical physiological processes, such as reproduction and lactation, growth and development, metabolism, immunomodulation and osmoregulation [1-3]. PRL acts as a growth, differentiating and survival factor in normal human mammary epithelial cells and has profoud positive effect on mammary gland carcinogenesis in rodent models [4-6]. The serum PRL levels and expression of its cognate class I cytokine family receptor (PRL-R) are increased in human breast cancer tissues [3, 7-8], where PRL-R signaling is implicated in tumor development, progression and resistance to chemotherapy, especially in the presence of various oncogenic mutations [9-11].

PRL-R lacks intrinsic enzymatic activity and transduces the signals inside the cell via pre-associated non-receptor tyrosine kinases of the Janus family, which in turn phosphorylate PRL-R, creating docking sites for downstream effectors that trigger intracellular signaling cascades [1]. The signaling branches have many points of convergence at different levels of signaling hierarchy and influence each other at different phases of signal propagation in both negative and positive manners, resulting in a dynamic and complex crosstalk. Hence a blockade of one signaling pathway may result in an activation of compensatory signaling, allowing tumors to evade cell death.

Research Objective

We sought to examine the topology of the PRL signaling network and to evaluate relative contributions of multiple signaling branches downstream of PRL-R to the activation of the extracellular signal-regulated kinases ERK1 and ERK2 (further reffered as ERK1/2) under endogenous conditions in human breast cancer cells.

To achieve this goal, we first examined the potency of PRL to activate Src/FAK, JAK/STAT, PI3K/Akt and Ras/MAPK signaling pathways in T47D and MCF-7 breast cancer cell lines, expressing different amounts of endogenous PRL-R. Thereafter, we performed successive inhibitory analysis of various signaling intermediates downstream of the PRL-R and subsequently measured the time-courses of ERK1/2 activation.

Materials and Methods

All common chemicals, solvents and reagents were of highest grade available from commercial sources. **Cell lines**: T47D and MCF-7 cells (ATCC) were grown in RPMI-1640 or DMEM medium (Mediatech Inc), respectively, supplemented with 10% FBS, 1% PennStrep solution, and (in case of T47D) 20 μg/ml of bovine insulin. **Stimulation**: Serum-starved cells cultivated on 60×15cm (for total cell lysates, TCL) or on 150×20 cm (for immunoprecipitation, IP) tissue culture dishes were either left untreated or were preincubated with inhibitors f or indicated periods of time prior to cell stimulation with 10 nM PRL (Peprotech Inc.) for various time intervals at 37°C. Inhibitors and PRL were diluted to final concentrations in respective starvation media. **Protein immunoprecipitation (IP), Ras-GTP and Rac1-GTP assays**: ~4 mln cells were lysed and TCL were preincubated with 5 μg of indicated primary (1°) Ab in the presence of protein A/G-agarose or with 30 μl of Ras-RBD or PAK1-PBD beads (Millipore), respectively, for 4 h at 4°C. Protein complexes were collected by brief centrifugation, washed with ice-cold IP buffer, supplemented with 10 mM $MgCl_2$ and with ice-cold PBS (twice/each). The proteins were released from beads at 95°C in 4× LDS s ample buffer. **Subcellular fractionation**: isolation of soluble (**Cyt**) and particulate (**Mem**) subcellular fractions was carried out in digitonin-permeabilized cells (150 mg/ml, 10 min) as described previously [12]. **Electrophoresis**: proteins were resolved by LDS-PAGE at 140V in 4-12% gradient Bis-Tris gels (Invitrogen) followed by transfer on nitrocellulose membranes (Bio-Rad) by conventional or **Multi-strip Western blotting [13-14]** at 30V for 90 min. The blots were kept in 3% BSA blocking buffer for 1h, incubated with 1°Ab to the phosphorylated or non-phosphorylated protein forms overnight at RT, washed (5x, 7 min/each) and treated with respective 2°Ab for 1 h followed by a final wash. Blocking, Ab dilution and washing solutions contained Tris-buffered saline with 0.5% Triton X-100. **Protein detection**: the chemiluminescent signals of protein bands were detected by ECL using a SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) and their signal net intensities were quantified by densitometry analysis software using KODAK Image Station 440CF. Signal intensities of a phosphorylated protein were normalized by signal intensities of total (phosphorylated and non-phosphorylated) protein forms at each time point and were expressed as fold changes over basal levels (in unstimulated cells). Kinetic curves and charts were plotted based on fold changes in SigmaPlot v.10. **Statistics**: All experiments were performed in triplicates. Only the representative blots and/or their quantitative values are shown. Student's t-test was used in order to determine statistically significant diffrences between each experimental group. **Wound-healing assay**: cells grown to confluence in 6-well plates were scratched using a pipette tip to create the wound, washed with serum-free medium (SFM) to remove loosened cells, and thereafter cultured in SFM that contained PRL with or without inhibitor, with a medium being replaced every 24 hours. Cells were photographed at 0 and 72 hours after wounding with by inverted light microscope at 4× magnification. Area of each wound surface was quantitated using Adobe Photoshop software. The mean percentage wound closure was calculated using the equation (S2–S1)/S2*100, where S2 is cell-free scratch area at 0 h after wounding, S1 - cell-free scratch area at 72 hours after wounding. **Proliferation** of cells cultured in PRL-supplemented SFM in the presence or absence of inhibitor was assessed after 72 h by AlamarBlue oxidation-reduction indicator assay (BD Biosciences). Fluorescence was measured in octuplets using microplate reader (BioTek) at 530Ex/590Em set.

Results

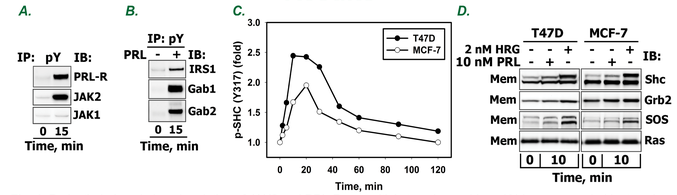


Fig. 1. Prolactin-induced phosphorylation of JAK2 and PRL-R on multiple tyrosine residues (**A**) is accompanied by tyrosine phosphorylation of adaptor proteins of IRS (**B**) and Shc (**C**) families, but less efficient recruitment of Shc, Grb2 and SOS to the plasma membrane as compared to heregulin-β1 (HRG), a potent growth and survival factor for breast cancer cells (**D**). SOS is a guanine nucleotide exchange factor, catalyzing an exchange of GDP to GTP in membrane-anchored Ras GTPase.

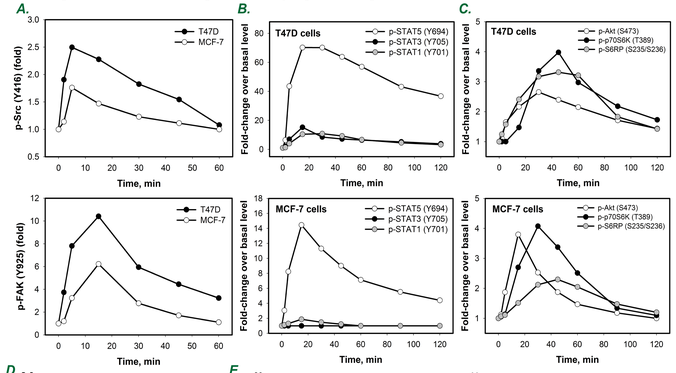


Fig. 2. PRL concomitantly activates c-Src (**A**, upper panel), FAK (**A**, lower panel), STAT family members (**B**) and effectors of the PI3K: Akt, p70 ribosomal protein S6 kinase (p70S6K) and S6 ribosomal protein (S6RP) (**C**). Despite a weak activation of Ras GTPase (**D**), PRL stimulates c-Raf/MEK/ERK signaling cascade and results in the activation of cytosolic p90 ribosomal S6 kinase (p90RSK) (**E**, left and right panels) in T47D and MCF-7 breast cancer cells.

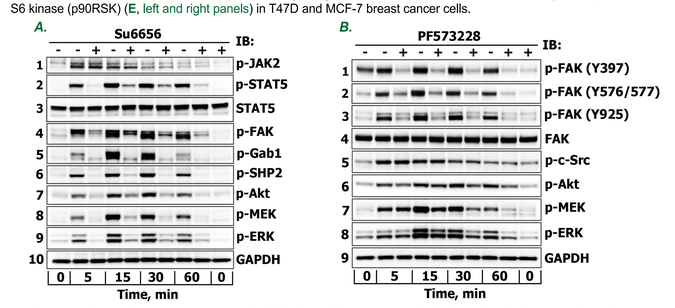


Fig. 3. A. Inhibition of Src family kinases by Su6656 (5 μM, 30 min) does not affect JAK2 activation (1), but greatly reduces tyrosine phosphorylation of STAT5 (2), FAK (4), Gab1 (5), protein tyrosine phosphatase SHP2 (6) and nearly abrogates the activation of Akt (7), MEK (8) and ERK1/2 (9) upon T47D cell stimulation with 10 nM PRL. **B.** Inhibition of FAK by PF573228 (0.5 μM, 2 h) prevents FAK autophosphorylation at Tyr397 (a binding site for SFKs, PI3K and PLCγ) (1) and Src-dependent phosphorylation at Tyr576/577 (2) and Tyr925 (a binding site for adaptor protein Grb2) (3) residues without affecting protein expression levels of FAK (4), but resulting in partially decreased activation of Akt (6), MEK (7) and ERK1/2 (8).

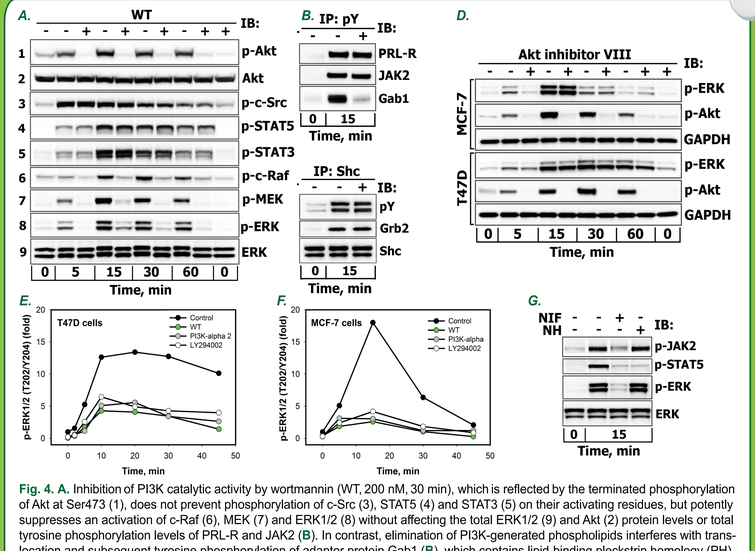


Fig. 4. A. Inhibition of PI3K catalytic activity by wortmannin (WT, 200 nM, 30 min), which is reflected by the terminated phosphorylation of Akt at Ser473 (1), does not prevent phosphorylation of c-Src (3), STAT5 (4) and STAT3 (5) on their activating residues, but potently suppresses an activation of c-Raf (6), MEK (7) and ERK1/2 (8) without affecting the total ERK1/2 (9) and Akt (2) protein levels or total tyrosine phosphorylation levels of PRL-R and JAK2 (**B**). In contrast, elimination of PI3K-generated phospholipids interferes with translocation and subsequent tyrosine phosphorylation of adaptor protein Gab1 (**B**), which contains lipid-binding pleckstrin homology (PH) domain. **C**. PI3K inhibition does not reduce total tyrosine phosphorylation levels of Shc or its binding to Grb2. **D-E**. Cell treatment with Akt inhibitor VIII (10 μM, 30 min) (**D**) or PKC inhibitor Gö 6850 (5 μM, 1 h) (data not shown) results in unchaged activation patterns of ERK1/2. **F-G**. Confirmation of a positive effect of PI3K on ERK1/2 responses by structurally different inhibitors of PI3K: PI3K-alpha-2 (2 μM, 30 min) and LY294002 (25 μM, 30 min). **G**. PRL-induced ERK1/2 activation depends on JAK2 activity, but is uncoupled from a STAT5/3/1-mediated signaling as shown by experiment employing STAT family inhibitor nicotinoylhydrazone compound (NH, 200 μM, 1 h) and JAK2 inhibitor nifuroxazide (NIF, 100 μM, 1h) in PRL-stimulated MCF-7 cells.

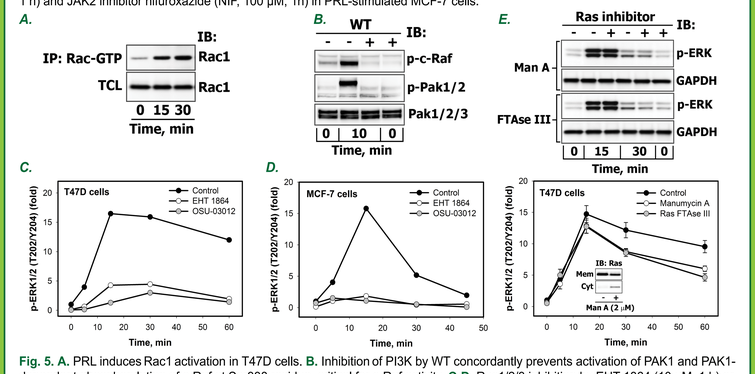


Fig. 5. A. PRL induces Rac1 activation in T47D cells. **B.** Inhibition of PI3K by WT concordantly prevents activation of PAK1 and PAK1-dependent phosphorylation of c-Raf at Ser338 residue, critical for c-Raf activity. **C-D.** Rac1/2/3 inhibition by EHT 1864 (10 μM, 1 h) or combined inhibition of PAK1 and PDK1 by OSU-03012 (25 μM, 30 min) greatly suppresses PRL-induced ERK1/2 activation in MCF-7 and T47D cells, whereas inhibition of Ras processing by the farnesyl transferase inhibitors manumycin A or FTase III (2 μM, 7 h each) only slightly attenuates the post-peak phosphorylation of ERK1/2 (**E, upper and lower panels**).

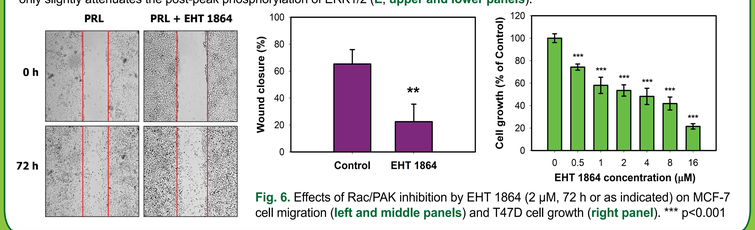
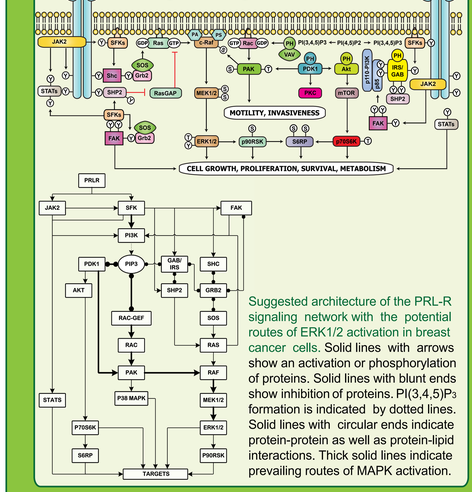


Fig. 6. Effects of Rac/PAK inhibition by EHT 1864 (2 μM, 72 h or as indicated) on MCF-7 cell migration (**left and middle panels**) and T47D cell growth (**right panel**). *** p<0.001

Flowcharts of PRL-R signaling network



Suggested architecture of the PRL-R signaling network with the potential routes of ERK1/2 activation in breast cancer cells. Solid lines with arrows show an activation or phosphorylation of proteins. Solid lines with blunt ends show inhibition of proteins. $PI(3,4,5)P_3$ formation is indicated by dotted lines. Solid lines with circular ends indicate protein-protein as well as protein-lipid interactions. Thick solid lines indicate prevailing routes of MAPK activation.

Conclusions

1. PRL-induced ERK1/2 activation requires activation of JAK2 and Src family kinases and partially depends on signaling downstream of FAK.

2. MAPK responses are potentiated by activation of PI3K in STAT, Akt, and PKC -independent manners.

3. PI3K-mediated ERK1/2 activation is predominantly controlled by the Rac/PAK signaling pathway, which promotes breast cancer cell growth and motility.

4. Signaling to c-Raf/MEK/ERK cascade through Ras GTPase is weak and may reflect inefficient recruitment of Shc/Grb2/SOS complex to the plasma membrane.

5. Our study revealed the limitations of using higher doses of inhibitors to eliminate all functional Ras from the plasma membrane. It caused a significant dephosphorylation of Akt followed by deactivation of MAPK, cell detachment and death. Therefore these approaches, including Ras siRNA, could not be used to quantify more accurately the contributions of Ras-dependent and Ras-independent inputs to hormone-, cytokine-, or growth factor-induced ERK1/2 activation.

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Acknowledgements

This research was supported by NIH Grant #GM059570